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Special Issue Reprint

Enzyme Production Using Industrial and Agricultural By-Products

Edited by
Heitor B. S. Bento and Ana Karine F. Carvalho

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Guest Editors

Heitor B. S. Bento
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Guest Editors

Heitor B. S. Bento
Department of Engineering of Bioprocess and Biotechnology
São Paulo State University
Araraquara
Brazil

Ana Karine F. Carvalho
Department of Basic and Environmental Sciences
University of São Paulo
Lorena
Brazil

Editorial Office

MDPI AG
Grosspeteranlage 5
4052 Basel, Switzerland

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About the Editors

Heitor B. S. Bento

Heitor B. S. Bento is an Assistant Professor at São Paulo State University, affiliated with the Department of Engineering of Bioprocess and Biotechnology at the School of Pharmaceutical Sciences. His research interests lie in biotechnology and chemical engineering, with emphasis on bioprocess development, microbial lipid production, and the use of agro-industrial residues as renewable feedstocks. He is involved in projects focused on the production and purification of bioproducts, as well as the scale-up of biotechnological processes, including work with genetically modified microorganisms for enzyme production. His expertise also includes chemical and biochemical reactors, chemical and enzymatic catalysis, enzyme immobilization, and material characterization. He holds a BSc and MSc in Chemical Engineering from the University of São Paulo, and a PhD in Industrial Biotechnology, with a focus on applied microbiology, also from the University of São Paulo. He additionally completed a postdoctoral project at São Paulo State University, working on the scale-up of L-asparaginase production.

Ana Karine F. Carvalho

Ana Karine F. Carvalho is a faculty member at the University of São Paulo, affiliated with the Department of Basic and Environmental Sciences at the School of Engineering of Lorena. Her research interests focus on bioprocess engineering, renewable energy systems, and biosystems, with particular emphasis on the development of integrated biorefinery platforms. She is involved in projects that combine microbiology, molecular biology, food technology, catalysis, and energy resources to design sustainable and efficient processes. Her work includes the application of chemical and biochemical reactors for the valorization of agro-industrial residues, aiming at the production of biofuels, fine chemicals, and biomaterials. She holds a degree in Food Engineering from the Federal University of Ceará, as well as an MSc in Chemical Engineering and a PhD in Industrial Biotechnology from the University of São Paulo, and has previously held academic positions at the University of São Paulo and the Federal University of Alfenas.

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Editorial

Special Issue: Enzyme Production Using Industrial and Agricultural By-Products

Heitor B. S. Bento [1,*] and Ana Karine F. Carvalho [2]

[1] Department of Bioprocess Engineering and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University-UNESP, Araraquara 14800-903, Brazil
[2] Department of Basic and Environmental Sciences, Engineering School of Lorena, São Paulo University, Lorena 12602-810, Brazil; anacarvalho@usp.br
* Correspondence: heitor.bento@unesp.br

The transition toward a circular bioeconomy has intensified the search for more sustainable and cost-effective strategies for enzyme production. Enzymes are essential biocatalysts across multiple industrial sectors, yet their large-scale production is still largely dependent on refined substrates, contributing significantly to overall process costs. In this context, the use of industrial and agricultural by-products as alternative feedstocks has emerged as a promising solution to simultaneously address economic and environmental challenges [1,2].

This Special Issue consolidates recent advances in this field by presenting a diverse set of studies that explore the production of enzymes using low-cost and abundant residues. The published works collectively demonstrate the feasibility of utilizing lignocellulosic materials, agro-industrial wastes, and other underutilized biomass streams as substrates for microbial cultivation and enzyme synthesis. These contributions reinforce a key paradigm shift in industrial biotechnology, where waste streams are increasingly regarded as valuable raw materials rather than disposal burdens.

A clear trend emerging from the Special Issue is the diversification of both substrates and microbial platforms. The studies encompass the production of cellulases, hemicellulases, lipases, oxidoreductases, and other industrially relevant enzymes using bacteria, filamentous fungi, and yeasts, highlighting the adaptability of different biological systems to heterogeneous feedstocks. In parallel, several works emphasize process optimization through statistical experimental design and cultivation strategies, contributing to improved yields and productivity under both submerged and solid-state fermentation systems.

Another important aspect addressed by the collected articles is the integration of enzyme production with downstream applications. Enzymes produced from by-products were successfully applied in biomass hydrolysis, biotransformation processes, and environmental remediation, supporting the broader concept of integrated biorefineries. This alignment between upstream production and practical application is essential for improving overall process feasibility and industrial relevance [3,4].

Despite these advances, it is important to highlight persistent challenges. One of the most critical limitations remains the scalability of these processes [5]. While laboratory-scale results are promising, the transition to industrial production requires addressing substrate variability, process control, and reactor design. Agro-industrial residues are inherently heterogeneous, and this variability can significantly impact microbial performance and enzyme yields. In addition, downstream processing remains a major bottleneck, as enzyme recovery, purification, and stabilization continue to contribute substantially to production costs [1,6].

Another gap identified is the limited integration of techno-economic and environmental assessments. Although the sustainability argument for waste-based enzyme production

is compelling, quantitative analyses such as life cycle assessment and techno-economic evaluation are still scarce, yet they are essential for guiding industrial implementation [4]. Furthermore, opportunities associated with advanced biotechnological tools remain underexplored in the context of complex waste-derived substrates, including metabolic engineering, omics approaches, and synthetic biology.

Within this framework, the present Special Issue contributes by advancing the understanding of how diverse by-products can be effectively converted into high-value enzymatic systems, while also demonstrating optimized cultivation strategies and application-oriented approaches. The studies collectively strengthen the foundation for integrating enzyme production into circular and biorefinery-based models.

Looking ahead, future research should move toward stronger integration of feedstock characterization, process optimization, and application development. Greater emphasis on scale-up studies, process robustness, and continuous operation will be necessary to bridge the gap between laboratory findings and industrial deployment. At the same time, combining enzyme production with other biorefinery processes can enhance resource efficiency and economic viability. Advances in strain engineering and data-driven process optimization are expected to play a key role in overcoming current limitations, while the development of efficient enzyme recovery and immobilization strategies will be crucial to improving process sustainability [1,7,8].

In conclusion, the contributions gathered in this Special Issue clearly demonstrate that enzyme production using industrial and agricultural by-products is a rapidly evolving field with significant potential to support sustainable bioprocessing. By aligning waste valorization with enzyme biotechnology, these studies contribute to the development of more resilient and resource-efficient industrial systems. Continued interdisciplinary efforts will be essential to translate these advances into scalable and economically viable solutions within the global bioeconomy.

Conflicts of Interest: The authors declare no conflicts of interest.

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Article

EPS from Activated Sludge: Prospection of Bioflocculation and Catalytic Properties

Sara Jaramillo Arvilla [1,2], Grazieli Pereira da Silva [1], Ismael Hernandes Pereira, Júnior [1], Sofia Kremer de Souza [1], Carolina Gommersbach [1], Guilherme Urbano [1], Rodrigo de Almeida Mohedano [1], Paulo Belli Filho [1], Rejane Helena Ribeiro da Costa [1] and Nelson Libardi, Junior [1,*]

[1] Department of Sanitary and Environmental Engineering, Federal University of Santa Catarina, Florianópolis 88040-970, SC, Brazil; hsu.guilherme09@gmail.com (G.U.); rejanehcosta@gmail.com (R.H.R.d.C.)
[2] EIDENAR School, Faculty of Engineering, Universidad del Valle, Santiago de Cali 76001, Colombia
* Correspondence: nelson.libardi@ufsc.br

Abstract

The recovery of extracellular polymeric substances (EPS) from activated sludge (AS) represents a promising strategy to transform wastewater treatment plants (WWTPs) into resource recovery facilities within a circular economy framework. In this study, EPS was extracted from an AS process in a full-scale WWTP, highlighting its catalytic and bioflocculant properties, which represent an innovation in the valorization of this biopolymer. The EPS was subsequently characterized in terms of polysaccharides, proteins, and enzymatic activities (amylase and lipase). The bioflocculation performance of the EPS was evaluated using activated sludge mixed liquor. Results showed that EPS recovery yields using 50 °C and 80 °C were 196.3 ± 38.2 mg EPS/g sludge and 283.5 ± 85.4 mg EPS/g sludge, respectively. Enzymatic assays confirmed amylase activity ranging from 100 to 350 U/g sludge according to the extraction temperature. Lipolytic activity (20 U/g sludge) was comparable to values reported in the literature for EPS from biological sludge. The addition of EPS significantly improved the sludge settling velocity (from 0.86 to 4.48 m/h) and the sludge volume index (from 118.6 to 35.5). However, EPS application also increased the resistance to filtration by 50% and reduced cellular respiration by approximately 40%. Overall, the findings demonstrate that EPS from activated sludge acts as an effective bioflocculant with relevant catalytic properties, highlighting its potential as a high-value biotechnological product while also pointing to operational challenges that require further optimization.

Keywords: amylase; lipase; extracellular polymeric substances; activated sludge; bioflocculation; resource recovery

1. Introduction

An increasingly studied and implemented strategy to enhance the relevance and value of wastewater treatment processes is to reconceptualize wastewater treatment plants (WWTPs) as resource recovery facilities (RRFs) rather than merely as treatment systems. Stricter regulations regarding effluent treatment levels and the need for self-sufficient or even energy- and resource-positive plants are the primary drivers for the research and development of new WWTP configurations [1]. New concepts in wastewater treatment, sometimes referred to as "biorefineries" or resource recovery facilities (RRFs), encompass circular economy principles and enable the creation and production of high-value-added co-products from residual biomass, which can meet niche market demands [2].

The extraction and recovery of biopolymers from biological sludge is a potential route for obtaining bioproducts, as described by several authors. In The Netherlands, two large-scale demonstration systems are already in operation to showcase the economic feasibility of biopolymer recovery [3]. These biopolymers have similar properties to alginate, which has various pharmaceutical, food, and textile industry applications. For biopolymers extracted from granular sludge, other applications arise, such as in the composition of construction materials, as flame retardants, bioflocculants, and biocatalysts. Their application directly at the WWTP can increase their economic potential, for example, by being used as a bioflocculant or adsorbent with catalytic activity, replacing commercial products, or as an adsorbent material for pollutant removal from water or for nutrient recovery, such as nitrogen and phosphorus [4].

According to Schambeck et al. [5], there is potential for biopolymer recovery from flocculent biomass in activated sludge systems due to the high sludge production of these systems. The extraction of biopolymers from the residual biomass of wastewater treatment systems emerges as an option for recovering a high-value-added product with a wide range of applications [6]. Biopolymer recovery represents an innovative concept for valorizing carbonaceous material instead of converting it into energy. According to the bioproduct recovery value pyramid, biomass should ideally first be transformed and recovered as a biomaterial (highest value) before being destined for final energy use (lowest value), such as its conversion into biogas or other forms of energy [7].

Studies, including those by Sun et al. [8] and Zhang et al. [9], have shown that residual activated sludge can be an effective source of EPS with bioflocculant properties, offering a low-cost and sustainable alternative for wastewater treatment. This approach has been considered an alternative to conventional inorganic and synthetic organic flocculants, such as alum, PAC, and commercial polymers, which are associated with drawbacks, including aluminum-related health concerns, the generation of chemically contaminated sludge, and potential toxic or carcinogenic effects from polymeric residues. In contrast, EPS-based bioflocculants are biodegradable, non-toxic, and do not cause secondary pollution, reinforcing their potential as environmentally favorable flocculation agents. Beyond their bioflocculation potential, EPS recovered from activated sludge can also act as a functional matrix for immobilized enzymes, enabling simultaneous pollutant adsorption and catalytic bioconversion.

More recent studies have been exploring the biomolecules present in EPS produced in activated sludge and aerobic granular sludge reactors. EPS produced by biological sludge consists mainly of proteins, humic compounds, polysaccharides, lipids, and nucleic acids. Adsorption and enzymatic bioconversion are the two main phenomena involved in pollutant removal by activated sludge, both of which occur within the EPS matrix. The diversity of functional groups present in EPS facilitates the adsorption of nutrients, organic micropollutants, heavy metals, and other pollutants of interest. The adsorption of these pollutants also increases their concentration and brings these molecules closer to the active sites of extracellular enzymes, facilitating enzymatic catalysis and their bioconversion [10].

Frølund et al. [11] studied the enzymatic activity contained in the EPS of activated sludge and found that extracellular enzymes are considered an integral part of this polymeric matrix, where they remain immobilized. Large molecular substrates/pollutants such as cellulose, starch, polypeptides, lipoproteins, glycoproteins, and fats are degraded upon contact with the matrix and can be absorbed by the microbial biomass. EPS not only protects enzymes against degradation but also allows proximity between their active sites and substrates. EPS acts as an immobilization agent for extracellular enzymes

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produced by the microbial biomass. These extracellular enzymes are retained within the EPS, interacting with polysaccharides and becoming immobilized, which increases their thermal stability and resistance to proteolysis [12]. Enzyme immobilization in the EPS matrix prevents their loss to the surrounding medium (mixed liquor), where they could be easily degraded by environmental conditions. Extracellular enzymes also appear to play an important role in degrading the EPS structure itself, to some extent, to maintain its porosity and, consequently, its high capacity for pollutant adsorption. Recovering EPS while preserving enzymatic activity can support the development of biotechnological products such as bioflocculants or catalytic adsorbents for environmental applications. According to Wasmund et al. [13] and Flemming et al. [14], carbohydrate-active enzymes, proteases, and lipases are key constituents of the EPS matrix.

Nabarlatz et al. [15] evaluated the activity of lipase extracted from activated sludge, using ultrasonication as an extraction method. The authors obtained 21 U/g sludge, highlighting that the obtained enzymatic extract could be used as an additive to enhance anaerobic digestion processes for biogas production. Liu and Smith [16] suggested that enzymes extracted from residual biomass from biological treatment processes could serve as an alternative and low-cost enzyme source for various environmental and industrial applications. The authors identified cellulase (13.5 U/g sludge), protease (8.4 U/g sludge), lipase (21 U/g sludge), and amylase activities (39 U/g sludge), using ultrasonication as the extraction method. Toja Ortega et al. [17] evaluated the hydrolytic activity of enzymes responsible for the hydrolysis of complex substrates during the anaerobic phase of a full-scale sequencing batch reactor. The authors state that the hydrolysis of particulate COD is the rate-limiting step for the bioconversion of complex substrates. According to these authors, flocculent sludge has higher hydrolytic activity compared to granular sludge.

Despite the well-documented bioflocculation capacity and enzymatic activity of EPS, these properties have so far been investigated separately, limiting the development of multifunctional bioproducts for wastewater treatment applications. In this context, the objective of this study was to recover EPS extracted from activated sludge of a full-scale WWTP and evaluate its potential as a bioflocculant with catalytic properties, aiming at a bioproduct applicable to sludge flocculation and biodegradation.

2. Materials and Methods

2.1. Materials

For the extraction of EPS, anhydrous sodium carbonate (purity 98.5%), supplied by Êxodo Científica (Sumaré, Brazil), was used. The evaluation of amylase enzymatic activity was carried out using isopropyl alcohol (isopropanol, purity 99.5%), 3,5-dinitrosalicylic acid (DNS, purity 99%), soluble starch (purity 99%), sodium chloride (purity 99%), glucose (purity 99%, supplied by LAFAN, São Paulo, Brazil), and maltose (purity 92%), with all reagents supplied by Êxodo Científica, unless otherwise specified. For buffer solution preparation, dipotassium phosphate (purity 92%), phosphoric acid (purity 85%), and sodium hydroxide (purity 98%, supplied by ALPHATEC, Rio de Janeiro, Brazil) were employed. The evaluation of lipase enzymatic activity was performed using p-nitrophenyl palmitate (purity 98%, supplied by Sigma-Aldrich, Waltham, MA, USA), together with Triton™ X-100 (purity 90%), tris(hydroxymethyl)aminomethane (Tris, purity 99%), and gum arabic (purity 99.5%), all supplied by Êxodo Científica.

2.2. EPS and ALE Recovery from Residual Biomass

Activated sludge (AS) samples were obtained from the aerobic treatment unit of a municipal WWTP operated by CASAN (Santa Catarina Sanitation Company, Florianópolis,

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Brazil). The facility, known as the Lagoa da Conceição WWTP, is located in Florianópolis, Santa Catarina, and is designed to treat a flow of 50–73 L/s. The biological process consists of a continuous-flow oxidation ditch activated sludge system configured for extended aeration. The mixed liquor sample was collected in the aeration tank and further centrifuged to remove the water. EPS was recovered following a modified version of the alkaline extraction protocol described by Felz et al. [18]. Briefly, 7.5 g of sludge was suspended in 150 mL of a sodium carbonate solution prepared with 0.625 g of Na_2CO_3 and subjected to constant agitation at 400 rpm at temperatures of 50 °C and 80 °C. After 35 min of extraction, the mixture was cooled to room temperature and subsequently centrifuged at 3000 rpm for 25 min; the supernatant containing solubilized EPS was collected while the solid fraction was discarded. The extracted EPS was subsequently used for physicochemical characterization, enzyme activity assessment, and bioflocculation experiments

Gelation tests were conducted to assess the hydrogel-forming capacity of alginate-like extracellular polymers (ALE) extracts obtained from EPS recovered at 50 °C and 80 °C. ALE was extracted via alkaline extraction with Na_2CO_3, followed by centrifugation (2150× *g*, 25 min) and dialysis. The dialyzed extract was acidified with 1 M HCl under gentle stirring (100 rpm, room temperature) to a final pH of 2.2 ± 0.05, yielding acidic ALE, which was subsequently recovered by centrifugation (4000× *g*, 4 °C, 20 min). The acidic gel was neutralized with 0.5 M NaOH to pH 8.5 to obtain Na-ALE. Ionic hydrogel formation was evaluated by slowly dripping the Na-ALE solution into a 2.5% (*w*/*v*) $CaCl_2$ solution. The formation of spherical Ca^{2+}–ALE beads was taken as evidence of ionic hydrogel formation [5].

2.3. Enzyme Activity Analyses

EPS samples collected on different dates were used for EPS extraction and for the evaluation of amylase activity. Lipase activity was assessed in a single EPS sample as a preliminary screening. Lipase activity was determined [19] through a spectrophotometric assay, employing p-nitrophenyl palmitate (p-NPP) as the substrate. During the reaction, the hydrolysis of p-NPP by the extracellular lipase promotes the release of p-nitrophenol, which, in an alkaline medium, exhibits a characteristic yellow coloration.

Amylase activity was determined based on starch hydrolysis and the quantification of reducing sugars released using the 3,5-dinitrosalicylic acid (DNS) colorimetric method. Initially, the buffers, the substrate solution, and a maltose standard curve were prepared and used as references for the analysis. For the evaluation of enzyme stability, a portion of the EPS was stored at 5 °C for six months. After this period, the EPS was brought to room temperature, and the amylase enzymatic activity was evaluated by incubating the enzyme with the starch solution at 37 °C for 10 min, followed by the addition of the DNS reagent as the colorimetric agent. The samples were boiled, cooled, diluted in distilled water, and analyzed at 540 nm using a spectrophotometer (DR3900, HACH, Jundiaí, Brazil). Amylase activity analysis was performed in triplicate.

Experiments were performed in triplicate (n = 3), and the results were expressed as mean values. Statistical analyses were carried out using analysis of variance (ANOVA), and mean comparisons were conducted using Tukey's test at a 5% significance level ($p < 0.05$). All statistical procedures were performed using Origin® 2017 software.

2.4. Polysaccharide and Protein Concentration in EPS

The concentration of polysaccharides in the extracted EPS was quantified by an adapted colorimetric assay using anthrone reagent [20]. Calibration curves were prepared using glucose (neutral sugars) and glucuronic acid (uronic acids) as standards. EPS samples were diluted (1:10) and analyzed in triplicate. Absorbance readings were performed in a spectrophotometer (HACH DR3900, Brazil) at 560 and 620 nm, and results were

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expressed as glucose equivalents (mg GLU/g sludge) and glucuronic acid equivalents (mg GLA/sludge).

Protein content in the EPS was determined using the modified Lowry method [21]. Calibration curves were prepared with bovine serum albumin (BSA) for protein quantification and humic substances to account for potential interference. EPS samples were analyzed in triplicate with a 1:10 dilution factor. Absorbance was measured at 750 nm in a spectrophotometer (HACH DR3900, Brazil). Final protein concentrations were expressed in mg BSA/g sludge after correction for humic substance interference [21].

2.5. Bioflocculation Properties of EPS

The jar tests were performed using six square-based jars with a volume of 2 L, each with six rotating propellers providing equal agitation and rest conditions for all jars. The experimental procedure for each jar test began with an intense mixing phase at 120 rpm for 15 s, followed by slow mixing at 50 rpm for 30 min, and subsequently a 15 min settling period. After the settling time, supernatant and settled solids samples were collected for further analysis.

Each jar was loaded with 1 L of activated sludge mixed liquor, which exhibited an initial pH of 6.73 and a temperature of 25.7 °C. The EPS volumes applied in the jars varied as follows: 0, 50, 100, 150, 200 and 250 mL.

Sludge settling velocity was determined by transferring 1 L of homogenized sample from each jar into graduated cylinder and monitoring the sludge blanket height at fixed time intervals (0–30 min). The sludge volume index (SVI) was calculated according to Standard Methods [22].

The Buchner funnel test was performed according to Lo et al. [23] and Zhang et al. [9]. This test is used to determine the Specific Resistance to Filtration (SRF) and the Filtration Time (FT) in order to identify dewatering characteristics of the treated effluent.

The oxygen uptake rate (OUR) test was performed to evaluate the impact of EPS on microbial respiration and on potential oxygen transfer limitations in the activated sludge mixed liquor, given that one possible application of EPS is its use as an additive to improve activated sludge bioflocculation. OUR measurements were conducted as described in [24]. For this procedure, the mixed liquor sample was aerated for 24 h without the addition of an exogenous substrate in order to reach the endogenous respiration phase. Glucose ($C_6H_{12}O_6$, 100 mg/L) and NH_4Cl (50 mg/L) were used as substrates for heterotrophic and autotrophic biomass, respectively, and allylthiourea was added as an inhibitor of autotrophic bacteria. Biomass fractionation was calculated according to Activated Sludge Model 1 (ASM1) [25].

3. Results and Discussion

3.1. EPS Recovery Yield and Biochemical Composition

The EPS extraction yields using 50 °C and 80 °C were 196.3 ± 38.2 mg EPS/g sludge and 283.5 ± 85.4 mg EPS/g sludge, respectively. The reduction in the recovery yields suggests that the extraction temperature influences recovery efficiency. Although higher temperatures increase the recovery yields, labile biomolecules like enzymes would suffer denaturation, and this was hypothesized in this work.

Schambeck et al. [5] found recovery yields for activated sludge flocs of 472 mg EPS/g sludge using the same extraction procedure at 80 °C. Higher recovery yields were found by the authors using aerobic granular sludge (495 mg EPS/g sludge), which is inherent to the structure of granules compared to activated sludge.

De Bruin et al. [26] analyzed the recovery of EPS from 16 Activated Sludge reactors located in 13 countries using the same alkaline extraction protocol with 80 °C used in

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this work and found yields varying from 28.1 to 185 mg EPS/g sludge. Li et al. [27] presented EPS and biopolymer recovery yields from 90 to 190 mg EPS/g sludge from activated sludge biomass. Future improvements could include adjustments in extraction temperature, pH control, or sequential recovery steps aimed at enhancing the solubilization of high-molecular-weight biopolymers without compromising their structural integrity.

Besides higher extraction yield, higher temperature (80 °C) also favored the polysaccharide and protein concentration (Figure 1). The glucuronic acid is the monomer responsible for the hydrogel properties, and its concentration increased 1.5 times when the temperature was higher. According to the hydrogel test shown in Figure 2, EPS beads extracted at 80 °C were more compact and structurally stable than those extracted at 50 °C, supporting the influence of glucuronic acid concentration. Polysaccharides, quantified as glucose and glucuronic acid equivalents, constituted a significant fraction of the EPS composition. This composition is consistent with previous studies reporting the prevalence of proteins and uronic sugars in EPS matrices. Schambeck et al. [5], using a comparable EPS extraction procedure at 80 °C, reported concentrations of 137 mg/g sludge for proteins, 18 mg/g sludge for glucose, and 48 mg/g sludge for glucuronic acid in activated sludge biomass.

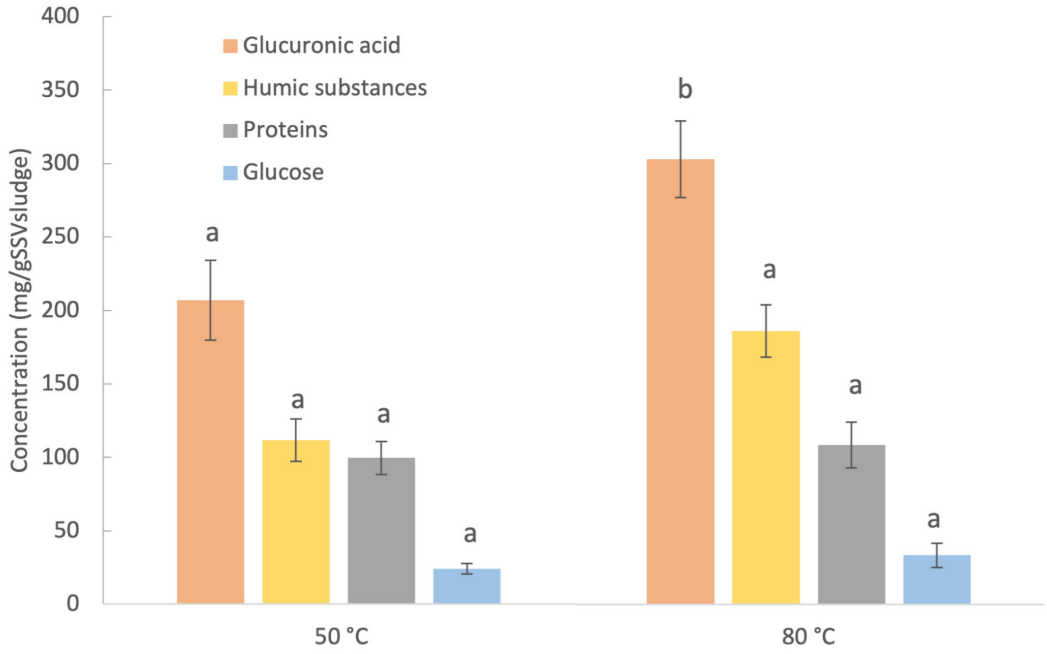

Figure 1. EPS biochemical composition from activated sludge after extraction at 50 °C and 80 °C. Bars represent mean values ± standard deviation (n = 3). Different letters indicate statistically significant differences between extraction temperatures according to Tukey's test ($p < 0.05$).

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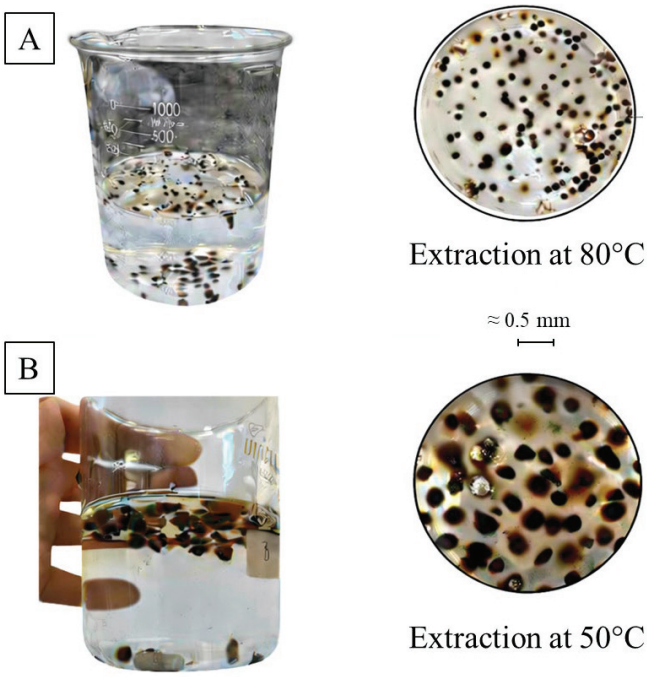

Figure 2. Hydrogel-forming properties of EPS recovered from activated sludge after extraction at 80 °C (**A**) and 50 °C (**B**).

One-way ANOVA revealed a significant effect of extraction temperature on glucuronic acid concentration ($p < 0.05$), with higher values obtained at 80 °C. No significant differences were observed for glucose or protein concentrations between temperatures ($p > 0.05$). Humic substances showed a borderline effect of temperature ($p \approx 0.05$), indicating a tendency toward higher values at 80 °C.

The ability of the extracted EPS to form hydrogels in the presence of divalent cations was demonstrated by the formation of stable beads upon contact with calcium chloride solution. This gelation behavior confirms the presence of alginate-like exopolymers (ALE), which are known to play a key role in EPS structuring and flocculation performance. The identification of ALE is particularly relevant to this study, as these polymers are directly associated with calcium-mediated cross-linking mechanisms that enhance particle aggregation and sludge settleability. Moreover, the extraction temperature significantly influenced the biopolymer composition and, consequently, the physicochemical properties of the recovered EPS. The predominance of proteins suggests an enhanced binding capacity through amino and amide functional groups, which promote electrostatic interactions and polymer bridging during bioflocculation. In parallel, the presence of humic substances may contribute to additional structural stability, given their resistance to biodegradation and their role in increasing EPS persistence within sludge systems [5]. From a functional perspective, humic substances may also affect the enzymatic activity associated with the EPS matrix, as these compounds are known to bind extracellular enzymes, stabilizing them while partially reducing their catalytic efficiency [28]. Overall, the combined presence of ALE, proteinaceous components, polysaccharides, and humic substances provides a coherent explanation for the observed bioflocculation performance of the EPS, supporting its potential application as a multifunctional bioproduct in wastewater treatment systems.

3.2. Enzyme Activity

Enzymatic analyses can help determine whether reducing the extraction temperature is an effective strategy to preserve the biological activity of proteins embedded within the EPS matrix. At a 5% significance level, the ANOVA and Tukey test results indicate that varying

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the temperature between 50 °C and 80 °C does not promote statistically significant changes in amylase activity. A high degree of intragroup variability was observed, which may have contributed to the absence of a significant temperature effect. Moreover, extraction at 80 °C did not reduce enzyme activity as would be expected due to thermal denaturation. Instead, amylase exhibited a degree of thermostability, given the absence of significant differences among replicates and between the 50 °C and 80 °C extraction temperatures.

This apparent thermostability of amylase, evidenced by the lack of significant differences between the 50 °C and 80 °C extraction temperatures (Figure 3), is consistent with previous findings reported in the literature. Liu and Smith [29] evaluated the use of ultrasonication for the extraction of amylase, protease, and cellulase from waste activated sludge and reported a maximum amylase activity of 52.2 U/g VS in samples collected from the aeration tank. Notably, comparable enzymatic activities were also observed in samples obtained from other stages of the treatment line, such as the thickening belt. These results support the hypothesis that enzymes remain protected within the EPS matrix throughout the treatment process, which may mitigate thermal and operational stresses and enable effective enzyme recovery even from thickened sludge. Florianópolis

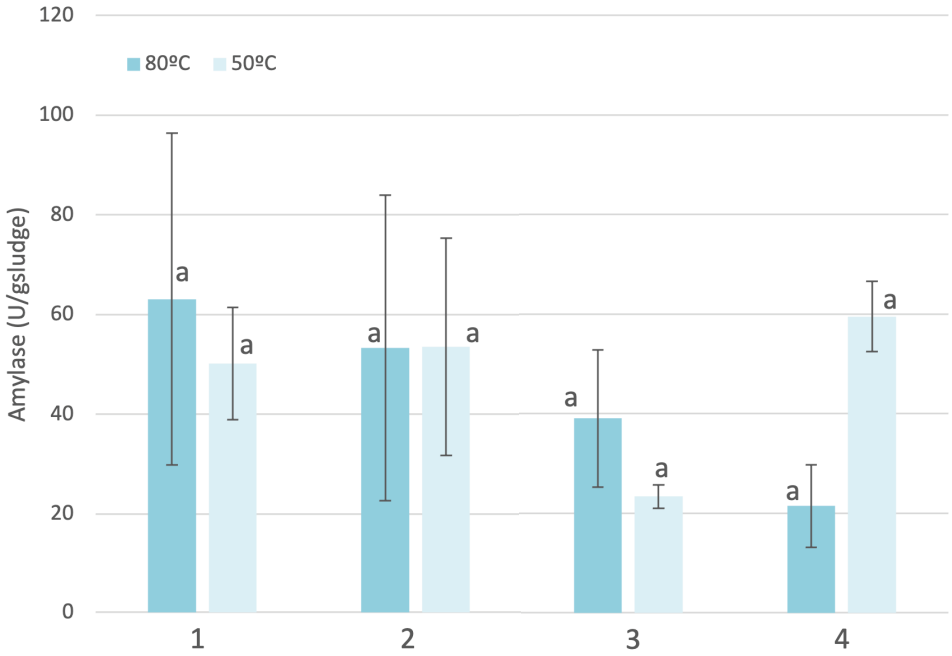

Figure 3. Specific amylase activity measured in EPS fractions extracted at 50 and 80 °C. Bars represent mean values ± standard deviation (n = 3). Same letters indicate not statistically significant differences between extraction temperatures according to Tukey's test ($p < 0.05$).

Yu et al. [30] evaluated several enzyme extraction procedures for activated sludge biomass, including ultrasonication, EDTA, formaldehyde, formaldehyde combined with ultrasonication, formaldehyde combined with NaOH, and cation-exchange resin. The authors reported amylase activities ranging from 11.4 to 14.9 U/g VS. Most of the amylase activity was recovered in the loosely bound EPS fraction, suggesting that carbohydrates present in the bulk liquid are adsorbed and subsequently degraded by amylases located in this compartment. These amylase activities were related to the loosely bound EPS obtained after the extraction procedure, similar to that obtained in this work.

Ortega and co-workers [31] studied amylase and protease activities in aerobic granular sludge through fluorescent activity assays. Amylase enzyme activity was identified in the outer layer of granules, indicating their relation to the substrates that will be hydrolyzed and the substrate mass transfer limitations.

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The EPS was stored frozen for six months, after which the stability of the amylase was evaluated. According to Figure 4, approximately 55% of the enzyme activity was lost during the freezing period for the EPS extracted at 50 °C, and about 45% was lost for the EPS extracted at 80 °C. The thawing process may have caused EPS precipitation and potentially exposed the amylase enzymes to denaturation.

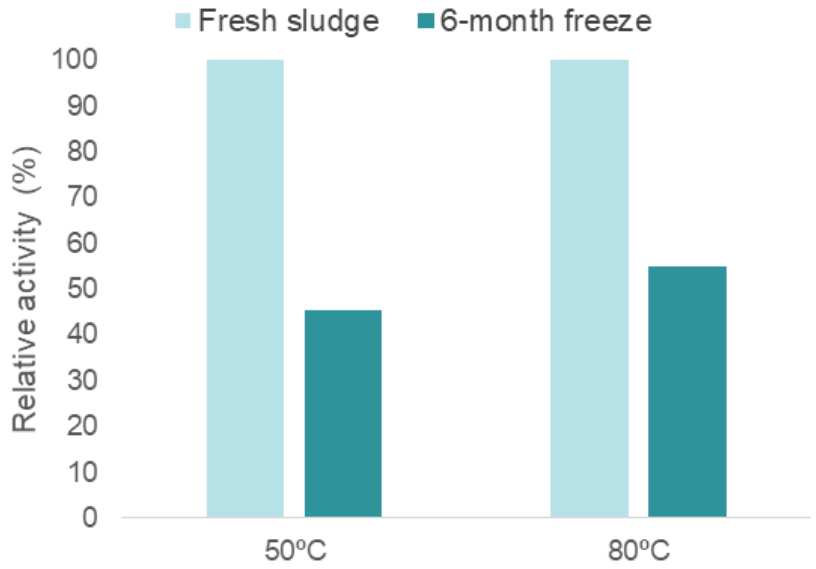

Figure 4. Effect of six-month frozen storage on specific amylase activity.

Lipase activity was measured in a single sample extracted at 50 °C, resulting in 20.8 U/g Sludge. The obtained activity is in the range of other authors' findings. The hydrolysis of complex substrates in aerobic granular sludge was studied by Toja Ortega et al [17]. The authors found that higher hydrolytic activities were found in flocculent biomass in comparison to the granular fraction. In the mixed sludge fraction the lipase activity of 34 μmol pNP/g sludge/h. Nabarlatz et al. [15] found lipase activities ranging from 2.3 to 22.9 U/g sludge, according to the extraction procedure, which included dialysis, precipitation and lyophilization. Gessesse et al. [32] showed that different extraction factors affect the lipase activity from activated sludge flocs, from 108 to 335 U/g sludge. High lipase activity was obtained using the non-ionic detergent Triton-X.

The majority of studies express enzymatic activity on a sludge mass basis. However, considering that EPS is intended to be used as a bioproduct in its liquid form, reporting activity on a volume basis becomes more appropriate. Moreover, EPS was applied in its liquid form in the bioflocculation tests. Future investigations may determine whether the enzymes embedded within the EPS matrix can withstand drying processes, such as spray drying or lyophilization, for the preparation of a dry product.

Biological macromolecules are abundant in domestic wastewater and can be found as particulates such as cellulose fibers, microbial cells, or colloidal and soluble polymers [17]. Raunkjær et al. [33] originally quantified that lipids and fats may account for up to 40% of the influent COD, while carbohydrates can contribute up to 18%. These ranges were later reiterated by Wasmund et al. [13], who emphasized the importance of these compounds as major nutrient sources in wastewater treatment plants. In this context, the work of Flemming et al. [14] provides a link in that extracellular enzymes embedded in the EPS matrix play a crucial role in hydrolyzing and degrading these macromolecules, thereby driving the biological transformation of organic matter in wastewater treatment systems.

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In line with this enzymatic perspective, recent genomic-based investigations have begun to unravel the microbial potential behind these processes. To better understand microbial functions in WWTPs, Wasmund et al. [13] predicted the secreted proteomes of the wastewater microbiota using more than 1000 high-quality metagenome-assembled genomes (MAGs) derived from 23 Danish WWTPs with biological nutrient removal. This genomic approach enabled the identification of secreted hydrolytic enzymes. Many of the MAGs encoding predicted secreted lipases were affiliated with taxa already known for lipase activity, thereby reinforcing the functional relevance of these predictions and supporting the enzymatic mechanisms previously proposed.

3.3. Flocculation Properties of the EPS

EPS addition markedly influenced the settling dynamics of mixed liquor activated sludge. The highest settling velocity was obtained in jar 6, which received 250 mL of EPS, reaching 4.48 m/h (Table 1). This value was substantially higher than that of the control and confirmed the capacity of EPS to accelerate biomass aggregation and compaction. Correspondingly, the sludge volume index (SVI) improved progressively with EPS dosage, with the best performance also observed in jar 6 (Figure 5). These findings demonstrate that EPS promotes denser flocs and enhances sludge settleability.

Table 1. Effect of EPS addition over settling velocity and SVI.

Jar	EPS Addition (mL)	Settling Velocity (m/h)	SVI
1	0	0.86	118.6
2	50	0.34	161.1
3	100	1.63	56.0
4	150	3.62	39.6
5	200	2.58	50.0
6	250	4.48	35.5

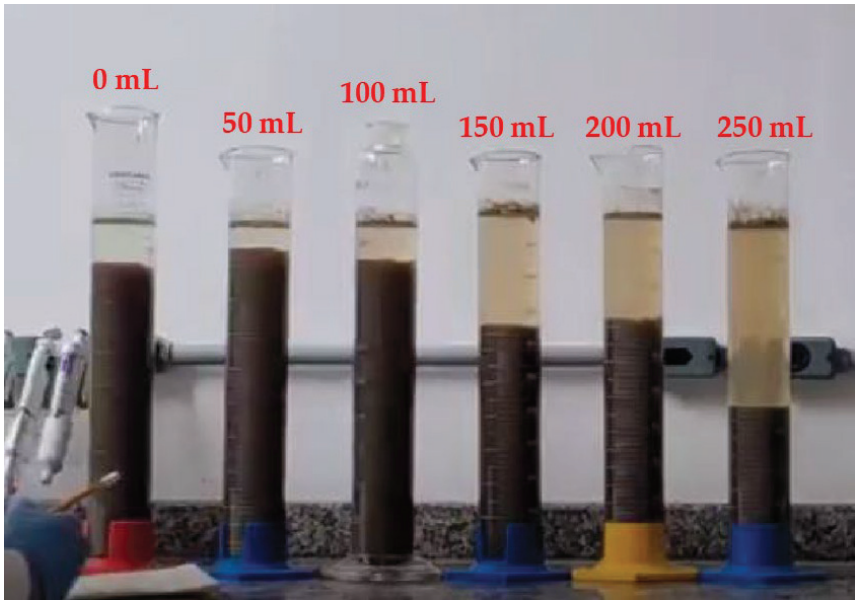

Figure 5. Sludge settling comparison with increasing EPS addition.

When applied to activated sludge mixed liquor, EPS demonstrated a bioflocculant effect, significantly improving aggregation and settling (Figure 6). The addition of EPS led to up to a fivefold increase in settling velocity compared to the control, with consistent

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improvements in the sludge volume index (SVI), which reached values classified as "optimal" under higher dosages for activated sludge mixed liquor. These findings confirm the capacity of EPS to promote stable floc formation and accelerate clarification in biological sludge. However, some side effects were observed. The EPS application increased the turbidity in the supernatant, suggesting the release of organic matter and nutrients during the process. Together, the results highlight the dual role of EPS: while it enhances flocculation and settling performance, its application also alters effluent quality and microbial activity, aspects that must be carefully evaluated for full-scale implementation.

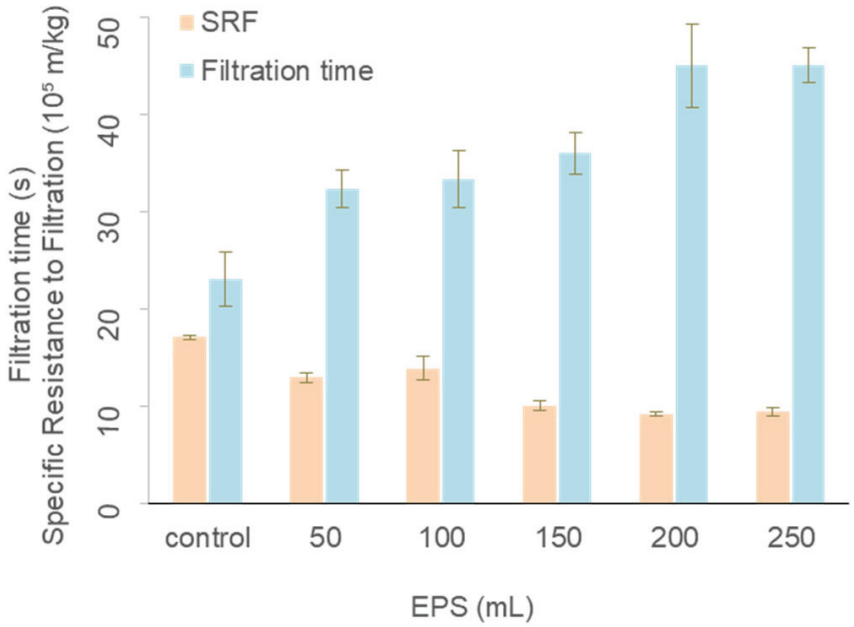

Figure 6. Specific resistance to filtration and filtration time for increasing EPS addition.

The specific resistance to filtration (SRF) allows identifying the influence that the addition of EPS has on the sludge filtration process. The SRF decreased with increasing EPS dosage, indicating improved floc cohesion and reduced passage of fine particles through the filter matrix. Conversely, the time required to filter 100 mL of mixed liquor with EPS after the jar test (filtration time) increased by nearly 50% at the highest EPS concentration compared to the control. This suggests that while EPS enhances particle retention and reduces SRF, it also tends to clog the filter pores, impairing water passage.

Overall, the results indicate that EPS improves settling and compaction of biological sludge but may worsen dewatering when filtration is the primary thickening or dewatering method. This trade-off emphasizes the importance of tailoring EPS application to treatment configurations where sedimentation rather than filtration is the key solid–liquid separation step.

Sun et al. [8] prepared a bioflocculant using residual activated sludge from a Chinese WWTP. The authors disintegrated the sludge with hydrochloric acid and used the supernatant as the bioflocculant in kaolinite clay suspensions, achieving a 99.5% flocculation rate. Zhang et al. [34] tested the use of a microbial bioflocculant produced by Proteus mirabilis TJ-1 screened out from activated sludge biomass. The bioflocculant resulted in SRF up to 27 (10^5 m/kg) and up to 8 min of time to filter during the Buchner funnel test. Compared to the control, the bioflocculant improved the sludge dewaterability, whichwas not observed in this study.

Inorganic coagulants and flocculants, such as aluminum sulfate and polyaluminum chloride (PAC), are widely applied due to their high efficiency in removing organic pollutants. However, their use is associated with important drawbacks, including potential

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health concerns related to aluminum exposure and the generation of large volumes of sludge that require further treatment and disposal. Commercial organic flocculants also pose environmental risks, as their polymeric residues may exhibit neurotoxic or carcinogenic effects [35]. In contrast, natural bioflocculants represent a more sustainable alternative. They are typically biodegradable, non-toxic, and do not lead to secondary pollution, offering economic and environmental advantages over conventional chemical flocculation. Bioflocculants can be produced by various microorganisms, such as *Bacillus*, *Pseudomonas*, *Serratia*, and *Azotobacter*, or recovered directly from mixed and non-sterile microbial cultures like waste activated sludge [36].

Sludge management is a major challenge for wastewater treatment plants due to its high moisture content (around 80%) and the associated operational costs. Consequently, sludge dewatering represents a critical step in sludge disposal [37]. In this context, enzymatic treatment has emerged as a competitive strategy because the sludge matrix is largely composed of extracellular polymeric substances (EPS) produced by bacteria, which can be disintegrated by specific enzymes. Importantly, the enzymes naturally embedded within the EPS matrix may themselves contribute to the degradation of EPS, potentially enhancing sludge disintegration and improving dewatering performance. On the other hand, the addition of exogenous EPS—as a bioflocculant—tends to increase sludge-bound water and structural resistance, thereby hindering sludge dewatering. This trade-off represents a key bottleneck for the practical application of EPS as a bioflocculant, particularly when considering the further dewatering steps required downstream. Nevertheless, if the use of EPS is envisioned as an in situ pre-treatment for bioflocculated sludge prior to its transfer to an anaerobic digester, this approach may still offer advantages, especially by facilitating hydrolysis and enhancing subsequent anaerobic conversion.

3.4. Impact of EPS on OUR

The impact of EPS addition on microbial activity was evaluated through oxygen uptake assays (Table 2). Compared to the control, the maximum specific oxygen uptake rate (OUR) of the sludge decreased by approximately 40% after the addition of 250 mL of EPS. This reduction suggests that EPS may increase the diffusional barrier surrounding microbial cells, thickening the liquid film, and thereby limiting oxygen transfer. Reduced oxygen availability at the cell surface likely explains the observed decline in metabolic activity.

Table 2. Oxygen uptake rate of activated sludge mixed liquor before and after EPS addition.

Sample	OUR ($mgO_2/L \cdot h$)	$OUR_{specific}$ ($mgO_2/L \cdot h \cdot X$)	$X_{Autotrophic}$ (gDQO/L)	$X_{Heterotrophic}$ (gDQO/L)
Sludge	15.13	147.6	15.7	38.8
Sludge + EPS	9.13	129.6		

Although the reduction in OUR was primarily attributed to an increased diffusional resistance caused by EPS accumulation around microbial cells, other mechanisms may also be involved. The addition of EPS can alter the physical and biochemical properties of sludge flocs, potentially affecting microbial accessibility to oxygen and substrates beyond simple diffusion limitations. Furthermore, EPS may induce short-term physiological stress or shifts in microbial community structure, temporarily reducing respiratory activity. Another possible contribution is the consumption of dissolved oxygen by biodegradable organic matter associated with EPS, which may compete with microbial respiration during OUR measurements. While these mechanisms were not individually quantified in the present study, their combined effects highlight the need for comprehensive OUR assessment when EPS is proposed as a multifunctional bioflocculant and catalytic additive in activated sludge systems.

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These results highlight the importance of carefully assessing the oxygen uptake rate of activated sludge when EPS is proposed as an additive. Since EPS is intended to act simultaneously as a bioflocculant and as a catalytic agent, its interaction with microbial metabolism becomes a critical factor for process stability. In wastewater treatment plants, flocculants and catalysts are typically supplied as separate commercial products, each with distinct operational purposes. The possibility of replacing both products with a single EPS-based additive represents a promising technological advancement. However, any benefits associated with improved flocculation and catalytic activity must be balanced against potential adverse effects on microbial respiration. Therefore, OUR measurements are essential to ensure that EPS application does not compromise biological performance, particularly under aerobic conditions where oxygen transfer is a key limiting factor.

4. Conclusions

The recovery of EPS from mixed liquor in activated sludge systems represents a promising strategy for the valorization of residual streams in wastewater treatment plants. Extraction temperature influenced EPS recovery yield and physicochemical characteristics, as well as the associated enzymatic activities. However, statistical analysis indicated that variations between 50 °C and 80 °C did not result in significant differences in amylase activity, suggesting a degree of enzymatic stability within the EPS matrix rather than thermal enhancement or denaturation.

When applied to activated sludge, the recovered EPS exhibited clear bioflocculant behavior. The observed increase in sludge settling velocity and reduction in SVI confirm the potential of EPS to act as a flocculation enhancer, with possible application in aerobic reactors to improve settling performance in secondary clarifiers. In addition, the presence of active enzymes within the EPS matrix highlights its potential added value as a functional additive for sludge pre-treatment prior to anaerobic digestion or for enzymatic hydrolysis processes aimed at resource recovery.

Nonetheless, some limitations were identified. Despite reducing filtration time, EPS addition increased the specific resistance to filtration, which may negatively affect downstream dewatering processes. Moreover, the reduction in oxygen uptake rate suggests that EPS may influence oxygen transfer under bench-scale conditions. These findings indicate that operational trade-offs should be carefully considered prior to full-scale application.

Overall, the recovery of enzyme-active EPS from activated sludge constitutes a viable pathway for producing a functional bioproduct from wastewater treatment residues. This approach aligns with circular economy principles by promoting resource recovery and reintegration within WWTPs, while emphasizing the need for further studies to optimize application conditions and assess process-scale implications.

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processes

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Circular Bioprocessing of *Chlorella* sp. Biomass via *Wickerhamomyces* sp. UFFS-CE-3.1.2 Fermentation for the Production of High-Value Enzymes, Glycerol, and Acetic Acid

Vitória Dassoler Longo [1,2], Marcelli Powzum Amorim [2,3], Nair Mirely Freire Pinheiro Silveira [1,2], Isabely Sandi Baldasso [1,2], Emanuely Fagundes da Silva [1,2], Arielle Cristina Fornari [4], Sérgio L. Alves, Jr. [5], Mateus Torres Nazari [6] and Helen Treichel [1,2,*]

[1] Undergraduate Program in Environmental and Sanitary Engineering, Federal University of Fronteira Sul, Erechim 99700-000, RS, Brazil; vitorialongo22@gmail.com (V.D.L.); nairfreire460@gmail.com (N.M.F.P.S.); isabelysandibaldasso@gmail.com (I.S.B.); emanuelyfagundesdasilva@gmail.com (E.F.d.S.)
[2] Laboratory of Microbiology and Bioprocess, Federal University of Fronteira Sul, Erechim 99700-000, RS, Brazil; marcellipowzum@gmail.com
[3] Graduate Program in Environmental Science and Technology, Federal University of Fronteira Sul, Erechim 99700-000, RS, Brazil
[4] Laboratory of Analytical Tests, Federal University of Fronteira Sul, Erechim 99700-000, RS, Brazil; arielle.fornari@uffs.edu.br
[5] Laboratory of Yeast Biochemistry, Federal University of Fronteira Sul, Chapecó 89815-899, SC, Brazil; slalvesjr@uffs.edu.br
[6] Graduate Program in Civil and Environmental Engineering (PPGEng), University of Passo Fundo (UPF), Passo Fundo 99052-900, RS, Brazil; nazari.eas@gmail.com
* Correspondence: helentreichel@gmail.com

Abstract

The transition to a circular economy and the pursuit of environmental sustainability are driving humanity to develop alternative technologies for producing a range of bioproducts. In this context, microbial-mediated fermentation processes have gained prominence. Although yeasts are well known for their ability to produce alcohols, they can also generate a wide range of value-added bioproducts. At the same time, microalgae emerge as an advantageous unconventional raw material, as their cultivation does not require arable land, thus avoiding competition with food production. To meet this demand, this study aimed to produce biocomposites through submerged fermentation using biomass from the microalgae *Chlorella* sp. Enzymatic hydrolysis was optimized using a 2^2 Central Composite Rotational Design (CCRD), with algal biomass and enzyme mass as independent variables. This step was followed by fermentation with the yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2. The enzyme alpha amylase employed is of commercial origin, commonly used in the brewing industry, characterized by its easy accessibility and lower environmental impact compared to chemical hydrolysis methods. The results demonstrated that the combination of microalgae biomass with the enzyme preparation led to the production of several compounds of interest, such as highly active enzymes, mainly protease (560 U/mL), catalase (3381 U/mL), and peroxidase (277 U/mL), as well as other compounds, such as glycerol (32.5 g/L) and acetic acid (22.8 g/L). These products have wide industrial applications and a strong market demand, reinforcing the potential of the yeast–microalgae synergy for the sustainable production of high-value biocompounds, which represents a matrix of environmentally friendly products.

Keywords: biorefinery; catalase; circular economy; microalgae; peroxidase; protease; yeast

1. Introduction

Microalgae are a low-cost biomass with high potential for the practical production of environmentally relevant compounds. They can serve as energy sources and do not require arable land for cultivation; moreover, they grow rapidly and with relatively low water demands [1,2]. Microalgal biomass is primarily composed of carbohydrates, including polysaccharides such as glycogen, starch, and cellulose, which can be further processed in industry to produce target compounds, such as enzymes, glycerol, and organic acids, including acetic acid [1,3]. According to industry market reports and published surveys, the enzymes market size was USD 5.6 billion in 2023 and is expected to reach USD 10.7 billion by 2032, growing at a compound annual growth rate (CAGR) of 7.5% during the forecast period from 2024 to 2032. Microorganisms dominated the specific enzymes market, accounting for approximately 72%, in 2023 (http://www.globenewswire.com).

Typically, these fermentation processes are carried out using strains of *Saccharomyces cerevisiae*, characterized by rapid growth and reproduction, a short metabolic cycle with minimal byproducts, ease of cultivation and separation, and tolerance to specific concentrations of ethanol, acetic acid, and other inhibitors. However, the exclusive use of *S. cerevisiae* reduces the biochemical complexity of the process, ultimately decreasing the diversity of compounds and biocompounds [4]. Fermentations using non-*Saccharomyces* yeasts, such as *Wickerhamomyces* sp., produce a wide range of specific extracellular enzymes, including proteases, pectinases, cellulases, glycosidases, and lipolytic hydrolases, that act on relevant substrates for the production of alcohol, esters, fatty acids, terpenes, and other metabolites [5–7].

Recent research has increasingly focused on the use of microalgal biomass as a fermentation substrate, providing experimental evidence that algal-derived carbohydrates can be efficiently converted into value-added metabolites by different microorganisms. For example, microalgal hydrolysates obtained from *Chlorella* and *Scenedesmus* species have been successfully fermented by yeasts and bacteria, yielding products such as ethanol, organic acids, and polyols, with performances strongly dependent on hydrolysis efficiency and fermentation conditions. Studies using *Saccharomyces cerevisiae* demonstrated that properly conditioned microalgal hydrolysates can achieve ethanol yields comparable to those obtained from conventional lignocellulosic feedstocks, highlighting the technical feasibility of algal biomass in fermentation-based bioprocesses [8].

Beyond ethanol, microalgae-derived substrates have also been explored for the microbial production of organic acids. Chen et al. reported efficient lactic acid production from *Chlorella vulgaris* hydrolysates using immobilized *Lactobacillus plantarum*, achieving high productivities under optimized operating conditions [9]. In addition, fermentation and wet preservation of microalgal biomass have been shown to promote the formation of short-chain organic acids, with acetic acid frequently identified as a dominant metabolite, reinforcing the suitability of microalgal carbohydrates for acidogenic fermentation pathways [10]. These results demonstrate that microalgae can serve as a versatile carbon source not only for single-target products but also for integrated biorefinery schemes generating multiple biocompounds.

In parallel, increasing attention has been given to the use of non-*Saccharomyces* yeasts to modulate fermentation outcomes and expand product diversity. Several studies have shown that non-conventional yeasts can redirect carbon flux toward metabolites such as glycerol and organic acids, owing to differences in redox balancing, stress tolerance, and enzyme secretion compared with *S. cerevisiae*. For instance, *Starmerella bacillaris* has been associated with enhanced glycerol formation and altered organic acid profiles under controlled fermentation conditions, illustrating how yeast selection can be strategically employed to tailor metabolite production [11,12]. Although many of these investigations

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are conducted in food- or beverage-related systems, the underlying metabolic principles are directly transferable to bioprocesses based on microalgal substrates.

Glycerol is a simple polyol widely used in the food and pharmaceutical industries for the production of cosmetics, paints, medicines, paper, textiles, leather, and explosives [13]. It is expected to become a key platform compound for the development of biopolymers and other high-value chemicals [14]. An alternative method for glycerol production involves microbial cells converting carbohydrates or CO_2 into glycerol. New strains of yeast, algae, and cyanobacteria, found in nature or genetically modified, can be used [15]. Under anaerobic conditions, glycerol production is consistently associated with the generation of byproducts, including ethanol, acetate, acetic acid, and acetoin, all of which have industrial value [16]. Acetic acid is an essential organic acid, widely used in the food, pharmaceutical, and chemical industries [17]. It is produced through an initial fermentation step and prepared from starchy or sugary raw materials [18]. Other compounds are generated during fermentation, such as enzymes, which can be applied in various areas, including sanitation, energy production, herbicide production, and hydrolysis [19].

Despite the growing body of literature on microalgae-based fermentation, there remains a lack of studies that systematically integrate microalgal biomass hydrolysis, fermentation with non-*Saccharomyces* yeasts, and operation in a controlled stirred-tank bioreactor (STR). In particular, limited attention has been given to how alternative enzymatic hydrolysis strategies affect downstream fermentation performance and the formation of targeted biocompounds, such as glycerol, organic acids, and enzymes, during scale-relevant bioreactor operation. Moreover, while non-*Saccharomyces* yeasts are increasingly recognized for their metabolic versatility, their application as biocatalysts in microalgae-based fermentation systems remains underexplored. The use of *Wickerhamomyces* sp., therefore, represents an additional distinguishing feature of this study, as this genus combines fermentative capacity with extracellular enzyme production and metabolic traits that may favor the formation of diverse biocompounds from complex substrates.

The integration of biotechnology with microalgal biomass has strong potential to contribute to the development of a low-carbon circular economy by enabling the conversion of renewable resources into multiple value-added products. Within this context, the novelty of the present work lies in combining an alternative enzymatic hydrolysis approach with fermentation optimization in a controlled stirred-tank bioreactor using *Wickerhamomyces* sp. as the production microorganism. Therefore, this study aimed to optimize fermentation performance for the production of targeted biocompounds from microalgae-derived substrates under STR conditions, providing insights into process behavior and metabolite formation relevant to the design and scale-up of microalgae-based bioprocesses.

2. Materials and Methods

2.1. Microalgal Biomass

Commercial microalgae of the genus *Chlorella* sp. were purchased from a commercial supplier (Brasbol, lot 10271, Sao Paulo, Brazil). They were stored at −60 °C until use. The moisture content was measured using a moisture analyzer to standardize the residual moisture in the biomass. According to the supplier, the microalgae biomass contains 2 g of carbohydrates, 5 g of protein, and 1.3 g of total fat per 10 g of biomass (https://www.zonacerealista.com.br, accessed on March, 2025).

2.2. Pretreatment and Saccharification

To optimize enzymatic saccharification, an experimental design was conducted with algal biomass mass and the volume of commercial alpha-amylase (200 U/mL) as inde-

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pendent variables. The experimental design was performed using a Central Composite Rotational Design (CCRD) (Table 1), with total reducing sugars as the response variable.

Table 1. CCRD for saccharification (absolute and coded values).

Assay	Mass (g)	Volume of Enzyme (mL)/Unit Concentration (U)
1	20 (−1)	2/200 (−1)
2	20 (−1)	7/1400 (1)
3	40 (1)	2/200 (−1)
4	40 (1)	7/1400 (1)
5	30 (0)	0.96/192 (−1.41)
6	30 (0)	8.04/1608 (1.41)
7	15.86 (−1.41)	4.50/900 (0)
8	44.14 (1.41)	4.50/900 (0)
9	30 (0)	4.50/900 (0)
10	30 (0)	4.50/900 (0)
11	30 (0)	4.50/900 (0)

The procedure began with a physical pretreatment to release carbohydrates from the microalgae. The experimental biomass masses (Table 1), corrected for moisture content, were diluted to 100 mL in 0.2 M sodium phosphate buffer (pH 5.5). The solution was frozen at −20 °C for 24 h, then incubated at 4 °C for 24 h, and finally placed in a thermostatic water bath at 100 °C for 10 min, as described by Rempel et al. [20].

Subsequently, saccharification was performed by adding α-amylase to the microalgal biomass (Table 1) and maintaining the mixture at 50 °C and 150 rpm in an orbital shaker. Samples were collected to determine available reducing sugars using the 3,5-dinitrosalicylic acid (DNS) method [21].

2.3. Microorganism and Inoculum

The yeast used in fermentation was *Wickerhamomyces* sp. UFFS-CE-3.1.2 [22]. It was maintained in YPD growth medium, composed of 1% yeast extract, 2% peptone, 2% glucose, and 2% agar. The strain was transferred to liquid YPD medium (without agar) and incubated at 30 °C for 24 h before inoculation into the wort at 10% (*v*/*v*) [23].

2.4. Shake-Flask Fermentation

The wort, with a volume of 250 mL and a working volume of 100 mL, was adjusted to pH 5.0. After inoculation, the experiments were maintained at 30 °C and 120 rpm for 48 h. Subsequently, the wort was subjected to fermentation with *Wickerhamomyces* sp. UFFS-CE-3.1.2 [24]. Aliquots of 2.5 mL were collected at 0, 12, 24, and 48 h for quantification of sugars, glycerol, carboxylic acids, and ethanol.

2.5. Scale-Up Fermentation

To scale up the process and assess the impact of volume on compound production, the experiment yielding the best results was fermented in a stirred tank reactor (STR) (BIO TEC, Tecnal, Piracicaba, Brazil) with a total volume of 7 L, as shown in Figure 1. A total of 800 g (dry biomass, corrected for moisture) of microalgae was weighed and diluted into 2 L of 0.2 M sodium phosphate buffer (pH 5.5). The wort was autoclaved at 121 °C and 1 atm to sterilize the medium and pre-treat the biomass. Due to the high temperature, the sterilization process also served as a pretreatment step, fractionating the structural polymers to enhance the accessibility of hydrolytic enzymes to carbohydrates in the subsequent step (hydrolysis/saccharification).

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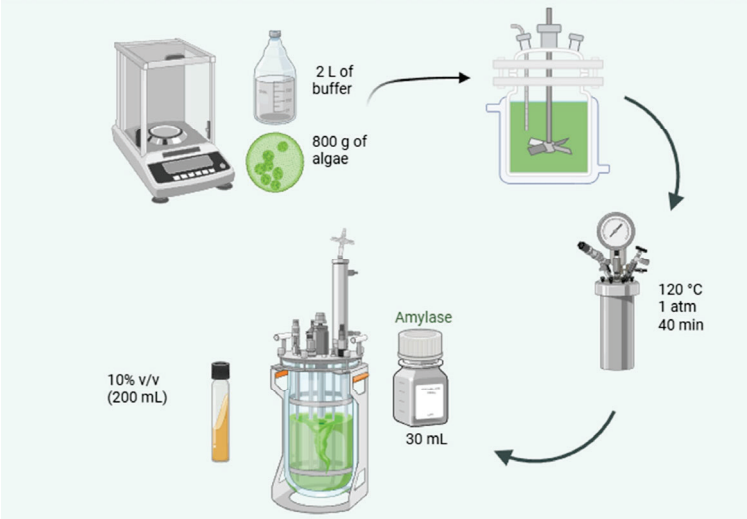

Figure 1. Scale-up of the process using an STR.

Then, 45 mL of the enzyme (activity as specified in Table 1) was added to the bioreactor to initiate saccharification. The amounts of microalgae and enzyme corresponded to the optimal conditions obtained in the shaken-flask fermentation but were scaled up to a final volume of 2 L. An additional 1 L was added, bringing the total to 3 L, to improve homogenization of the medium and facilitate subsequent steps, given the medium's viscosity. After one hour, yeast was inoculated at 10% (v/v) and transferred into the fermentation tank, as described by Bonatto et al. [24]. Fermentation was conducted for 72 h at 30 °C and 80 rpm. Samples were collected at 0, 12, 24, 48, and 72 h for further analysis.

2.6. Enzymatic Analysis

To analyze and quantify the enzymes present in the reactor fermentation, the methods described by Kubeneck et al. [19] were used. Blank assays containing reaction buffer and substrate without cell extract were included in all measurements to account for background signals. For amylase (with starch as the substrate) and cellulase (cellulose as the standard substrate), the methodology adopted was to quantify total reducing sugars, with readings taken on a spectrophotometer at 540 nm using the DNS method [21]. The enzymatic activities of amylase and cellulase were calculated from the glucose standard curve and expressed in units per milliliter (U/mL).

Peroxidase activity, using 8% hydrogen peroxide as the standard substrate, was determined by measuring absorbance at 470 nm and expressed as units per milliliter (U/mL). The substrate for the laccase enzyme reaction was 2,2′-azino-di-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and U was defined as the amount of enzyme capable of forming one µmol of ABTS+ per minute under the reaction conditions. Protease activity with casein as the substrate was determined by spectrophotometry at 660 nm, and U was defined as the amount of enzyme required to release 1 µg of tyrosine per minute under the test conditions.

Catalase activity, using 0.0125 M hydrogen peroxide as the substrate, was measured at 240 nm for 3 min, with readings taken every 30 s, to monitor the formation of oxidized products. For ascorbate peroxidase, a wavelength of 290 nm was used, with readings taken every 15 s for 1 min, using 0.008 M ascorbic acid as the substrate. One unit (U) of catalase activity was defined as the amount of enzyme catalyzing the conversion of 1 µmol of hydrogen peroxide per minute under the assay conditions. Finally, superoxide dismutase activity was measured under light exposure, using a 15 kW fluorescent lamp, except for the control, which was kept in the dark. Enzymatic activity was quantified at 560 nm every

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1 min for 15 s. Superoxide dismutase activity is determined by the amount of enzyme required to inhibit 50% of the photoreduction of NBT (4-Nitro blue tetrazolium chloride).

2.7. Analytical Methods

Quantification of glucose, cellobiose, arabinose, and fructose, as well as ethanol, glycerol, citric acid, and acetic acid, was performed by High-Performance Liquid Chromatography (HPLC). Samples were diluted with 0.005 M sulfuric acid, vacuum-filtered using a Millipore® (Merck Millipore, Burlington, MA, USA) 0.45 μm membrane, and degassed in an ultrasonic bath for 15 min [18]. The chromatographic system consisted of a Shimadzu instrument equipped with a refractive index detector (RID-10A) and an Aminex Biorad HPX-87H column (Shimadzu do Brasil, Barueri, Brazil). Chromatographic conditions were as follows: mobile phase (H_2SO_4), flow rate 0.6 mL/min, and temperature 45 °C [22].

2.8. Statistical Analysis

Statistical analysis of the CCRD 2^2 was performed using Protimiza Experimental Design software (https://experimental-design.protimiza.com.br/, accessed in 2025), with a 95% confidence level ($p < 0.05$). The central point of the experimental design, as recommended by the tool, was used to determine the standard deviation.

3. Results and Discussion

3.1. Optimization of Enzymatic Hydrolysis

The results of the experimental trials demonstrated the high potential of commercial amylase to convert biomass polysaccharides into fermentable sugars, as shown in Table 2, thereby catalyzing the hydrolysis of starch and other α-glucans [25]. In Assay 3, with 40 g of microalgae biomass and 2 mL of enzyme, approximately 31.8 g/L of total reducing sugars (TRS) were obtained after saccharification. This condition was selected for scale-up because it was identified as the most promising for producing compounds of interest through fermentation in a bioreactor. Given the amount of sugar available, this condition underscores the importance of enzyme loading in process feasibility. Among the compounds analyzed, ethanol was produced at low concentrations under the tested conditions, whereas acetic acid reached 23 g/L after 48 h of fermentation. This concentration is technically relevant, as acetic acid is a key platform chemical in biorefinery concepts and can be integrated as an intermediate for downstream valorization routes, rather than being considered solely as a low-value end product [26,27]. In addition, acetic acid is increasingly recognized as a versatile building block for the synthesis of fuels, solvents, polymers, and esters, which supports its inclusion in integrated bioprocess schemes that generate multiple products from renewable feedstocks [26]. Reported acetic acid titers in microalgae-based or mixed-substrate fermentation systems vary widely depending on microorganism and process configuration, typically ranging from a few grams per liter to tens of grams per liter, placing the value obtained in this study within the upper range of reported concentrations for biologically derived acetic acid [27]. Compounds such as citric acid and arabinose were not detected at any time.

Assay 1 showed an attractive sugar content; however, these sugars were not consumed during fermentation, which does not indicate the formation of bioproducts of this consumption, such as sugars and acids. The optimized model was evaluated using Analysis of Variance (ANOVA), as shown in Table 3, where the calculated F-value exceeded the tabulated F-value, and the R^2 value was 86%. These results indicate that the experimental data are well explained by Equation 1, where A represents the enzyme volume, and B represents the algal biomass mass.

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$$\text{TRS}\ (\text{g/L}) = 26.55 - 3.14 \times \text{A} - 8.16 \times \text{A}^2 + 0.41 \times \text{B} - 5.52 \times \text{B}^2 + 4.62 \times \text{A} \times \text{B} \quad (1)$$

where A: enzyme volume; B: microalgal biomass mass

Table 2. Total reducing sugar production from enzymatic hydrolysis.

Assay	Mass (g)	Volume of Enzyme (mL)	Total Reducing Sugars (g/L) *
1	20 (−1)	2 (−1)	29.86
2	20 (−1)	7 (1)	26.36
3	40 (1)	2 (−1)	31.83
4	40 (1)	7 (1)	30.72
5	30 (0)	0.96 (−1.41)	18.14
6	30 (0)	8.04 (1.41)	15.42
7	15.86 (−1.41)	4.50 (0)	23.62
8	44.14 (1.41)	4.50 (0)	30.01
9	30 (0)	4.50 (0)	39.82 **
10	30 (0)	4.50 (0)	34.22 **
11	30 (0)	4.50 (0)	37.46 **

* Standard deviation of each experiment was less than 5% (n = 3). ** Standard deviation of 2.81 g/L.

Table 3. Analysis of variance (ANOVA) for total reducing sugar (TRS) production.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F-Test
Regression	601.7	5	120.3	6.1
Residual	98.6	5	19.7	
Lack of fit	97.7	3		
Pure error	0.9	2		
Total	700.3	10		

Regression Coefficient: R = 0.85; $F_{(0.95;5;5)}$ = 5.05.

Between enzyme concentration and algal biomass, intermediate concentrations were favorable for the process. In this study, the enzyme used was readily available, allowing feasible concentration ranges; however, using smaller amounts of enzyme is preferable. A contour plot was constructed based on the model validation, as shown in Figure 2, highlighting the central region.

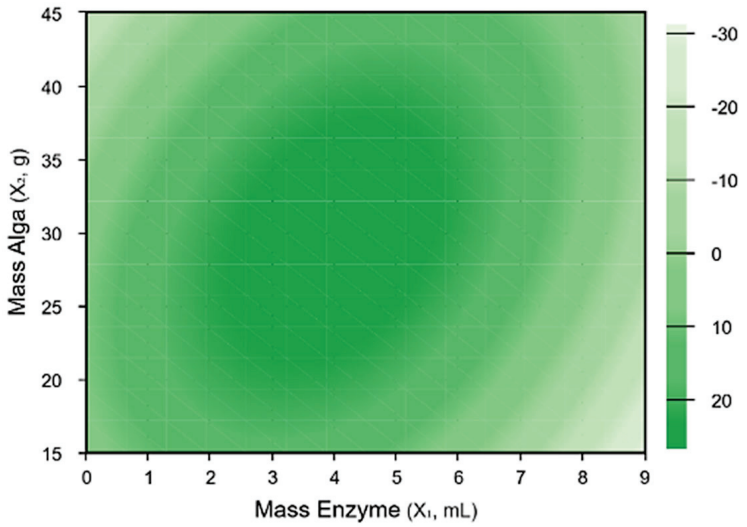

Figure 2. Reducing sugar obtained by enzymatic hydrolysis, represented by the CCRD 2^2 response surface.

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Some assays, such as 5 and 6, demonstrated significant sugar consumption during fermentation, 80% and 84%, respectively. However, in Assay 5, the low availability of sugars released during hydrolysis suggests a potentially lower product yield. On the other hand, Assay 6, which used 30 g of biomass and 8.04 mL of enzyme, achieved considerable sugar conversion; however, its enzyme volume was 4 times that of Assay 3, making the process less economically viable.

As shown in Figure 3, Assay 3 produced 2.44 g/L of glycerol and 0.67 g/L of acetic acid in 12 h, representing the best condition for total reducing sugars (TRS), as it contained 15.84 g/L at the start of fermentation. Next, Assay 4 stands out for producing 32.5 g/L of glycerol in 24 h and 18.86 g/L of TRS, while Assay 1 produced 22.8 g/L of acetic acid in 48 h with 36 g/L of TRS.

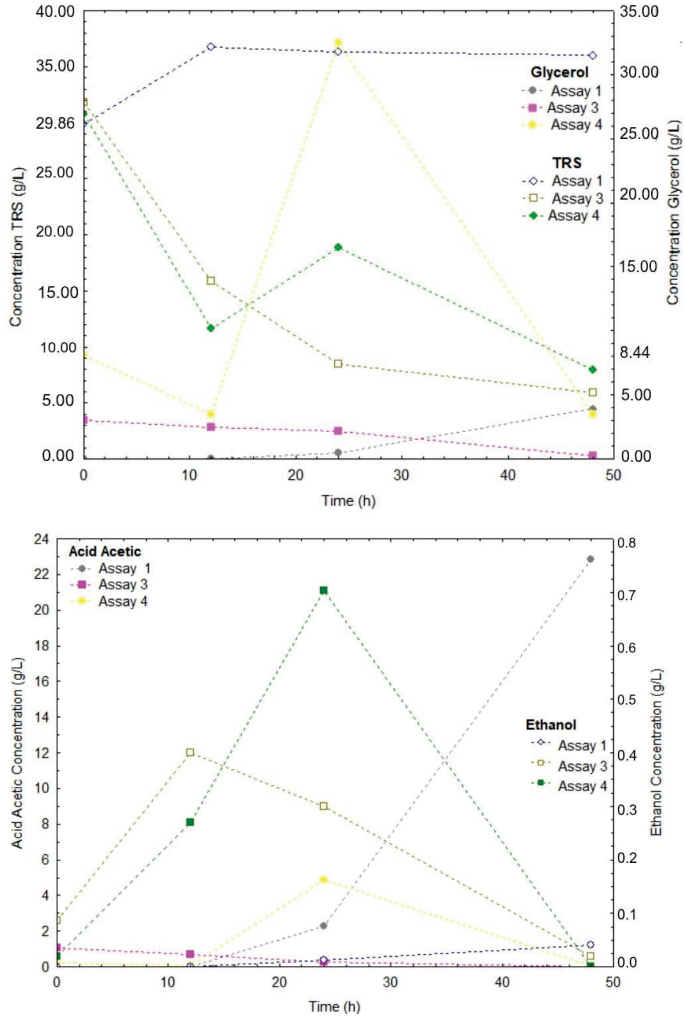

Figure 3. Sugar consumption and production of different compounds during fermentation. Standard deviation of each experiment was less than 5% (n = 3).

Studies demonstrate a relationship between high glycerol production and low ethanol yield, caused by the diversion of dihydroxyacetone phosphate from glycolysis toward glycerol-3-phosphate via the enzyme glycerol-3-phosphate dehydrogenase. This process consumes NADH and reduces the fermentative flux, thereby decreasing ethanol production. However, due to redox imbalance, acetic acid accumulates [28], a primary fermentation inhibitor that impairs yeast growth and metabolism [29,30]. Consequently, in experiment 1, ethanol production was hampered by the high acetic acid concentration resulting from sugar degradation [31].

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3.2. Scale-Up

During scale-up, it was observed that 2 L of buffer was insufficient for fermentation, as the medium became dense, hindering reactor agitation and other operational parameters. Therefore, an additional 1 L of buffer was added before sterilization and fermentation. It is important to note that scale-up is not necessarily linear; bioprocess scaling typically relies on empirical and semi-empirical approaches to maintain consistency in key process variables [32].

The fermentation results are shown in the Supplementary Material (Table S1). The central values found were: 36.34 g/L of TRS at zero h, 2.10 g/L of glycerol at zero h, 1.47 g/L of ethanol at 24 h, and 0.68 g/L of acetic acid at 48 h. The ethanol concentration was lower compared to optimized fermentations with *Chlorella* sp., where values above 10 g/L were reported [33] and up to 20 g/L with efficient pretreatment and the use of *Saccharomyces cerevisiae* [34]. This difference may be related to the limited availability of fermentable sugars.

Regarding acetic acid, the accumulation of 0.68 g/L is considered moderate. In fermentations with *S. cerevisiae* or *Zymomonas mobilis*, concentrations between 5 and 10 g/L already exert strong inhibitory effects, while under extreme conditions (7.5–15 g/L), significant reductions in growth and sugar consumption occur. Thus, the value obtained in this study is below the critical threshold, suggesting it did not substantially compromise fermentation [35–37].

3.3. Evaluation of Enzymatic Activities

This study successfully documented the presence of various enzymes during fermentation in a reactor using the added microorganism. The yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2 has been previously used for ethanol production; however, different studies are expanding the range of products derived from its metabolism. This study highlights its potential for producing a fermented composite rich in enzymes with diverse applications. A significant increase in the production of various enzymes was observed after 24 h of fermentation. This is because decomposition processes drive the synthesis and secretion of enzymes [38].

The results demonstrate that the enzyme composite is rich in catalase, protease, and peroxidase, as shown in Figure 4. Crucial antioxidant enzymes, such as superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase, play a vital role in scavenging free radicals. Catalase is responsible for the degradation or reduction of hydrogen peroxide into water and oxygen [39,40], reaching an activity of 3381 U/mL in 48 h, highlighting that catalase activity was already elevated at the beginning of fermentation when compared to baseline levels typically reported for yeasts under non-stressed or early growth conditions [41]. Catalase is a key oxidative stress-response enzyme in yeasts, and its activity increases in response to reactive oxygen species generated during aerobic cultivation, substrate complexity, or metabolic shifts, which may explain the early activation observed in this study [41,42].

In this study, a protease production of 560.55 U/mL was achieved by the end of fermentation. Protease facilitates the hydrolysis of proteins into amino acids, making them more readily available for absorption [43]. The synergistic action of other enzymes present in the fermentation matrix can enhance protease activity. Cofactors, coenzymes, or activators derived from plant extracts and yeast strains can facilitate protease activation and enhance overall proteolytic activity [44,45]. Egbune et al. [46] reported a protease activity of 39.2 U/mL. Consequently, the biomass and microorganisms used in this fermentation produced a substantially higher protease yield than in other studies. Enzymes such as ascorbate peroxidase, cellulase, and laccase showed consistently low activities (<2 U/mL)

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at all sampling times (Figure 5). Blank assays confirmed that these values were close to background signal levels.

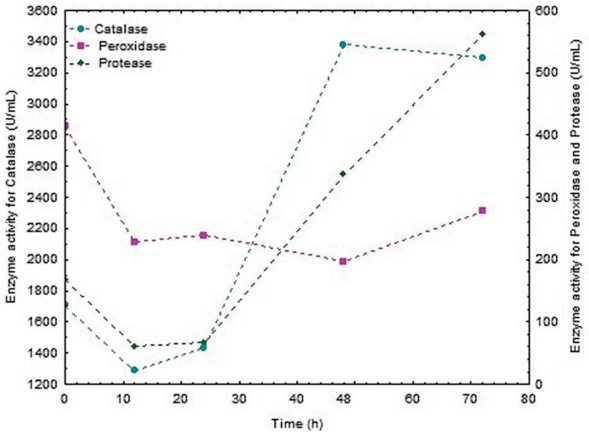

Figure 4. Enzymatic activities during fermentation. Standard deviation of each experiment was less than 5% (n = 3).

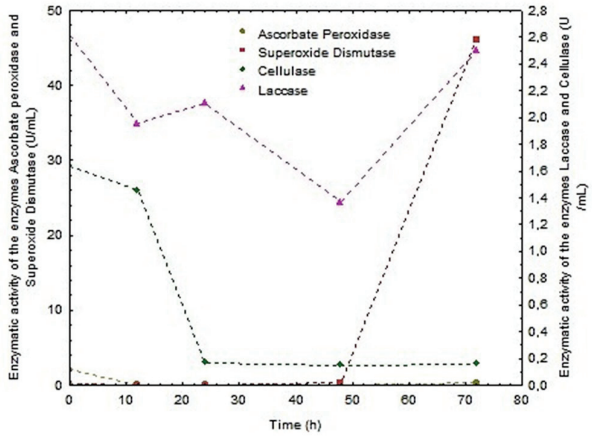

Figure 5. Other enzymes quantified in fermentation. Standard deviation of each experiment was less than 5% (n = 3) (ascorbate peroxidase and superoxide dismutase refer to the left *Y*-axis, and the other enzymes to the right *Y*-axis).

The enzyme superoxide dismutase showed a significant activity level at 72 h, with a value of 46 U/mL. Specifically, this enzyme facilitates the dismutation of the superoxide anion (O_2^-) into O_2 and H_2O_2, while peroxidase limits the accumulation of free radicals in plant cells, mitigating oxidative stress. Ascorbate peroxidase further detoxifies H_2O_2, thereby protecting plant cells from oxidative stress-induced damage [47,48]. For peroxidase, the highest activity was 277.78 U/mL at 72 h of fermentation. This value is based on the initial amount of enzyme present in the must, which was not produced by the microorganism. This enzyme requires organic hydroperoxides or H_2O_2 as co-substrates to stimulate oxidative reactions [33].

4. Conclusions

This study investigated the conversion of microalgae-derived substrates into targeted biocompounds via fermentation with *Wickerhamomyces* sp. in a stirred-tank bioreactor (STR) using an alternative enzymatic hydrolysis strategy. The results demonstrated that microalgal biomass can be effectively converted into a fermentable medium and processed under controlled bioreactor conditions, yielding organic acids, polyols, and enzyme-related activities. The observed fermentation profiles highlighted the importance of integrating

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upstream hydrolysis conditions with downstream fermentation performance to control metabolite distribution.

The use of a non-*Saccharomyces* yeast was a critical aspect of this work, as *Wickerhamomyces* sp. exhibited metabolic versatility when cultivated on microalgae-derived substrates, supporting the concept of diversified bioproduct generation in microalgae-based biorefineries. Moreover, the application of the STR operation enabled a more realistic assessment of process behavior, contributing to the understanding of fermentation performance under scale-relevant conditions.

From a future perspective, further development of this type of research may focus on optimizing operational parameters, such as aeration, agitation, and feeding strategies, to enhance process robustness and selectively steer metabolite formation. In addition, integrating techno-economic analysis and life cycle assessment would provide valuable insights into the feasibility and sustainability of microalgae-based fermentation routes. Exploring different microalgal species, hydrolysis strategies, and microbial systems, including co-cultivation approaches, may further expand the portfolio of value-added products and advance integrated microalgae biorefinery concepts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr14010111/s1, Table S1. Compounds monitored during 72 h STR fermentation.

Author Contributions: Conceptualization: V.D.L. and H.T.; Formal analysis and investigation: V.D.L., M.P.A., N.M.F.P.S., I.S.B., E.F.d.S. and A.C.F.; Writing—original draft preparation: V.D.L., M.P.A., N.M.F.P.S., I.S.B., E.F.d.S. and A.C.F.; Writing—review and editing: M.T.N., S.L.A.J. and H.T.; Supervision: H.T. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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Valorization of Green Arabica Coffee Coproducts for Mannanase Production and Carbohydrate Recovery

Raquel Coldibelli Ribeiro [1,2,†], Leonardo João Bicalho de Moraes de Barros [1,†], Laura Braga de Menezes [1], Claudia Moraes de Rezende [2], Ayla Sant'Ana da Silva [3], Elba Pinto da Silva Bon [1] and Ricardo Sposina Sobral Teixeira [1,*]

[1] Laboratório Bioetanol (LB), Instituto de Química, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro 21941-630, Brazil; raquel.coldibelli@pos.iq.ufrj.br (R.C.R.); leonardojbmb@gmail.com (L.J.B.d.M.d.B.); lauramenezes@eq.ufrj.br (L.B.d.M.); elba1996@gmail.com (E.P.d.S.B.)

[2] Laboratório de Análise de Aroma (LAROMA), Instituto de Química, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro 21941-630, Brazil; claudia.rezendeufrj@gmail.com

[3] Laboratório de Biocatálise, Bioprocessos e Bioprodutos, Divisão de Catálise, Biocatálise e Processos Químicos, Instituto Nacional de Tecnologia, Ministério da Ciência, Tecnologia, Inovações, Rio de Janeiro 70067-900, Brazil; ayla.santana@int.gov.br

* Correspondence: ricardot@iq.ufrj.br; Tel.: +55-21-987524829

† These authors contributed equally to this work.

Abstract

Agro-industrial residues rich in carbohydrates represent low-cost and sustainable feedstock for enzyme production. This study demonstrates that green Arabica coffee press cake, a mannan-rich coproduct of oil extraction, is an efficient carbon source for *Aspergillus niger* (CFAM 1234) cultivation and for inducing mannanase production. Furthermore, the enzymes obtained were tested for mannose recovery in the enzymatic hydrolysis of healthy and defective coffee beans to investigate their hydrolytic potential. Mannanase production was investigated using various carbon sources—including ground coffee beans; coffee press cake; different particle sizes of coffee press cake; aqueous coffee cake extract (prepared at 30 $g \cdot L^{-1}$ under constant stirring (300 rpm) at 80 °C for 2 h, followed by filtration.); and a commercial galactomannan, locust bean gum (LBG). CNHSO analysis was performed in the best carbon source (coffee press cake) and LBG. Statistical optimization (Plackett–Burman and Central Composite Rotatable Design) simplified the culture medium composition to coffee press cake (48.78 $g \cdot L^{-1}$), yeast extract (4 $g \cdot L^{-1}$), and potassium phosphate (0.25 $g \cdot L^{-1}$, pH 5.5) and increased mannanases productivity to 22.4 ± 0.6 $U \cdot mL^{-1}$ within only 3 days (a 42.9% improvement compared to non-optimized conditions, which were 30 $g \cdot L^{-1}$, carbon source, 4 $g \cdot L^{-1}$ yeast extract, 1 $g \cdot L^{-1}$ Al_2O_3, 0.5 $g \cdot L^{-1}$ potassium phosphate buffer (pH 5.5), 0.5 $g \cdot L^{-1}$ of $MgSO_4 \cdot 7H_2O$, and 0.05 $g \cdot L^{-1}$ of $CaCl_2 \cdot 2H_2O$, which resulted in a maximum of ~20 $U \cdot mL^{-1}$ in 7 days). The crude extract also exhibited β-mannosidase activity (1.39 ± 0.06 $U \cdot mL^{-1}$). When applied to the hydrolysis of untreated healthy and defective coffee beans, the enzyme preparation enabled ~25% mannose recovery (considering the value obtained through acid hydrolysis as 100%), highlighting its potential as a mannose resource. The results demonstrate that coproducts from the coffee production chain can be used as an efficient carbon source (coffee cake) for mannanase production, as well as sugar recovery (defective coffee beans), offering an integrated strategy to strengthen the circular bioeconomy and generate carbohydrates with potential industrial and nutritional applications.

Keywords: mannanase; β-mannosidase; *Aspergillus niger*; coffee cake; agro-industrial coproducts; submerged cultivation; enzymatic hydrolysis; mannose

1. Introduction

The inappropriate disposal of agro-industrial waste from agriculture and food processing constitutes a significant environmental problem. However, these residues hold remarkable potential for biotechnology applications, since they are sources of cellulose, hemicellulose, and lignin [1]. Their application in bioprocesses, being used as a carbon source for microbial enzyme production, can simultaneously reduce environmental impacts while adding value to by-products that would otherwise be discarded [2–4]. Moreover, the conversion of agro-industrial residues into high-value-added products is aligned with the concept of the circular bioeconomy, a growing trend in global sustainable development [5], and contributes directly to the United Nations' Sustainable Development Goals (SDGs) 9 (Industry, Innovation and Infrastructure) and 12 (Responsible Consumption and Production) [6].

Among agro-industrial residues, those generated by the coffee industry are particularly noteworthy, given that, according to the International Coffee Organization (2023) [7], only 5% of the coffee pulp remains in the final beverage for consumers. Waste from the coffee sector accounts for approximately 40% of the total production volume, consisting of coffee husks, pulp, mucilage, parchment, silverskin, and coffee grounds [8–11]. In addition, other residues can be considered, such as coffee press cake (which represents approximately 90% of the residual biomass from green coffee oil extraction [12]) and defective beans, which represent up to 20% of green coffee production [13]. Such biomass is often disposed of in landfills or incinerated, which can pose a significant risk to the environment, or incorporated into fodder and animal feed [14].

Brazil, as the world's largest producer and exporter of coffee, generated 55.1 million processed 60 kg bags in 2023 [15], leading to a substantial volume of residues. Arabica coffee (*Coffea arabica* L.), the most widely cultivated species [16], contains about 50% polysaccharides in its dry matter (mainly mannans, galactomannans, and type II arabinogalactans) [17] and 7–17% lipids [16]. Approximately 70% of these lipids are triglycerides, which are widely exploited for coffee oil production [18–20]. Oil extraction by mechanical pressing yields a solid by-product known as coffee press cake [21]. This by-product is rich in the polysaccharide fraction of green coffee, mainly composed of cellulose, type II arabinogalactans, and galactomannans [22]. Due to its high mannan content (making up about 50%) [17,22], a hemicellulose composed of mannose chains linked by β-1,4 glycosidic bonds, this residue represents an attractive carbon source for microorganisms capable of producing mannan-degrading enzymes, such as mannanases (EC 3.2.1.78 and EC 3.2.1.25) [23].

Mannanases constitute a group of enzymes with diverse catalytic action. Endo-β-mannanases cleave internal (1,4-β-D-mannosidic) glycosidic bonds in the main chain of mannans, while β-mannosidases hydrolyze terminal mannose residues, releasing monosaccharides. Additionally, there are other accessory enzymes, including α-galactosidase, which catalyze the hydrolysis of galactose groups from galactomannans, as well as acetyl esterases and feruloyl esterases, which catalyze the hydrolysis of acetyl and ferulic groups, depending on the type of mannan present [23,24]. These enzymes have wide-ranging industrial applications, including juice clarification, wastewater treatment, papermaking, and improving oil and sugar extraction from plant matrices [25]. Furthermore, β-mannanases can perform partial hydrolysis of plant mannans, generating β-mannan-oligosaccharides (β-MOS), which exhibit prebiotic activity that stimulates the growth of beneficial gut bac-

teria in vitro [26–28]. Owing to these properties, mannanases have attracted significant interest from the food and pharmaceutical industries [29].

The production of mannanases can be carried out by different microorganisms, with fungi such as *Aspergillus niger* being among the most efficient producers due to their ability to secrete large amounts of extracellular enzymes [25,30]. This fungus is widely employed in fermentation processes due to its capacity to grow on a wide range of carbon sources [24,31]. Moreover, *A. niger* exhibits high resistance to pH and temperature variations, making it suitable for large-scale industrial applications [3,24], particularly in the production of enzyme cocktails for the degradation of plant polysaccharides into oligosaccharides and monosaccharides [30,32,33].

Submerged fermentation (SmF) is suitable for fungi growth, offering precise control of environmental parameters and nutrient availability, which ensures uniform and efficient microbial growth [3]. Even though SmF is a cultivation in liquid medium, there is the possibility of using solid components. In this context, the use of agro-industrial residues rich in mannan as carbon and energy sources in SmF represents an effective strategy to boost mannanase production. When optimized, SmF can yield significantly higher enzyme levels, requiring studies to define optimal cultivation conditions such as nutrient concentrations, pH, and incubation time [25,34]. Statistical approaches, including Plackett–Burman (PB) design and the Response Surface Methodology (RSM), are particularly useful, as they enable the identification of critical variables and fine-tuning of fermentation parameters to maximize enzyme production [29,35].

There are few studies reporting the production of mannanases using coffee grounds or husks [3,36–38], while green coffee press cake, the solid residue generated after oil extraction, remains largely underexplored for enzyme production, even though it is particularly rich in mannans. Thus, this is the first study to report the use of Arabica coffee press cake as a carbon source for the submerged fermentation of *Aspergillus niger* aimed at mannanase production. Furthermore, few studies address the production of β-mannosidase, which is essential for the complete hydrolysis of mannans into fermentable sugars such as mannose. The use of such low-cost substrates not only reduces enzyme production costs but also provides an environmentally sustainable route for agro-industrial waste valorization [29].

In this context, the present study investigates coffee press cake as a mannan-rich carbon source for submerged fermentation with *Aspergillus niger*, aiming at the induction of endo-β-mannanase and β-mannosidase production. Statistical optimization approaches were employed to enhance enzymatic yield, while the enzymes obtained were further evaluated in the hydrolysis of healthy and defective coffee beans to examine their hydrolytic potential, thus integrating waste valorization, process optimization, and circular bioeconomy opportunities.

2. Materials and Methods

2.1. Materials

2.1.1. Sample Preparation

The green Arabica coffees of the Catuaí Amarelo variety were obtained by semi-mechanized harvesting and post-harvesting by dry processing on a terrace. The coffee beans were obtained from the 2018 harvest from a farm located in São José do Vale do Rio Preto, Rio de Janeiro, Brazil (22°11′35.2″ S, 42°59′8.6″ W).

The coffee beans were ground using a Pulverisette 19 knife mill (Fritsch, Pittsboro, NC, USA) equipped with a 1 mm sieve, followed by lipid extraction through pressing (CA59O, IBG Monforts, Mönchengladbach, Germany) without heating, at 18 rpm, and with a 5 mm outlet diameter, according to a previously reported method for optimizing lipids extraction from green Arabica coffee beans [39]. The solid residue from pressing

(coffee press cake) was reground and subjected to granulometry adjustment using a sieving system (Analysette 3 Spartan, Fritsch, Pittsboro, NC, USA) with 80- and 20-mesh sieves, amplitude of 2 mm, for 15 min.

Ground coffee beans, coffee cake, and commercial galactomannan (locust bean gum, LBG, Sigma-Aldrich, St. Louis, Missouri, USA) were tested as carbon to evaluate their effect as a carbon source for fungal mannanase production under submerged cultivation. The influence of particle size was also investigated by using two coffee cake fractions (80/20 and 20 mesh). In addition, an aqueous extract of coffee cake was also tested as a carbon source, prepared at 30 $g \cdot L^{-1}$ under constant stirring (300 rpm) at 80 °C for 2 h, followed by filtration.

For enzymatic hydrolysis, the coffee beans were ground using a Pulverisette 19 knife mill (Fritsch, Pittsboro, NC, USA) equipped with a 1 mm sieve.

2.1.2. Elementary Organic Analysis (CHNSO) in the Best Carbon Source

Elemental analyses were carried out on the selected carbon source (coffee cake) and on commercial mannan (LBG) for comparison, using an Unicube organic elemental analyzer (Elementar, Germany) equipped with a thermal conductivity detector and using helium as carrier gas. The analysis determined the mass percentage of carbon (C), nitrogen (N), hydrogen (H), sulfur (S), and oxygen (O) and allowed for the calculation of the C/N ratio in the culture media.

2.1.3. Microorganism

The microorganism used was a wild strain of *Aspergillus niger* from the Amazon Cultivation Collection (CFAM—Fiocruz), code 1234, isolated from drinking water in the community of Serra Baixa, Iranduba, Amazônia, Brazil. This strain was selected due to its high potential for mannanase production compared to other *Aspergillus* strains [40]. It was propagated on potato agar dextrose agar (PDA, Sigma-Aldrich) at 27 °C for 7 days in an incubator (Incucell 111, MMM Group, Planegg/München Germany). Spores were suspended in 0.9% NaCl solution and centrifuged at 9000 rpm for 15 min. The supernatant was discarded, and the conidia were resuspended in 20% glycerol to achieve 10^8 spores.mL^{-1}, and stored in cryogenic vials at −18 °C (for later use in inoculation).

2.2. Enzyme Production

2.2.1. Preliminary Growth Medium Composition and Cultivation

The initial medium for mannanase production (Table 1) was formulated based on previous reports [41–45]. Potassium phosphate buffer was prepared by mixing 9.55 g of KH_2PO_4 with 0.24 g of K_2HPO_4, dissolving it in 800 mL of distilled water, and adjusting the pH to 5.5 with KOH or HCl. The final solution was brought to a volume of 1 L using a volumetric flask. Aluminum oxide (Al_2O_3) was used to prevent excessive growth of filamentous fungi, acting to reduce the size of fungal pellets and, consequently, helping to control hyphal development and minimize cell leakage [44]. Submerged cultivations were carried out in 1000 mL Erlenmeyer flasks containing 250 mL of medium, incubated in a shaker (Innova 44R, New Brunswick, NJ, USA) at 200 rpm and 30 °C. The cultivation time varied according to the purpose of each test. The pre-inoculum was prepared by inoculating 1% (v/v) of the spore suspension into the medium and incubating under the same conditions for 48 h. Each cultivation was then inoculated with 10% (v/v) of the pre-inoculum.

Table 1. Initial cultivation media formulated based on scientific prospecting [41–45].

Compound	Concentration ($g \cdot L^{-1}$)
Carbon Source *	30.0
Yeast extract	4.0
Potassium phosphate buffer (pH 5.5)	0.5
$CaCl_2 \cdot 2H_2O$	0.05
$MgSO_4 \cdot 7H_2O$	0.5
Al_2O_3	1.0

* ground coffee bean, coffee cake, coffee cake 80/20 mesh, coffee cake 20 mesh, coffee cake extracts, or LBG.

2.2.2. Determination of Mannanase Activity

Mannanase activity was performed using LBG as substrate, prepared at 0.5% (*w*/*v*) in 50 mM sodium citrate buffer (pH 4.8) [46]. The reaction mixture, which consisted of 0.25 mL of enzyme extract and 0.25 mL of substrate solution, was incubated at 50 °C for 10 min. The reaction was stopped by adding dinitrosalicylic acid (DNS), followed by heating at 100 °C for 5 min. The released reducing sugars were quantified according to Teixeira and coauthors [47]. One unit of enzymatic activity (U) was defined as the amount of enzyme required to release 1 µmol of reducing sugar (D-mannose-based) per minute at 50 °C.

2.2.3. Statistical Experimental Design for Mannanase Production

Plackett–Burman Design (PB)

The PB was carried out to evaluate the significance of six independent factors, tested at levels from −1 to +1, as shown in Table 2. Data analysis was performed in the Statistica 12.0 software, considering a 10% error level.

Table 2. Coded levels and corresponding values of the independent factors in the Plackett–Burman design.

ID	Independent Factor ($g \cdot L^{-1}$)	Level		
		−1	**0**	**1**
X_1	Coffee cake	20	30	40
X_2	Yeast extract	2	4	6
X_3	Phosphate buffer (pH 5.5)	0.25	0.5	0.75
X_4	$CaCl_2 \cdot 2H_2O$	0	0.005	0.1
X_5	$MgSO_4 \cdot 7H_2O$	0	0.5	1
X_6	Al_2O_3	0	1	2

Central Composite Rotatable Design (CCRD)

The factors identified as significant in PB design were further evaluated using CCRD, tested at levels from −1.41 to +1.41, as shown in Table 3. Data analysis was carried out in the Statistica 12.0 software, considering a 10% error level.

Table 3. Coded (−1.41, −1, 0, +1, +1.41) and decoded values of central composite rotatable design factors.

ID	Independent Factor ($g \cdot L^{-1}$)	Level				
		−1.41	**−1**	**0**	**+1**	**+1.41**
Z_1	Coffee cake	25	30.82	45	59.18	65
Z_2	Yeast extract	4	5.16	8	10.84	12

This approach generated a predictive model equation for mannanase production, which was validated by comparing theoretical values (calculated by substituting the optimal conditions into the model equation) with the experimental results.

2.2.4. Determination of β-mannosidase Activity

The specific production of β-mannosidases was evaluated in the culture medium optimized for mannanase production after 3, 5, and 7 days of cultivation. β-mannosidase activity was carried out according to an adapted method from Gottschalk and coauthor [48], using 4-nitrophenyl-β-D-mannopyranoside (PNP-m) as substrate. The reaction mixture contained 0.1 mL of enzyme (diluted in 50 mM sodium acetate buffer, pH 5.0), 0.6 mL of ultrapure water, 0.2 mL of 500 mM sodium acetate buffer (pH 5.0), and 0.1 mL of substrate solution (10 mM). Reactions were incubated at 50 °C for 10 min in a water bath and stopped by adding 0.5 mL of 100 mM sodium carbonate solution. Absorbance was measured at 405 nm in a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). An analytical curve of *p*-nitrophenol (0.02 to 0.20 mM) was used for quantification. One unit of β-mannosidase activity (U) was defined as the amount of enzyme required to release 1 μmol of PNP per minute under the assay conditions.

2.3. Hydrolysis Experiments of Coffee Beans

2.3.1. Enzymatic Hydrolysis

A crude mannanase preparation (culture supernatant) was used for the enzymatic hydrolysis of healthy and defective milled Arabica coffee beans to recover their monomeric sugars. Enzymatic hydrolysis was performed with 12% (*w*/*v*) dry biomass, enzymatic activity of 15 $U \cdot g^{-1}$ of dry biomass, and 100 mM sodium citrate buffer (pH 4.8) to complete 10 mL medium. Reactions were carried out at 50 °C and 200 rpm in a shaker incubator (Innova 44R, New Brunswick, NJ, USA). Aliquots were withdrawn in 0, 6, 24, and 48 h, heated at 100 °C for 5 min to stop the enzymatic reaction, and centrifuged at 8000 rpm for 10 min.

Monosaccharides were analyzed by HPLC-RI, using a Dionex Ultimate 3000 system (Thermo Fischer Scientific, Germany) equipped with a RefractoMax 521 refractive index detector (Thermo Fischer Scientific, Germany), and an Aminex HPX-87P column (300 mm × 7.8 mm, 9 μm, Bio-Rad, Hercules, CA, USA). The mobile phase was Milli-Q water at a flow rate of 0.6 $mL \cdot min^{-1}$, with a total run time of 25 min. Quantification was carried out using an external analytical curve with a mixture of sugar standards such as: D-glucose (6.2 $mg \cdot mL^{-1}$), D-xylose (2.98 $mg \cdot mL^{-1}$), D-galactose (2.12 $mg \cdot mL^{-1}$), L-arabinose (2.95 $mg \cdot mL^{-1}$), and D-mannose (4.91 $mg \cdot mL^{-1}$). Mannose and fructose content were reported together due to co-elution.

2.3.2. Acid Hydrolysis

To determine the yield of enzymatic hydrolysis (Equation (1)), acid hydrolysis was carried out on healthy and defective coffee beans according to the NREL Protocol [44], including extractives determination and hydrolysis with sulfuric acid. Monosaccharides were quantified as described for enzymatic hydrolysis.

$$Yield\ (\%) = 100\% \times \frac{manose\ content\ (enzymatic\ hydrolysis)}{manose\ content\ (acid\ hydrolysis)} \quad (1)$$

2.4. Statistical Analyses

Statistical analyses were performed using Statistica 12 (StatSoft) and Microsoft Excel. All experiments were carried out in triplicate. Data were analyzed by one-way ANOVA, followed by Student's *t*-test (for comparisons between two variables) and Tukey's test (for comparisons with more than two variables), and differences were considered not significant for $p \leq 0.05$.

3. Results

3.1. Enzyme Production

3.1.1. Influence of Carbon Source Arrangement

Ground coffee bean, coffee cake, coffee cake fractions (80/20 and 20 mesh), coffee cake extracts, and LBG were tested as carbon sources to evaluate their effect on mannanase production. Cultivations were conducted at 30 °C and 200 rpm, conditions commonly reported as optimal for *Aspergillus* [43,48–50]. The pH was adjusted to 4.8, based on previous studies on mannanase production from *Aspergillus* [41–45].

Figure 1 shows the mannanase production profile under these conditions with different carbon sources. The highest mannanase production was observed on day 7 of cultivation, consistent with the results reported by Saleh and coauthors [29], who obtained 20.92 U·mL^{-1} using *A. niger* MSSFW in submerged fermentation of coffee powder waste over 7 days.

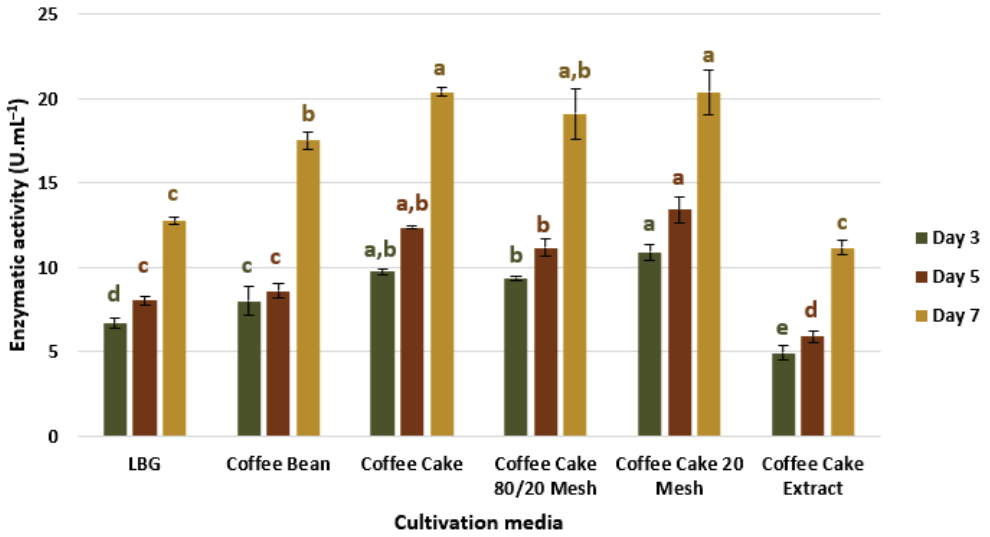


Figure 1. Mannanase production profile measured on days 3, 5, and 7 of cultivation using the initial medium described in Table 1 and different carbon sources, incubated in a shaker at 30 °C and 200 rpm. The comparison of mannanase activity between cultivation days was performed using a one-way ANOVA. Identical letters, for the same day, indicate no significant difference ($p \leq 0.05$) between carbon sources for mannanase production, according to Tukey's test.

The medium containing only LBG, a commercially available galactomannan, resulted in lower mannanase activity compared to media containing coffee cake or green coffee beans. These results indicate that coffee beans are an excellent substrate for mannanase production. Furthermore, since coffee cake is an industrial waste product, its use in the enzyme production process could be highly cost-effective.

The lowest mannanase activities were observed in media containing coffee cake extracts, indicating that the nutrient extraction process from the coffee cake was inefficient. The presence of lipids in green coffee beans negatively affects enzymatic activity compared to coffee cake. A hypothesis for this is that the fungus may be using the lipids present in the substrate as a source of carbon, redirecting its metabolism toward the production of enzymes related to lipid degradation, such as lipases, to the detriment of mannanases. Lin et al. (2004) [51] indicate that the presence of oil in copra (a fat-rich matrix) may depress mannanase production.

The highest activities were obtained in media containing coffee cake of different particle sizes, with no significant differences among them ($p \leq 0.05$). Therefore, subsequent

experiments were conducted using unprocessed coffee cake (20.39 ± 1.50 U·mL^{-1}), as this simplifies the procedure and avoids a 3% mass loss.

3.1.2. Influence of Cultivation Time

Mannanase production using coffee cake and LBG was evaluated over several days, as shown in Figure 2. On all sampling days, LBG showed significantly lower activity compared to coffee cake ($p \geq 0.05$).

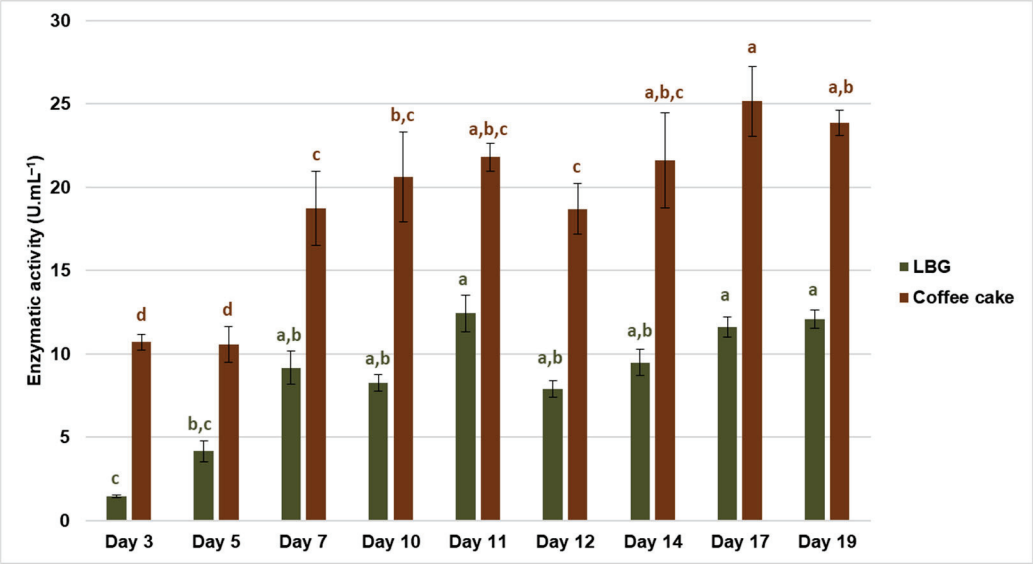


Figure 2. Mannanase production profile over 19 days of cultivation using coffee cake and LBG as carbon sources, in the columns, with the initial medium described in Table 1, incubated in a shaker at 30 °C and 200 rpm. The comparison of mannanase activity between cultivation days was performed using a one-way ANOVA. Identical letters, in the same carbon source, indicate no significant difference ($p \leq 0.05$) between cultivation days for mannanase production, according to Tukey's test.

Peak mannanase production in the LBG-based medium occurred on day 11 (12.44 U·mL^{-1}), followed by stabilization or a slight decline, suggesting substrate depletion. In contrast, the coffee cake medium maintained high activity for longer periods, peaking on day 17 (25.15 U·mL^{-1}), which may be related to the greater structural complexity of the substrate and the gradual release of nutrients. In short, a notable discrepancy was observed between the maximum activity levels: the coffee cake medium reached an average of 25.15 ± 2.08 U·mL^{-1}, while the LBG medium reached only 12.44 ± 1.09 U·mL^{-1}, consistent with the preliminary tests. Considering productivity, the optimal cultivation day was day 7, which may reflect a characteristic of *Aspergillus niger,* as this trend was observed in both media.

One possible explanation for this activity difference between media is that coffee cake provides more favorable and sustained conditions for mannanase production, possibly because it contains components that act as inducers or because it offers a richer and more diverse nutritional environment. Coffee cake exhibited higher carbon, nitrogen, and sulfur content compared to LBG (Table 4), highlighting its potential as a low-cost source of mannan, compared to the commercial galactomannan extracted from *Ceratonia siliqua* seeds (LBG).

The availability of carbon and nitrogen in the culture medium is fundamental for the growth and metabolite production of *Aspergillus niger*. According to Chauhan and coauthors [52], carbon serves as both an energy source and an essential structural element. It can also influence the production of specific enzymes, such as mannanases, depending on the type and concentration present in the medium.

Table 4. Elementary organic analysis (CHNSO) of the coffee cake and locust bean gum (LBG).

Carbon Source	%C	%H	%N	%S	%O
LBG	38.85 ± 0.12 [a]	6.64 ± 0.04 [a]	0.87 ± 0.01 [b]	0.15 ± 0.03 [a]	53.49 ± 0.17 [a]
Coffee cake	43.42 ± 0.05 [a]	6.36 ± 0.00 [b]	2.61 ± 0.00 [a]	0.23 ± 0.01 [a]	47.38 ± 0.04 [a]

The comparison of elementary organic analysis between LBG and coffee cake was performed using one-way ANOVA. Identical letters, in the same element, indicate no significant difference ($p \leq 0.05$) between the carbon sources, according to Student's *t*-test.

Although nitrogen is supplied to the medium through yeast extract, the nitrogen content in the biomass also contributes to fungal growth and enzyme synthesis. Different nitrogen sources may enhance the yield of specific enzymes. Moreover, the carbon-to-nitrogen (C/N) ratio plays a key role in regulating fungal growth and metabolite production [53]. Considering that the yeast extract used in the medium has 38.15 ± 0.07% carbon (%C) and 10.85 ± 0.24% nitrogen (%N), the C/N ratio was calculated for the medium containing coffee cake and LBG, resulting in 11.96 and 18.96, respectively. These results indicate that the C/N ratio of the coffee bean medium favors mannanase production, in agreement with findings reported by Gottschalk and coauthors [48] for *Aspergillus awamori* 2B.361 U2/1 cultivated on wheat bran and yeast extract (C/N equal to 10.3).

3.1.3. Validation of Preliminary Tests

Once the best arrangement of the carbon source had been determined, a new test was carried out, in a shorter time, to validate the results obtained, as shown in Figure 3.

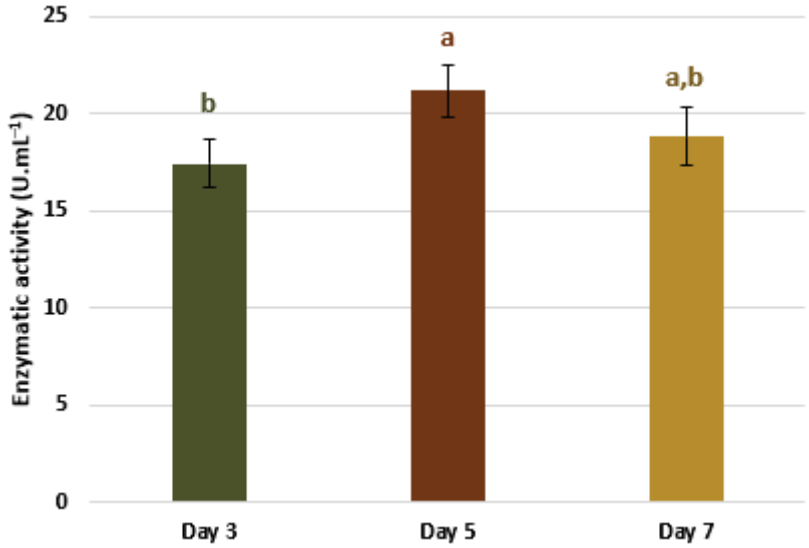


Figure 3. Mannanase production profile over 7 days of cultivation using coffee cake as a carbon source, with the initial medium described in Table 1, incubated in a shaker at 30 °C and 200 rpm. The comparison of mannanase activity between cultivation days was performed using a one-way ANOVA. Identical letters indicate no significant difference ($p \leq 0.05$) between cultivation days for mannanase production, according to Tukey's test.

In this experiment, the peak enzymatic activity of mannanases did not differ significantly ($p \leq 0.05$) between the 5th and 7th days. It is common for biotechnological processes such as this to vary in terms of the ideal time, as everything depends on the development of the strain under the cultivation conditions [54]. As the peak of mannanase enzyme activity occurred on the fifth day of cultivation, this was considered the ideal day for enzyme production in subsequent studies.

3.1.4. Statistical Experimental Design

Data from the preliminary tests supported the application of statistical experimental designs to optimize mannanase production by modifying the nutritional composition of the culture medium. A Plackett–Burman (PB) design was employed to evaluate the influence of six independent variables and identify those with significant effects on mannanase production. Subsequently, a Central Composite Rotatable Design (CCRD) was applied to optimize the significant variables, enabling a more detailed evaluation of their individual and interactive effects through a full factorial approach. The statistical analyses were performed using response surface methodology (RSM) and analysis of variance (ANOVA), following established procedures for bioprocess optimization [54,55]. Results were expressed as means ± standard deviations, and variance analysis was performed using Tukey's test at the 5% significance level.

Table 5 presents the characteristics of each test (12 experimental points and 4 central points), along with the results of the dependent variable under study. The results showed very close mannanase activities from 19 to 21 U·mL^{-1}, which may indicate that the concentrations studied had no effect on the production of mannanases. Only assays 8 and 12 presented values below 15 U·mL^{-1}.

Table 5. Results of the Plackett–Burman design, represented in codified variables, for mannanase production on the fifth day of cultivation.

ID	X_1	X_2	X_3	X_4	X_5	X_6	VI_1	VI_2	VI_3	VI_4	VI_5	Mannanase Activity (U·mL^{-1})
1	1	−1	1	−1	−1	−1	1	1	1	−1	1	21.87 ± 1.64
2	1	1	−1	1	−1	−1	−1	1	1	1	−1	21.49 ± 0.24
3	−1	1	1	−1	1	−1	−1	−1	1	1	1	19.42 ± 0.61
4	1	−1	1	1	−1	1	−1	−1	−1	1	1	20.44 ± 0.41
5	1	1	−1	1	1	−1	1	−1	−1	−1	1	21.67 ± 0.77
6	1	1	1	−1	1	1	−1	1	−1	−1	−1	20.77 ± 0.93
7	−1	1	1	1	−1	1	1	−1	1	−1	−1	19.96 ± 0.81
8	−1	−1	1	1	1	−1	1	1	−1	1	−1	13.56 ± 0.83
9	−1	−1	−1	1	1	1	−1	1	1	−1	1	19.53 ± 1.34
10	1	−1	−1	−1	1	1	1	−1	1	1	−1	20.79 ± 0.43
11	−1	1	−1	−1	−1	1	1	1	−1	1	1	20.65 ± 0.30
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	14.52 ± 0.22
13	0	0	0	0	0	0	0	0	0	0	0	20.74 ± 0.14
14	0	0	0	0	0	0	0	0	0	0	0	20.57 ± 0.14
15	0	0	0	0	0	0	0	0	0	0	0	20.20 ± 0.17
16	0	0	0	0	0	0	0	0	0	0	0	20.52 ± 0.07

ID—identification assay number; X_1—Coffee cake; X_2—Yeast extract; X_3—Phosphate buffer (pH 5.5); X_4—$CaCl_2 \cdot 2H_2O$; X_5—$MgSO_4 \cdot 7H_2O$; X_6—Al_2O_3; IV_{1-5}—dummy variables, included to complete the design matrix, estimate the experimental error, and allow identification of the real factors that have a significant effect on mannanase production.

After analyzing the results with Statistica 12 software, the information from each assay was consolidated to generate the Pareto chart (Figure 4), which enabled the statistical interpretation of the studied variables. Pareto chart analysis revealed that only two variables were statistically significant: coffee cake (carbon source) and yeast extract (nitrogen source). Consequently, all independent factors with statistically insignificant effects—except for phosphate buffer—were excluded from the medium in the subsequent CCRD. The buffer was retained due to its essential role in adjusting the initial pH to levels suitable for *Aspergillus niger* growth. Since the phosphate buffer exhibited a negative effect, it was maintained at the minimum concentration tested (level −1, corresponding to 0.25 g·L^{-1}, expressed as phosphate).

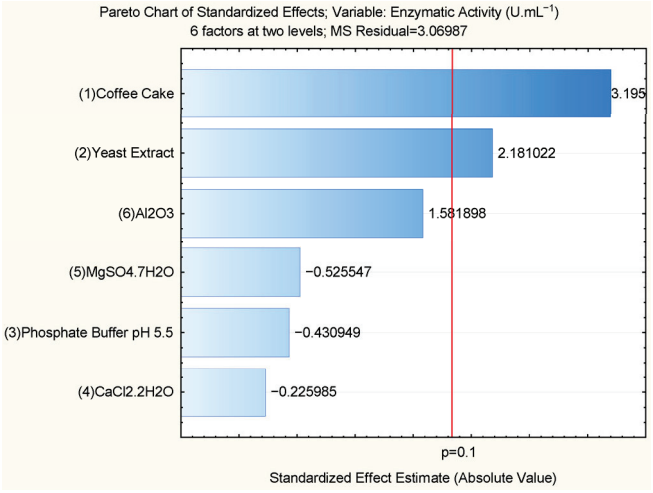


Figure 4. Pareto chart of effects for mannanase production (U·mL^{-1}).

The CCRD was conducted with eight factorial points and four central points to improve the reliability of the results, as shown in Table 6. The highest mannanase activities were obtained in assays 8, 9, and 10, of which two tests correspond to the central points of the design.

Table 6. Results of the Central Composite Rotatable Design for mannanase production, on the fifth day of cultivation, where Z_1 is the coffee cake concentration, and Z_2 is the yeast extract concentration, represented in codified variables.

ID	Z_1	Z_2	Mannanase Activity (U·mL^{-1})
1	−1	−1	21.01 ± 0.27
2	1	−1	22.97 ± 0.45
3	−1	1	21.30 ± 0.18
4	1	1	22.65 ± 0.27
5	−1.41	0	21.07 ± 0.59
6	1.41	0	23.07 ± 0.43
7	0	−1.41	22.02 ± 0.61
8	0	1.41	25.43 ± 0.84
9	0	0	25.63 ± 0.48
10	0	0	25.33 ± 0.30
11	0	0	23.64 ± 0.29
12	0	0	24.50 ± 0.52

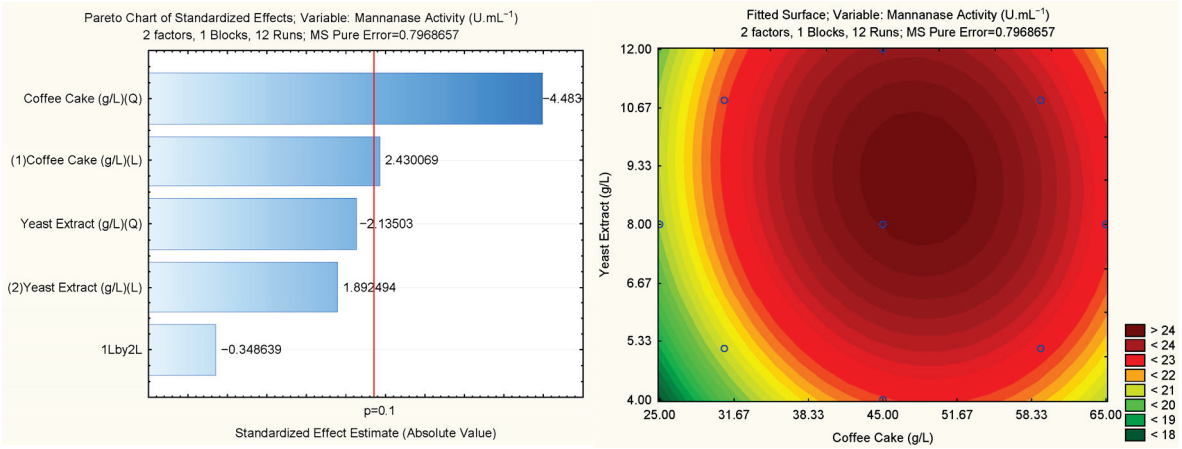
Based on the results processed in Statistica 12, a Pareto chart was generated (Figure 5a) to interpret the significance of the studied variables, along with a response surface plot predicting the tested conditions and their corresponding responses (Figure 5b). Both the linear and quadratic terms for coffee cake were statistically significant, whereas neither term for yeast extract showed significance, leading to its exclusion from the model.

The mathematical model (Equation (2)), obtained for the Pareto chart, was used even though the variables corresponding to yeast extract (L and Q) were not statistically significant, due to the necessity of a nitrogen source to support fungal growth. Although yeast extract did not have a significant effect on the evaluated response, it is well known to be an essential nutrient source. Therefore, since the central composite rotatable design (CCRD) did not include experiments with zero concentration of this component, the

minimum studied value was adopted. The model predicted a mannanase production (Man_{prod}) of 24.32 U·mL^{-1}.

$$Man_{prod}\ (U{\cdot}mL^{-1}) = -1.58866Z_1{}^2 - 0.75652Z_2{}^2 + 0.76809Z_1 + 0.59818Z_2 + 24.77747 \quad (2)$$

The validation assay of the statistical experimental design was performed using the yeast extract at the lowest level studied (4 g·L^{-1}), since it had no significant effect. After five days of cultivation, the experimental activity was 21.85 ± 0.73 U·mL^{-1}, close to the theoretical value of 24.32 U·mL^{-1} predicted by the CCRD model, with an error of approximately 10%. This confirmed the validity of the statistical model. It is important to emphasize that the equation enables the calculation of mannanase activity under varying cultivation conditions, allowing for the prediction of potential outcomes based on different concentration levels.



(**a**) (**b**)

Figure 5. (**a**) Pareto chart of effects and (**b**) contour curves graph obtained through the two-factor CCRD conditions (coffee cake and yeast extract content) from culture medium for mannanase production (U·mL^{-1}).

Additional assays under the optimized condition showed activities of 22.43 ± 0.59 U·mL^{-1} at three days and 17.26 ± 0.56 U·mL^{-1} at seven days of cultivation. The activities on days three and five were not significantly different ($p \leq 0.05$), indicating that the peak of enzymatic activity shifted to the third day of cultivation.

The maximum activity obtained in the present was higher than that reported by Agu and coauthors [56], who used wild-type *Aspergillus* strains and agro-industrial residues as carbon sources. In that study, three species (*A. niger*, *A. flavus*, and *A. tamari*) were isolated from decomposing palm kernel cake and cultivated under submerged fermentation with coconut yam powder as the sole carbon source. *A. niger* produced the highest mannanase activity (0.35 U·mL^{-1}), followed by *A. flavus* (0.18 U·mL^{-1}) and *A. tamari* (0.15 U·mL^{-1}).

The medium composition optimization allowed an increase in mannanase production of approximately 10% and a 42.9% increase in productivity, going from 20.39 ± 1.50 U·mL^{-1} (at seven days) to 22.44 ± 0.61 U·mL^{-1} (at three days). The reduction in cultivation time from seven to three days is particularly relevant for industrial applications, since shorter fermentations reduce energy consumption, contamination risks, and downstream processing costs. Moreover, the elimination of inorganic salts ($CaCl_2$, $MgSO_4$, Al_2O_3) from the medium highlights the robustness of the process, enabling a simplified and cost-effective formulation that relies almost exclusively on the coffee coproduct. Thus, the optimized cultivation medium consisted only of 48.78 g·L^{-1} of coffee cake, 4 g·L^{-1} of yeast extract, and 0.25 g·L^{-1} of potassium phosphate buffer (pH 5.5).

3.1.5. Determination of β-mannosidase Enzyme Activity

In addition to the high production of mannanases, the specific production of β-mannosidases was evaluated under the optimized culture medium using coffee cake. Enzyme activity was assessed after 3, 5, and 7 days of cultivation, resulting in 0.52 ± 0.01 U·mL^{-1}, 0.88 ± 0.06 U·mL^{-1}, and 1.39 ± 0.06 U·mL^{-1}, respectively. The maximum β-mannosidase activity was observed on the seventh day, differing from β-mannanase production, which peaked on the third day. This value was 5.6-fold higher than that previously reported for *A. niger* (0.25 U·mL^{-1}) cultivated with soya flour [57].

These results suggest that the strain used in this study has considerable potential for β-mannosidase production, even in a culture medium that has not been optimized for its production. β-Mannosidase catalyzes the hydrolysis of β-1,4-mannosidic bonds in mannose-containing polysaccharides such as galactomannan, playing a key role in converting complex carbohydrates into simpler sugars. Its applications extend to the food industry, where it improves ingredient digestibility and produces prebiotics, as well as to biotechnology, biofuel production, and animal feed, where it enhances nutritional efficiency and supports sustainable bioconversion processes [58–60]. A noteworthy aspect of the present study is the concomitant production of β-mannosidase, which broadens the applicability of the crude enzymatic cocktail. While most reports focus exclusively on β-mannanases, the simultaneous presence of β-mannosidase increases hydrolytic efficiency, particularly in generating fermentable mannose. Although this is a very positive result, the addition of an external β-mannosidase (either commercial or laboratory-concentrated) to balance the β-mannosidase/β-mannanase ratio could enhance the enzymatic hydrolysis of this material, enabling the complete conversion of polysaccharides into monosaccharides.

Using coffee press cake as a carbon source for β-mannosidase production also aligns with the principles of the circular economy and agro-industrial residue valorization. By-products of the coffee industry contain galactomannan and have been shown to support enzyme production by fungi of the *Aspergillus* species [61,62]. Leveraging coffee cake reduces production costs, promotes environmental sustainability, and repurposes waste that would otherwise be discarded, reinforcing its potential as an efficient and sustainable carbon source for enzyme production.

To expand this study, the enzymes produced were applied to the enzymatic hydrolysis of defective coffee beans, aiming to release fermentable sugars that could potentially be used for the generation of higher-value-added products.

3.2. Hydrolysis Experiments of Coffee Beans

It is estimated that approximately 20% of global coffee production consists of defective beans, which, due to their poor sensory quality, are unsuitable for consumption [8]. These beans, therefore, represent a potential worldwide residue, rich in mannan, whose processing faces significant challenges. To explore alternative uses, this study investigated the potential of the produced enzyme for mannose recovery from defective beans through enzymatic hydrolysis, with comparisons to the hydrolysis of healthy beans. Valorizing defective beans as a substrate is particularly relevant, as mannose can serve as a precursor for diverse bioproducts, such as immunostimulants [63], anti-tumor agents [64], vitamins [65], and D-mannitol [66].

Polysaccharides, which account for 50–70% of the dry weight of green coffee beans, are mainly composed of galactomannans, arabinogalactans, and cellulose [17]. During hydrolysis, these polysaccharides are depolymerized into monosaccharides, mainly mannose, whose release was monitored at 0, 6, 24, and 48 h, as shown in Figure 6. Since the chromatographic column used did not separate mannose and fructose, their contents were quantified together. The reactions were conducted at 50 °C and 200 rpm in buffer

at pH 4.8—conditions commonly reported as suitable for enzymatic hydrolysis—while agitation enhanced enzyme–substrate contact and activity [67–71].

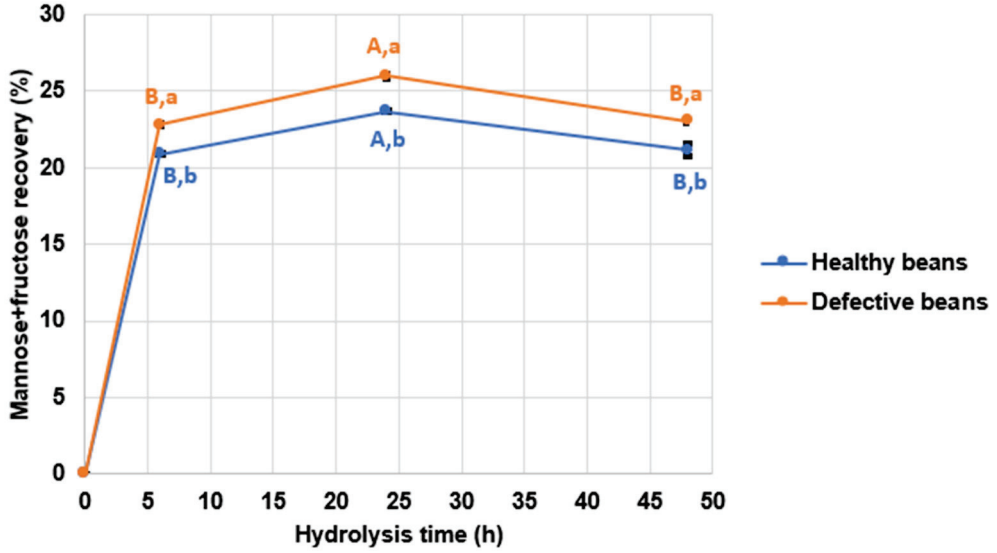


Figure 6. Mannose plus fructose recovery (%) obtained from the enzymatic hydrolysis of healthy and defective coffee beans at 50 °C and 250 rpm, using the optimized enzyme productized. The mannose value obtained by acid hydrolysis was considered 100%. The comparison of mannose + fructose recovery between cultivation days and between biomass was performed using a one-way ANOVA. Identical uppercase letters indicate no significant difference ($p \leq 0.05$) between hydrolysis time, in the same biomass, for mannose recovery, according to Tukey's test. Identical lowercase letters indicate no significant difference ($p \leq 0.05$) between biomass, in the same hydrolysis time, for mannose recovery, according to Student's *t* test.

The best mannose yield was obtained after 24 h, as shown in Figure 6. The mannose value obtained by acid hydrolysis was considered 100%. The efficiency of mannose recovery by enzymatic hydrolysis was 23.68 ± 0.03% for healthy beans and 25.98 ± 0.10% for defective beans, in 24 h of reaction. Although relatively low, these values demonstrate the enzyme's potential, achieving ~25% mannose recovery directly from untreated biomass. Limited yields are attributed to the recalcitrant structure of coffee beans, where crystalline mannans hinder enzymatic access. To improve recovery, pretreatments—physical, chemical, or combined—are required to disrupt the cell wall, solubilize hemicelluloses and lignin, and increase surface area for enzymatic action [32,33,71–73]. Examples include extrusion, hydrothermal processing, acid or alkaline hydrolysis, and treatments with organic solvents [74]. Although only monosaccharides were quantified, presented in Table 7, mannanoligosaccharides (MOS) may also have been formed and remained in the reaction medium without being converted to mannose, due to the imbalance between β-mannosidase and β-mannanase activities.

In a related study [75], coffee grounds were used as a mannose-rich biomass (19.3 g 100 g^{-1}). After enzymatic hydrolysis carried out with 50 mM sodium citrate buffer (pH 4.8), 10% dry matter, and an enzyme cocktail (cellulase—17.74 mg·g^{-1} of dry biomass; pectinase—16 mg·g^{-1} of dry biomass) for 24 h, a mannose recovery of approximately 47% was achieved. The higher yield observed in roasted beans was likely due to the previous processing, such as roasting, grinding, and hot-water extraction, which may remove enzyme inhibitors and promote biomass deconstruction, facilitating enzymatic access.

Table 7. Monosaccharides from the acid and enzymatic hydrolysis of healthy and defective coffee beans. Enzymatic hydrolysis was performed at 50 °C and 200 rpm.

Samples	Monosaccharides (g·100 g^{-1})			
	Glucose	Galactose	Arabinose	Mannose + Fructose
Acid hydrolysis				
Healthy beans	6.56 ± 0.21	13.53 ± 0.70	1.91 ± 0.38	19.69 ± 4.06
Defective beans	5.52 ± 0.53	12.59 ± 0.76	1.68 ± 0.13	18.50 ± 2.07
Enzymatic hydrolysis (24 h)				
Healthy beans	5.01 ± 0.33	0.18 ± 0.00	0.00	4.66 ± 0.01
Defective beans	3.96 ± 0.39	0.17 ± 0.01	0.00	4.81 ± 0.02

Xylose was not detected for any sample.

The enzyme produced in this study also demonstrated good potential for recovery of glucose (76% in healthy beans and 72% in defective beans) but limited recovery of galactose and arabinose. To maximize sugar release, integrated strategies should be adopted, including appropriate pretreatments, optimization of hydrolysis parameters (enzyme loading, pH, and temperature), and the use of tailored enzymatic cocktails containing accessory enzymes (e.g., β-galactosidase and β-glucosidase) to enhance the conversion of complex polysaccharides into fermentable sugars [74].

Furthermore, optimizing enzymatic hydrolysis conditions is essential, considering adjustments in solid matter concentration, enzyme proportion, and precise control of operational parameters such as pH and temperature. Another critical aspect is the use of tailored enzymatic cocktails containing accessory enzymes such as β-galactosidase and β-glucosidase, which work synergistically to enhance the conversion of structural biomass components into fermentable sugars, like mannose and glucose. These integrated approaches can significantly improve the efficiency of biotechnological processes.

4. Conclusions

This study demonstrated that green Arabica coffee press cake is an efficient and sustainable substrate for β-mannanase and β-mannosidase production by *Aspergillus niger*. The comparison of enzyme productivity was performed using the same carbon source (coffee press cake). After statistical optimization, the medium composition was simplified to 48.78 g·L^{-1} coffee cake, 4 g·L^{-1} yeast extract, and 0.25 g·L^{-1} potassium phosphate buffer (pH 5.5), which increased mannanase productivity to 22.4 ± 0.6 U·mL^{-1} within 3 days, representing a 42.9% improvement. The simplified medium composition—based solely on coffee press cake, yeast extract, and phosphate buffer—further enhances economic feasibility by eliminating the need for additional supplements (Al_2O_3, $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$). Although mannose recovery from untreated coffee beans reached only ~25% (using the acid hydrolysis value at 100%), the results demonstrate the enzyme's capacity to act on recalcitrant biomass, with higher efficiencies expected through pretreatment strategies and tailored enzymatic cocktails.

From an industrial and sustainability perspective, these findings align with circular bioeconomy principles. A low-value by-product (coffee press cake) was used as a carbon source for *A. niger* cultivation and production of a high-value enzymatic mixture, capable of valorizing another waste stream (defective beans), enabling the recovery of functional monosaccharides for food, nutritional, and pharmaceutical applications.

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processes

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Article

Bioconversion of Ferulic Acid to 4-Vinylguaiacol by Ferulic Acid Decarboxylase from *Brucella intermedia* TG 3.48

Sylvia Patricia de Carvalho, Mohammed Anas Zaiter, Karine Sousa Dantas, Érike Jhonnathan Pereira, Ronivaldo Rodrigues da Silva, Maurício Boscolo, Roberto da Silva, Maitê Bernardo Correia dos Santos and Eleni Gomes *

Laboratory of Unesp's Bioenegy Institute IPBEN, São Paulo State University, UNESP, São José do Rio Preto 15054-000, SP, Brazil; sylvia.patricia@unesp.br (S.P.d.C.); anas.zaiter@unesp.br (M.A.Z.); karine.dantas@unesp.br (K.S.D.); jhonnathan.pereira@unesp.br (É.J.P.); ronivaldo.r.silva@unesp.br (R.R.d.S.); mauricio.boscolo@unesp.br (M.B.); roberto.silva@unesp.br (R.d.S.); maite.bernardo@unesp.br (M.B.C.d.S.)
* Correspondence: eleni.gomes@unesp.br; Tel.: +55-17-3221-2393

Abstract

4-vinylguaiacol (4-VG) is a commercially important compound due to its characteristic clove-like aroma and its use as a flavoring in the food, beverage, and cosmetics industries. However, its extraction from natural sources or by a chemical method is expensive. The bioconversion of ferulic acid (FA) to 4-VG via microorganisms is an alternative, considering the market trend toward biotechnological and environmentally friendly processes and products. This study aimed to evaluate the tolerance of the bacterial strain *Brucella intermedia* (basonym *Ochrobactrum intermedium*) TG 3.48 to FA, its bioconversion to 4-VG, and the activity of the FA decarboxylase enzyme (FADase), which is key to the 4-VG production process. The strain tolerated FA concentrations up to 700 mg L^{-1}. When the microorganism grew at 300 mg L^{-1} FA in Mineral Liquid Medium (MLM), it converted 99.5% of FA to 4-VG within 12 h. The FADase activity was cell-associated with 5.17 U mL-1 in the whole cell, 4.40 U mL^{-1} in the intracellular extract, and 3.54 U mL^{-1} in the cell wall fragments, while the specific activity was 778.90 U mg^{-1}.

Keywords: ferulic acid; 4-vinylguaiacol; decarboxylase; *Brucella intermedia* TG 3.48; *Ochrobactrum intermedium* TG 3.48

1. Introduction

Several processes that use lignocellulosic raw material result in an excessive amount of lignin that is not optimally utilized. This polymer and its derivatives with high content of aromatic compounds have potential applications in the biosynthesis of various products [1]. Ferulic acid (FA) is present in high concentrations in hydrolyzed lignocellulosic biomass and serves as a substrate for the synthesis of flavor compounds such as vanillic acid (VA), vanillin, and 4-vinylguaiacol (4-VG) [2,3].

4-VG is a phenolic volatile substance with a spicy clove-like aroma, which makes it a key aroma compound in the beverages and beer industries [4], and as an additive in flavors and fragrances industries [5]. On the other hand, 4-VG is a well-documented volatile phenolic compound that occurs in roasted coffee beans during roasting process by thermal decarboxylation of FA [6,7], while in citrus, research reports 4-VG among identified aroma compounds in citrus peels and dried citrus products, especially when peel is processed (drying, extraction) or when bound precursor is hydrolyzed, as well as in

cold-pressed grapefruit oil [8,9]. In addition, its antioxidant activity makes it a potential pharmaceutical agent [10,11]. 4-VG can be extracted from plants, yet the low concentration of this compound makes the process economically unfeasible. Chemical synthesis is an alternative representing around 80% of total 4-VG production [12,13].

The biological synthesis of 4-VG by fungi and bacteria using FA as a substrate has been reported [2,5,13,14]. These biocatalytic routes use low-cost renewable FA, operate under mild aqueous conditions with high chemo-selectivity and reduced by-product formation, simplifying downstream processing [15]. In contrast, classical chemical routes such as Wittig-type alkene formation produce stoichiometric phosphine-oxide waste, typically require harsher conditions and organic solvents, and exhibit poor green chemistry metrics [16].

The biotransformation of FA to 4-VG is catalyzed by FA decarboxylase enzyme (FADase, EC 4.1.1.102) via non-oxidative decarboxylation of FA to 4-VG with release of CO_2 from the substrate's terminal carboxyl carbon [12] (Figure 1). FADas has been reported in various microorganisms such as *Aspergillus luchuensis*, *Candida guilliermondii*, *Bacillus* sp., and *Klebsiella pneumoniae*, where it generally involves in culture media detoxification processes and, in some species, forms part of the metabolic pathway for assimilating xenobiotic compounds [2,11].

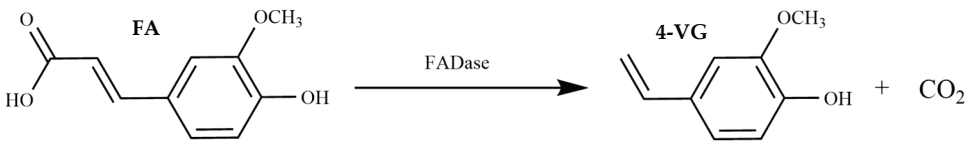

Figure 1. FADase non-oxidative decarboxylation of FA to 4-VG with release of CO_2.

The bacterium *Brucella intermedia* TG 3.48 demonstrates tolerance to compounds that typically inhibit microbial growth, such as lead and chromium VI, and can degrade xenobiotic substances including diuron and piracetam (2-(2-oxopyrrolidin-1-yl) acetamide) [17–22]. A 16S rRNA gene partial sequence (1329 bp) of *B. intermedia* TG 3.48 is available in GenBank under accession number MG214517, as reported by Egea et al. [21].

Previous studies report large inter- and intra-species variation in FA tolerance, *Klebsiella pneumoniae* TD4.7 showed growth and activity only at 0.3–0.5 g L^{-1} [2], as for *Bacillus subtilis* B7-S lost growth and bioconversion at levels around 1.3 g L^{-1} [23], while adaptive laboratory evolution (ALE) of *Pseudomonas putida* KT2440 markedly increased tolerance up to the 30 g L^{-1} range, showing that baseline tolerance is strain- and condition-dependent and can be substantially improved by ALE [24].

Unlike many fungal and yeast ferulic acid decarboxylases that require the prenylated-FMN (prFMN) cofactor and its biosynthetic partner (UbiX/PAD1) for activity [25], the enzyme described here is cofactor independent, which simplifies heterologous expression and process design, avoiding the co-expression or supplementation of accessory cofactor pathways, and thereby facilitates robust single step bioconversion of FA from lignocellulosic hydrolysates to 4-VG [5,26].

The objective of this work is to assess the FA tolerance, study the potential of *B. intermedia* TG 3.48 to produce 4-VG via decarboxylation of the FA and evaluate the expression and characterization of the FADase enzyme.

2. Materials and Methods

2.1. Microorganism

The bacterium *Brucella intermedia* (basonym *Ochrobactrum intermedium*) TG 3.48 [27], previously isolated [21], is part of the working collection of the Biochemical and Applied

Microbiology laboratory, Unesp Bioenergy Research Institute, São José do Rio Preto, SP, Brazil. The strain has been maintained at −80 °C in a 15% glycerol solution. Identification was carried out by DNA extraction using the commercial AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen, Union City, CA, USA) from a colony obtained after cultivation on Luria-Bertani agar medium (LB) for 12 h at 30 °C. The partial sequence of the 16S rRNA gene is available on NCBI website https://www.ncbi.nlm.nih.gov/nuccore/MG214517.1/, accessed on 20 August 2025.

2.2. Culture Medium

The Liquid Nutrient Medium (LNM) [28] consisted of 0.2% glucose, 0.1% sodium chloride, 1% tryptone, and 0.5% yeast extract. An adaptation of the Mineral Liquid Medium (MLM) proposed by Bushnell and Haas [29], composed of 11% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 0.002% $CaCl_2$, 0.1% $KHPO_4$, and 0.1% KH_2PO_4 was also used. The solid medium contained the same nutrients as LNM, with the addition of 1.5% agar.

2.3. Bacterial Tolerance to FA and Potential Biotransformatio of FA to 4-VG

For all cultivation procedures, the pre-inoculum was prepared by suspending bacterial cells in a liquid nutrient medium at pH 6.0. Pre-inoculum cultures were incubated in an orbital shaker at 28 °C and 150 rpm. The optical density (O.D) of the culture was measured at 600nm using a spectrophotometer until an O.D of 0.8 was reached.

Bacterial tolerance to FA was assessed in 96-well microtiter plates containing 196.0 µL of LNM, with FA concentrations ranging from 100 up to 700 mg L^{-1}, and after addition of ferulic acid the medium pH was readjusted to pH 6.0 with 1 M NaOH (or HCl where required) prior to inoculation to avoid pH-driven effects on bacterial growth, incubated at 28 °C and 190 rpm.

For the decarboxylation of FA to 4-VG, the methods described above were followed, with 250 mL of LB medium containing 300 mg L^{-1} of FA in a 500 mL Erlenmeyer flask, which was inoculated with one mL of inoculum obtained as previously described and left for 48 h. During the first 24 h of cultivation, samples were collected every two hours, and subsequently every eight hours, for the microbial growth evaluation by O.D at 600 nm. The concentrations of glucose, FA, 4-VG, and other metabolites were determined. The initial FA concentration was used to calculate product yields (%).

FA, 4-VG, VA, catechol and vanillin, metabolites commonly reported in the FA bioconversion pathways, were assayed by HPLC. The formation of 4-VG during cultivation was verified by mass spectrometry (MS) analysis.

Two controls were employed in this work, an abiotic control (medium supplemented with FA but not inoculated); and a biotic control (medium inoculated with the microorganism but without FA addition).

Unless indicated otherwise, all reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Analytical Methods

Metabolites potentially derived from FA including catechol (benzene-1,2-diol), guaiacol (2-methoxyphenol), 4-VG (2-Methoxy-4-vinylphenol), VA (4-hydroxy-3-methoxybenzoic acid) and vanillin (4-Hydroxy-3-methoxybenzaldehyde) were screened and analyzed by HPLC and LC-MS. Filtered samples were prepared using a 0.22 µm syringe filter (analitica 2202213 300 Cw syringe filter, hydrophilic PTFE membrane, filter diameter 13 mm, pore size 0.22 µm, pre-cleaned, with polypropylene (PP) pre-filter.), and 20 µL aliquots were injected into a 1220 Infinity liquid chromatograph (LC) (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, autosampler, column oven, and a UV-VIS detec-

tor. Chromatographic separation was achieved on a ZORBAX Eclipse Plus C18 column (4.6 × 250 mm) (Agilent Technologies, Santa Clara, CA, USA), and preserved at 25 °C.

Chromatographic separation was performed using solvent A (methanol:acetic acid:water, 10:2:88 v/v) and solvent B (methanol:acetic acid:water, 90:2:8 v/v). [30]. Retention times were determined using analytical standards, and calibration curves of FA and its metabolites were plotted at concentrations between 2.5 and 300 mg L^{-1}. The correlation coefficient was 0.999 for analyzed compounds, and the peak areas were quantified according to the calibration curves.

Sugars were quantified by ion-exchange chromatography using a Dionex ICS-5000 HPAEC-PAD (Thermo Scientific, Hampton, NH, USA) equipped with a CarboPac® PA-1 anion exchange column at a temperature of 25 °C. Eluents were prepared with ultrapure deionized water (18 MΩ) and degassed with N_2. The flow rate was set to one mL min^{-1} with solvent A (ultrapure water) and solvent B (500 mM NaOH), under isocratic condition of 96% A and 4% B for 25 min. The lint sample was centrifuged at 15,000× g for 15 min, and the supernatant was filtered through same previously mentioned 0.22 µm syringe filter, diluted 50-fold, and injected into the chromatograph.

2.5. FA Decarboxylase Activity (FADase)

FADase activity was evaluated in three different crude enzyme fractions: extracellular crude enzyme (culture medium), intracellular extract, and cell wall-associated enzymes (fragments). To obtain the biomass needed, 2 L of medium MLM (as described previously) with the supplemented with 0.05% of glucose (w/v) and 300 mg L^{-1} of commercial FA was inoculated and incubated at 28 °C and 150 rpm. Cell growth was monitored until OD600 reached 0.80. The entire culture volume was used to obtain the crude enzyme fractions previously mentioned as follows.

- Extracellular crude enzyme: Culture medium was centrifuged at 15,000× g for 15 min at 4 °C and the supernatant was filtered through a 0.22 µm filter and retained as the extracellular enzyme fracture.
- Intracellular crude enzyme: Cell biomass was washed twice with 50 mM sodium phosphate buffer (pH 6.0), the supernatant was discarded, and the pellet was resuspended in the same buffer at a concentration of 0.2 g mL^{-1} wet biomass (0.2 g wet cell per mL buffer). The resulting cell suspension was sonicated 4 times for 30 s at 20 kHz with 15 s cooling intervals in an ice bath. The lysate was centrifuged at 15,000× g for 15 min at 4 °C, and the supernatant was collected and used as the intracellular enzyme fraction.
- Cell wall-associated enzyme: The pellet obtained from the previous centrifugation step was resuspended in 50 mM sodium phosphate buffer (pH 6.0) and centrifuged at 15,000× g and 4 °C for 15 min. The supernatant was discarded, and the pellet resuspended in the same buffer at a concentration of 0.2 g mL^{-1} (wet weight), the method was described by dos Santos et al. [2].

The reactional mixture consisted of 1:9 ratio of enzyme solution to substrate, one mmol L^{-1} of dithiothreitol, and a FA (0.5 g L^{-1}) prepared in sodium phosphate buffer 50 mM (pH 6.0). Reaction was maintained at 30.0 °C for five minutes, and the enzymatic activity was then halted by adding ethanol 96 ºGL in a 1:4 ratio (reaction mixture: ethanol).

FA decarboxylase activity was determined by FA reduction and 4-VG formation (µmol). One unit (U) of enzyme activity was defined as the amount of enzyme required to produce one µmol of 4-VG per minute under the assay conditions. Specific activity was calculated by relation of total enzyme unit (U) to the total proteins (mg) of the intracellular

fraction used in the reaction. The total protein content was determined using the Bradford method, with bovine serum albumin (BSA) as the standard.

2.6. FA Decarboxylase Characterization

2.6.1. Effect of pH and Temperature on Enzyme Activity and Stability

During the cell wall-associated crude enzyme characterization, the enzyme activity was evaluated across a pH range of 4.0 to 9.0, using 0.1 M sodium acetate (pH 4.0–5.0); MES (pH 5.5–6.5); HEPES (pH 7.0–8.0); BICINE (pH 8.5–9.0) at 30 °C. The temperature effect on the enzyme activity was determined in the range of 20 and 60 °C, at pH 5.5, following the previously described enzyme reaction. Enzyme stability was assessed by incubating the enzyme without substrate, while for pH stability, samples were incubated in buffers ranging from pH 4.0 to 9.0 at 4 and 25 °C for 60 min. Thermal stability was determined by incubating the crude enzyme solution at a temperature range from 20 to 50 °C for 60 min. The remaining decarboxylase activity was determined under standard assay conditions described above at 40 °C and pH 5.0.

2.6.2. Stability of Enzyme on Storage at Different Temperatures

The enzyme was stored at 4 °C and −20 °C for 30, 90, and 180 days, and the remaining activities were determined as described in the previous section after filtration in a 0.22 μm syringe filter.

2.7. Data Analysis

At least three independent replicates were included for each measurement, with a control experiment performed under the same conditions. Results are expressed as the mean of replicate values with their corresponding standard deviations (mean ± SD).

Statistical analyses were conducted through one-way ANOVA and followed by Tukey's post hoc test for multiple comparisons in GraphPad Prism 10.6.0, with significance defined at $p < 0.05$.

3. Results and Discussion

3.1. Studies on Bacterial Tolerance to FA and Its Transformation to 4-VG

There was no significant difference in bacterium growth at FA concentrations between 100 and 700 mg L^{-1}. (Figure 2A), although a slight decrease was observed at concentrations above 500 mg L^{-1}. For more details check Figure S9 in Supplementary material.

Figure 2B shows that during cultivation with 300 mg L^{-1} of FA, there was a rapid decrease in FA concentration within the first 6 h, during which no bacterial growth was observed. Once the FA level reached its minimum, glucose was rapidly consumed, which marked the onset of growth. Glucose concentration continued to decrease until 12 h of cultivation when the maximum biomass and the highest 4-VG concentration (236.8 ± 76.3 mg L^{-1}) were achieved. The 4-VG concentration remained stable for up to 24 h of cultivation. See Figure S10.

LC-MS chromatography revealed a proton ion peak at 151.1 m/z (Figure S2), consistent with the mass of 4-VG (150.17 g mol^{-1}). A yield of 99.5% was achieved for the bioconversion of FA to 4-VG, starting with an initial concentration of 258.7 ± 4.0 mg L^{-1} (1.71 mmol L^{-1}).

HPLC did not detect any FA-derived metabolites, except in samples collected between 12 and 14 h of cultivation when VA was observed (Figure 3).

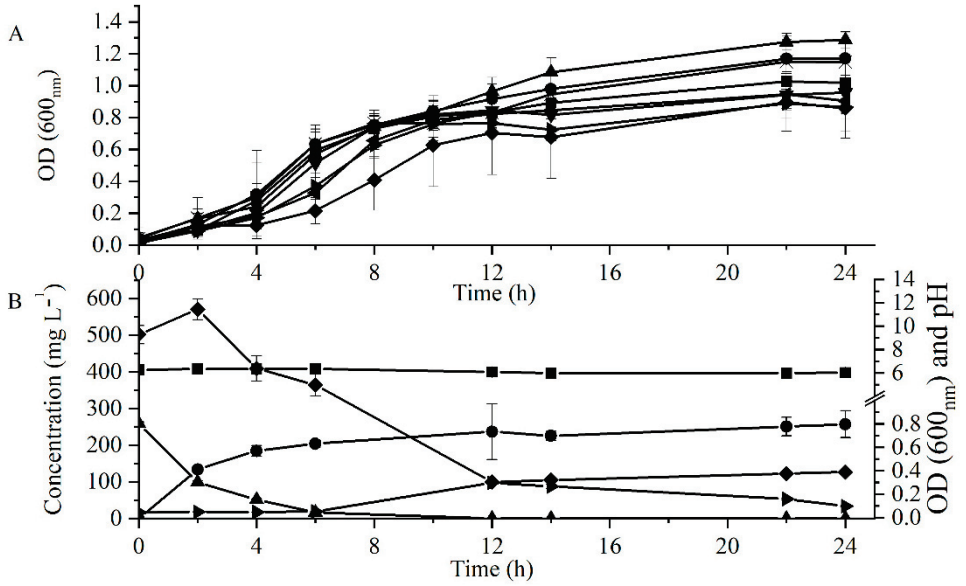

Figure 2. (**A**) Growth of *B. intermedia* TG 3.48 in nutrient medium containing 100 to 700 mg L^{-1} of FA. ■ 100 mg L^{-1}; ● 200 mg L^{-1}; ▲ 300 mg L^{-1}; ▼ 400 mg L^{-1}; ▸ 500 mg L^{-1} and ◂ 600 mg L^{-1}; ♦ 700 mg L^{-1} of FA. × = biotic control; and (**B**) *B. intermedia* TG 3.48 growth in a medium with 300 mg L^{-1} of FA. Left y-axis (concentration mg L^{-1}): ▲ = FA; ♦ = Glucose; ● = 4-VG. Right y-axis (pH up/OD down): ■ = pH; ▸ = microbial biomass. Data are mean ± SD of three replicates (n = 3).

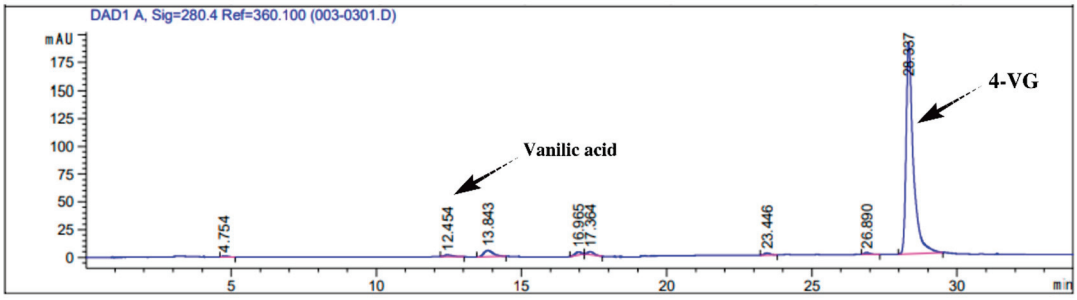

Figure 3. Chromatogram using a sample from 12 h of cultivation of *B. intermedia* TG 3.48 in a medium with 300 mg L^{-1} of FA, only showed the presence of VA as a FA derivative. The compounds were confirmed by LC-MS.

B. intermedia TG 3.48 has been shown to be capable of degrading xenobiotic compounds [21,31,32]. FA has been characterized as an antimicrobial agent with bacteriostatic effect, impacting cell mobility, biofilm formation, and energy metabolism. On the other hand, the microbial transformation of this compound could be seen as a detoxification action [33,34].

A concentration of FA around 500 mg L^{-1} inhibited the growth of *Pseudomonas aeruginosa* ATCC 10145 and *Escherichia coli* CECT4342, while *Staphylococcus aureus* CECT 976 was only inhibited at 5000 mg L^{-1} of FA [35]. *B. intermedia* TG 3.48 tolerated FA concentrations up to 700 mg L^{-1} without a significant decrease in biomass production (Figure 2A). The simultaneous consumption of glucose and FA suggests that the bacteria utilize the sugar as an energy and carbon source, while FA is biotransformed into 4-VG as a detoxification mechanism.

For the bioconversion of FA to 4-VG, 300 mg L^{-1} of FA was used to avoid inhibition in bacterial growth and to optimize the process. The concurrent decrease in FA concentration and formation of 4-VG (Figure S3) confirms the use of decarboxylation as a detoxifica-

tion mechanism. Similar results have been reported for *Enterobacter* sp. Px6-4 [36] and *K. pneumoniae* [2].

3.2. FA Decarboxylase Activity (FADase)

FADase activity was examined in the intracellular extract (lysed cell) using the whole cell, the cell wall fragments, and the culture medium. It was considered an extracellular enzyme in all the samples, except the intracellular extract. When the whole cell was used, an activity of 5.17 U mL^{-1} was determined. With the intracellular extract and the cell wall fragments, the activity was 4.40 U mL^{-1} and 3.54 U mL^{-1}, respectively (Figure 4 FADase Activity, B and C), while the specific activity was 778.90 U mg^{-1}. These findings indicate that the enzyme is associated with the cell. A small amount of 4-VG detected in the culture medium (0.35 U mL^{-1}) could be the result of cell disruption during cultivation.

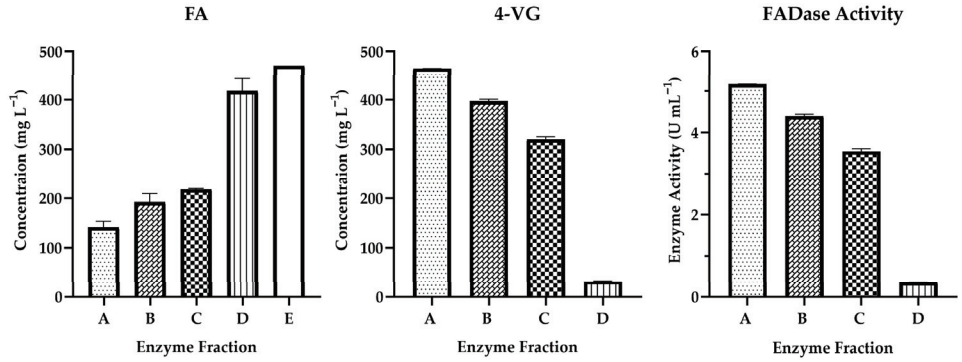

Figure 4. Residual ferulic acid (FA), 4-vinylguaiacol production (4-VG), and FADase activity. A: whole cell; B: intracellular extract; C: cell wall fragments; D: culture medium; E: control. Data are mean ± SD of three replicates (n = 3) and statistical significance (ANOVA/Tukey's post hoc test) was set at $p < 0.05$.

A one-way ANOVA confirmed the highly significant difference among the enzyme fractions for all tested parameters, including FADase activity, 4-VG production, and FA consumption (all $p < 0.0001$). Post hoc analysis with Tukey's test showed that the whole-cell fraction (A) had significantly higher FADase activity and produced significantly more 4-VG product compared to the culture medium (D) ($p < 0.0001$ for both comparisons). The negligible activity and product formation in the culture medium confirm that the FADase enzyme is cell-associated and is secreted into the medium only at low concentrations.

Our results confirm that the conversion of FA to 4-VG occurs via decarboxylation. The detection of cell-associated FADase activity supports this hypothesis. Data indicates that FADase is associated with the whole cell and cell wall fragments, suggesting it is an extracellular enzyme, but it was not released into the medium. This profile is consistent with enzymes involved in medium detoxification [37]. Similar findings were reported by Li et al. [36], where the intracellular activity of FADase was directly associated with an increase in 4-VG concentration in *Enterobacter* sp. Px6-4.

3.3. Enzyme Characterization

FADase exhibited maximum activity at pH 5.5 (Figure 5A) and at 50 °C, with activity in temperature range between 40 and 55 °C (Figure 5B). In the absence of any substrate, FADase remained stable within a pH range of 4.5 to 6.5 (Figure 5C), while remaining stable between 30 and 45 °C, it lost 40% of its initial activity at 50 °C and was inactivated at 55 °C (Figure 5D).

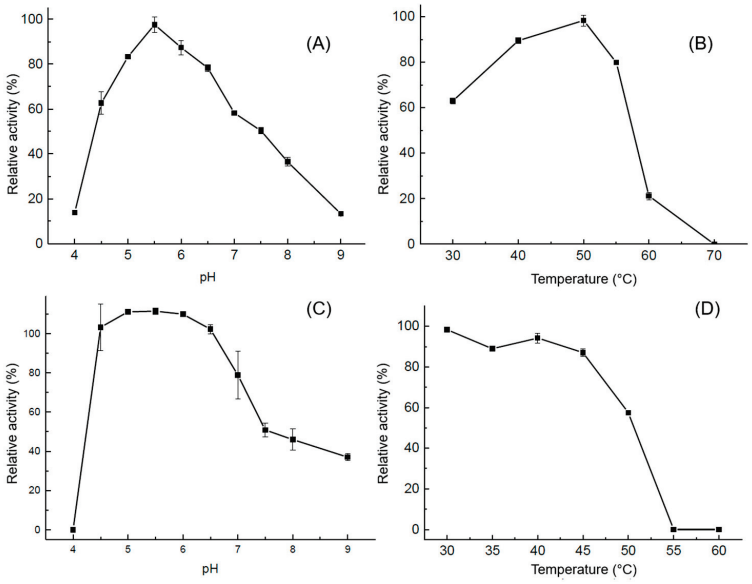

Figure 5. Effect of pH (**A**) and temperature (**B**) on enzyme activity. Effect of pH (**C**) and temperature (**D**) on the enzyme stability in absence of substrate. The maximal activity among the data was considered as 100%. Data are mean ± SD of three replicates (n = 3).

The enzyme remained stable for 180 days when stored at −20 °C. At 4 °C, it retained 60% of its initial activity after 90 days and 40% after 180 days (Figure 6).

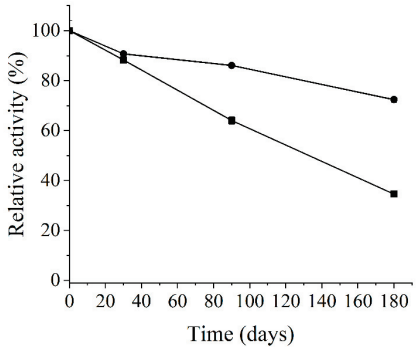

Figure 6. Effect of temperature on enzyme storage. ■ = 4 °C; ● = −20 °C. 100% of activity was considered based on the unincubated enzyme.

The physicochemical properties of FADase, including maximum activity at pH 5.0 and 6.0, are consistent with other FADases, such as that described by Santos et al. [2] from *K. pneumoniae* TD 4.7 and by Maeda et al. [38] from *Bacillus* sp. BP-7. However, the highest activity observed at 40 and 55 °C indicates that these enzymes are more thermostable than those from *Candida guilliermondii*, which has an optimal temperature at 25 °C, *P. fluorescens* UI 670 at 27 to 30 °C, *B. pumilus* PS213 at 37 °C, and *K. pneumoniae* TD 4.7 at 40 °C [2,37,39,40].

3.4. Products of FADase Activity

The degradation of FA via 4-VG is a pathway commonly employed by bacteria, filamentous fungi, and yeasts, but with differences in efficiencies and resulting metabolites. In many studies, 4-VG is reported as an intermediate metabolite resulting from the decarboxylation of FA. This intermediate can be further biotransformed into acetovanillone or vanillin. Oxidation of vanillin produces VA, which can be subsequently bioconverted into protocatechuic acid or catechol [2,13,41,42].

In this study, only trace amounts of VA were detected, indicating that *B. intermedia* TG 3.48 can convert 4-VG to vanillin and subsequently to VA, as suggested in the literature [43]. The non-detection of vanillin may be due to its rapid conversion to VA. The maintenance of 4-VG concentration after the maximum production peak at 12 h of cultivation suggests that this metabolite was not used as a carbon source and was minimally converted to other metabolites (vanillin, VA, and catechol) [11]. However, it is possible that, with cultivation exceeding 24 h, further metabolization of 4-VG to vanillin and VA may occur, as observed in *Streptomyces setonii* by Salgado et al. [44]. These findings are interesting from a biotechnological perspective, indicating the potential use of this bacterium for single step production of 4-VG at high yield.

4. Conclusions

To our knowledge, this study is the first report that *B. intermedia* TG 3.48 biotransforms FA to 4-VG via a cell-wall-associated FADase. The strain tolerates high FA concentrations and achieves a high conversion yield, while the enzyme is stable between 30 and 40 °C and operates without added cofactors, features that simplify reactor design and reduce operating costs. Although vanillin was not detected in the culture, likely due to rapid conversion, the strain can further convert 4-VG into downstream derivatives such as vanillic acid, indicating useful substrate scope for valorizing lignocellulosic FA into value-added aroma and chemical intermediates. When compared with conventional biotransformation routes that require cofactor supplementation or regeneration, or to free enzyme approaches, which typically demand purification, can be less stable, and suffer greater sensitivity to substrate and product toxicity, *B. intermedia* TG 3.48 stands as a practical and competitive biocatalyst for valorizing FA from lignocellulosic biomass, because of the combination of species-level novelty, cell-wall-associated localization, cofactor independence, thermal stability in a mesophilic range, high substrate tolerance, and elevated bioconversion rates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr13103367/s1, Figure S1. Chromatogram obtained from a sample of medium after 24 h of cultivation of the bacterium *B. intermedia* TG 3.48, where the peak at 28.6 min represents 4-VG. Figure S2. Chromatogram (A) and mass spectrum (B) for 4-VG, determined by LC-MS. Figure S3. FA consumption profile and 4-VG formation at different incubation times. Figure S4. Chromatogram corresponding to the retention time of the FA standard. Figure S5. Chromatogram corresponding to the retention time of the 4-VG standard. Figure S6. Chromatogram corresponding to the retention time of the catechol standard. Figure S7. Chromatogram corresponding to the retention time of the vanillic acid standard. Figure S8. Chromatogram corresponding to the retention time of the vanillin standard. Figure S9. Growth profiles of the bacterium *Brucella intermedia* TG 3.48 at concentrations of (A) 100 to 500 mg L^{-1} FA and (B) between 500 and 700 mg L^{-1} FA. Figure S10. *B. intermedia* TG 3.48 growth in a medium with 300 mg L^{-1} of FA. Left y-axis (concentration mg L^{-1}): ▲ = FA; ♦ = Glucose; ● = 4-VG. Right y-axis (pH up/OD down): ■ = pH; ■ = microbial biomass.

Author Contributions: Conceptualization: E.G.; methodology: E.G., M.B.C.d.S., M.A.Z., É.J.P. and M.B.; investigation: S.P.d.C. and K.S.D.; data curation: E.G., M.B.C.d.S., M.B. and R.d.S.; formal analysis: E.G. and M.B.C.d.S.; writing—original draft preparation: S.P.d.C., E.G. and M.A.Z.; writing—review and editing: E.G., M.A.Z. and É.J.P.; visualization, R.R.d.S.; supervision: E.G. and M.B.C.d.S.; project administration: E.G., M.B. and R.d.S.; funding acquisition: E.G., M.B. and R.d.S. All authors have read and agreed to the published version of the manuscript.

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processes

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Review

From Waste to Wonder: Valorization of Colombian Plant By-Products for Peroxidase Production and Biotechnological Innovation

John J. Castillo

Escuela de Química, Universidad Industrial de Santander, Bucaramanga 680002, Colombia; jcasleon@uis.edu.co; Tel.: +57-76344000 (ext. 2893)

Abstract

The valorization of agricultural by-products represents a sustainable strategy to reduce waste and create high-value biotechnological products. This review highlights Colombian plant-derived peroxidases (PODs) obtained from Guinea grass, royal palm, African oil palm, lemongrass, sleepy plant, and sweet potato. These enzymes catalyze oxidative reactions and show potential in biosensing, polymer synthesis, environmental remediation, and health monitoring. We summarize extraction and purification strategies while addressing current challenges such as operational stability, scalability, and cost. Special emphasis is given to applications like cross-linked enzymatic aggregates (CLEAs) and electrochemical biosensors, where Colombian PODs demonstrate superior stability and sensitivity compared to horseradish peroxidase (HRP). This review frames these advances within the circular bioeconomy, presenting insights into waste reduction and CO_2 savings. By integrating local biodiversity into innovative processes, Colombian PODs can drive sustainable technologies and provide industrial and environmental solutions.

Keywords: agricultural by-products; peroxidases; biotechnological applications

1. Introduction

The global shift toward sustainability has intensified interest in innovative strategies to repurpose agricultural residues [1–3]. These by-products, including crop residues, peels, leaves, and stems, are often discarded or burned, contributing to pollution and greenhouse gas emissions [3–7]. However, this organic waste contains valuable biomolecules such as enzymes, proteins, and fibers that can be repurposed for high-value applications. The concept of waste valorization, particularly in the context of the circular bioeconomy, involves transforming these by-products into new products that can be reintegrated into the economy, thus extending the lifecycle of the original resource [5,8,9]. The circular bioeconomy is a model that emphasizes the sustainable management of biological resources through the reuse, recycling, and regeneration of materials [8,10]. In the context of agriculture, this means finding innovative ways to utilize by-products that are often discarded, thereby reducing the environmental footprint of agricultural activities while adding value to waste materials [2,3,11]. Agricultural by-products, which are often rich in bioactive compounds, represent a promising source of raw materials for the production of enzymes, biopolymers, and other high-value chemicals [5,12].

Colombia, recognized for its biodiversity and strong agricultural economy, generates large volumes of plant-based residues that remain underutilized. Extracting peroxidases

(PODs) from these materials represents a dual opportunity: reducing waste and developing sustainable enzymes for industry [13]. In recent years, PODs have garnered significant attention for their role in various industrial and environmental processes [14]. These enzymes have been employed in biosensing technologies, where they facilitate the detection of H_2O_2, a critical marker in many biochemical reactions. Additionally, as is shown in Figure 1, PODs have shown promise in the synthesis of advanced materials, such as conducting polymers like polyaniline (PANI) [15], further extending their utility in material science. Their capacity to form cross-linked enzymatic aggregates (CLEAs) [16] also makes them valuable tools for bioremediation, particularly in the removal of industrial pollutants such as dyes, as well as their use in chemiluminescence assays. The biotechnological potential of PODs extracted from Colombian plant by-products represents a convergence of waste valorization and innovation. Plants such as Guinea grass (*Panicum maximum*), royal palm (*Roystonea regia*), African oil palm (*Eleais guineensis*), lemongrass (*Cymbpogon citratus*), sleepy plant (*Mimosa pudica*), and sweet potato (*Ipomea batatas*) produce significant amounts of agricultural waste [17–20]. By extracting and purifying PODs from these plants, researchers can harness their enzymatic properties for a variety of applications, contributing to both a reduction in waste and the development of sustainable technologies.

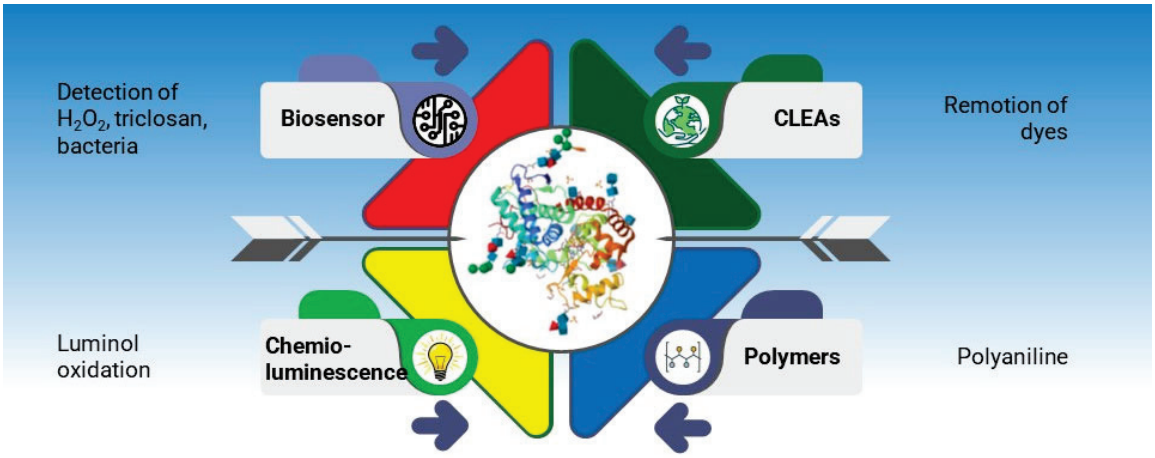

Figure 1. Novel biotechnological applications of Colombian plant-derived PODs from agricultural by-products, emphasizing their use in biosensing, advanced materials, and environmental remediation.

In Colombia, where agriculture plays a vital role in the national economy, the potential for waste valorization is particularly high. The country's diverse ecosystems and wide variety of crops generate substantial amounts of plant-based by-products that could be repurposed for biotechnological applications. By leveraging the biochemical properties of enzymes like POD, Colombia can contribute to global efforts in sustainability while also fostering innovation in sectors such as environmental remediation and health monitoring [1,21].

PODs are a class of enzymes that catalyze the oxidation of various substrates in the presence of H_2O_2. These enzymes are widely distributed in plants and play an important role in defense mechanisms against environmental stress [22,23]. In biotechnological contexts, PODs are valued for their ability to facilitate oxidative reactions, making them useful in a range of applications including biosensors, polymer synthesis, and bioremediation [24,25].

As is shown in Figure 2, Colombian plants such as Guinea grass (*Panicum maximum*), Royal palm (*Roystonea regia*), African palm (*Elaeis guineensis*), lemongrass (*Cymbopogon citratus*), sleepy plant (*Mimosa pudica*), and sweet potato (*Ipomoea batatas*) are rich sources of PODs.

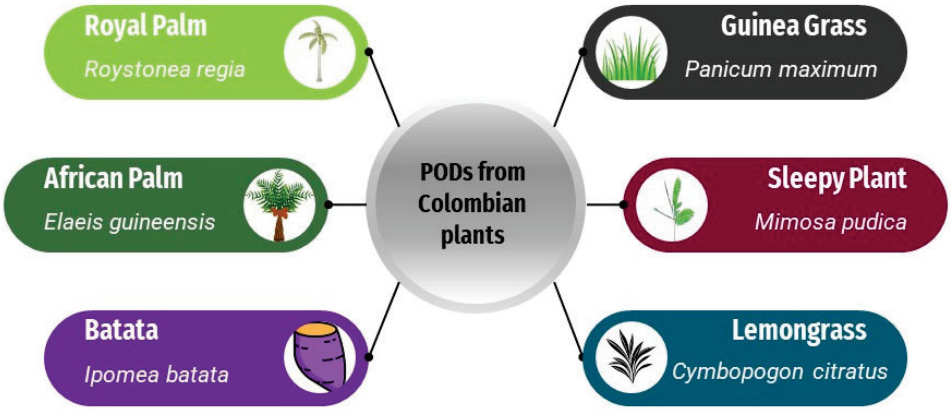

Figure 2. Main Colombian plant sources of PODs with high potential for sustainable enzyme production.

These plants produce significant agricultural by-products that can be exploited for enzyme extraction, turning waste into valuable biotechnological resources. The extraction and purification of PODs from these plants not only contribute to waste valorization but also provide a renewable source of enzymes for industrial applications [26].

Finally, the valorization of Colombian plant by-products for POD production represents a powerful example of how waste can be transformed into valuable resources within the framework of the circular bioeconomy. By harnessing the biochemical properties of peroxidases, researchers can develop innovative solutions to pressing environmental and industrial challenges. From biosensing technologies to environmental remediation, the applications of these enzymes are vast and diverse.

The purpose of this review is to provide a comprehensive overview of the current state of the research on peroxidase extraction from Colombian plant by-products, showcasing their potential as a renewable and sustainable source of enzymes for various biotechnological applications. This review highlights the potential of Colombian plant by-products as a renewable source of PODs, encouraging further research and development in this promising field. Despite the promising potential of Colombian plant-derived PODs, significant research gaps remain. First, there is a lack of comprehensive kinetic modeling to better understand the catalytic mechanisms of these enzymes, which is crucial for optimizing their performance in industrial and biosensing applications. Second, structural characterization studies, such as X-ray crystallography and molecular dynamics simulations, are limited, hindering insights into the relationship between enzyme structure and function. Finally, there is an evident scarcity of large-scale application studies aimed at transitioning these findings from laboratory settings to real-world industrial or environmental processes. Addressing these gaps is essential to fully unlocking the biotechnological potential of Colombian PODs and to fostering innovations aligned with the circular bioeconomy framework.

2. Agricultural By-Products as a Source of Peroxidases

Colombia's rich and diverse agricultural landscape is a key pillar of the country's economy, contributing significantly to both domestic markets and international trade. With its favorable climate and vast ecosystems, Colombia produces a variety of crops, generating large quantities of agricultural by-products [13,27–30]. These by-products, traditionally considered waste, are now recognized as valuable resources that can be valued for biotechnological and industrial applications. Waste valorization of these by-products not only contributes to sustainability but also adds value to agricultural production, supporting the circular bioeconomy. Several key plants and their by-products stand out for their potential in areas like energy, food, and biotechnology. Sakharov y cols., conducted a preliminary

study to analyses the enzymatic activity of various Colombian plant species, including their leaves, fruits, and roots (Table 1) [31]. This research marked one of the earliest explorations into the potential of Colombian flora for biotechnological applications. The study focused on the extraction of enzymes such as PODs. The findings highlighted the rich enzymatic diversity present in Colombian plants and opened the door to further investigations into their potential for waste valorization and sustainable production. Building on this foundational research, the focus on agricultural by-products as a source of valuable enzymes has continued to grow, driving advancements in both scientific understanding and practical applications. The study identified several plant species that exhibited particularly promising enzymatic activity, making them strong candidates for further exploration in biotechnological applications. Among these, Guinea grass, royal palm, African palm, lemongrass, sleepy plant, and sweet potato stood out for their significant potential in enzyme production and waste valorization.

Table 1. Comparison of peroxidase activity across plant parts (adapted from [31]).

Plant Part	Source of POD	POD Activity (U/g)
Fruits	Almond (*Terminalia catappa*)	<1.0
	Cocoa (*Theobroma cacao*)	11.6
	Coffee (*Coffea arabica*)	22.9
	Cocoa palm (*Cocos nucifera*)	1.2
	Tree tomato (*Cyphomandra betacca*)	16.2
	Totumo (*Crescentia cujete*)	5.8
Roots	Celery (*Apium graveolens*)	58.0
	Arracacha (*Arracacia xanthorrhiza*)	<1.0
	Sweet potato (*Ipomea batatas*)	1800.0
	Coriander (*Coriandrum sativum*)	35.0
	Bore (*Colocasa esculenta*)	370.0
	Ginger (*Zingeber officinale*)	11.6
	Red radish (*Rapharus sativas*)	121.3
	Horseradish (*Armoracia rusticana*)	2600.0
	Cassava (*Manihot esculenta*)	1.7
Leaves	Oleander (*Nerium oleander*)	98.3
	Pear cactus (*Monstera delisiosa*)	179.0
	Banana (*Musa sapientum*)	49.7
	Bamboo (*Bambusa guadua*)	<1.0
	Spanish moss (*Tillandsia recurvata*)	5.2
	Boojum tree (*Cereus hexagonus*)	19.0
	Marigold (*Calendula oficionales*)	231.2
	Bottlebrush (*Callistemon lanceolatu*)	<1.0
	Sugar cane (*Sacharum officinarum*)	104.0
	Sleepy plant (*Mimosa pudica*)	460.0
	Fique (*Agave fourcroides*)	19.6
	Fern (*Adiamtum obliguum*)	<1.0
	Castor bean plant (*Ricinum communis* L.)	440.0
	Lemongrass (*Cymbopogon citratus*)	390.0
	Fan palm (*copernica pectori*)	220.0
	African oil palm (*eleais guineensis*)	566.0
	Date palm (*Phoenix dactilera*)	580.0
	Royal palm (*Roystonea regia*)	694.0
	Coconut palm (*Cocos nucifera*)	48.6
	Corozo palm (*Acrocomia aculeata*)	570.0
	Wine palm (*Scheelea butyracea*)	173.4
	Thatch palm (*Astrocarium* sp.)	220.0
	Macaw palm (*Bactris* sp.)	196.0
	Palma mararai (*Aiphanes cariotifolia*)	1145.0
	Guinea Grass (*Panicum maximum*)	980.0
	Parsley (*Petroselinum sativum*)	35.0

2.1. Guinea Grass (Panicum maximum)

It is widely cultivated in Colombia, particularly in the livestock sector. It serves as high-quality fodder for cattle, but the leftover biomass, such as stems and leaves, often remains underutilized [17]. Valorization of these by-products can include the production of bioenergy through anaerobic digestion, turning waste into biogas, and extraction of fibers for biodegradable packaging materials. Additionally, Guinea grass has been explored for its potential in phytoremediation, where it is used to clean contaminated soils, contributing to environmental sustainability.

2.2. Royal Palm (Roystonea regia)

This is another species that produces significant agricultural by-products, primarily from its fronds and seeds. Traditionally, these materials have been discarded or used as mulch, but recent advances have explored their use in biochar production, which can serve as a soil enhancer and carbon sequestration tool. Royal palm by-products can also be transformed into activated carbon, which is highly effective in water purification processes, adding environmental and economic value to this previously wasted material [18,32,33].

2.3. African Palm (Elaeis guineensis)

This plays a crucial role in Colombia's economy as a major source of palm oil. However, the industry generates large amounts of by-products, including empty fruit bunches, palm kernel shells, and fibers. These residues can be valorized in various ways, such as converting them into biofuels or using them in the production of biocomposites [18]. Palm oil mill effluent, a liquid by-product, is rich in nutrients and can be treated to produce biogas or used in the production of organic fertilizers, promoting a closed-loop system in agriculture.

2.4. Lemongrass (Cymbopogon citratus)

This is known for its essential oils, which are widely used in the fragrance and food industries. The remaining biomass, such as leaves and stalks, can be repurposed through extraction of bioactive compounds, including antioxidants and antimicrobial agents. Lemongrass waste has also shown promise in bioenergy production, where it can be converted into bioethanol or used as a raw material to produce natural fibers [33–36].

2.5. Sleepy Plant (Mimosa pudica)

This species, with unique movement in response to touch, is often used for medicinal purposes in Colombia. Its leaves, stems, and roots contain compounds with antibacterial, antifungal, and anti-inflammatory properties, making it a potential source for pharmaceutical and cosmetic applications. Waste valorization efforts can focus on extracting these bioactive compounds for commercial use, reducing environmental waste while creating high-value products [37–39].

2.6. Sweet Potato (Ipomea batata)

This is a staple crop in Colombia, and its peels and other by-products can be repurposed in various ways. Sweet potato peels are rich in starch and antioxidants, making them valuable for food additives or as a substrate for microbial fermentation in bioethanol production. The valorization of sweet potato by-products aligns with the circular bioeconomy by reducing food waste and creating new value chains in food, energy, and material science industries [20,40–43].

3. Peroxidases: Definition, Purification and Biochemical Properties

3.1. Peroxidases

PODs are a diverse group of enzymes that catalyze the oxidation of various substrates by H_2O_2 as an electron acceptor. These heme-containing enzymes play critical roles in both plants and animals, though their functions in plants are particularly significant. In plants, peroxidases are primarily involved in defense mechanisms, growth regulation, and the metabolism of reactive oxygen species (ROS) [14,44]. The biological role of peroxidases is closely linked to their ability to detoxify H_2O_2, a by-product of various metabolic processes that can cause oxidative damage to cells. By breaking down H_2O_2 into water and oxygen, PODs help mitigate oxidative stress, protecting cellular components such as lipids, proteins, and DNA from damage. In addition to their role in oxidative stress management, peroxidases are also essential for lignin biosynthesis, a process critical for cell wall formation and the structural integrity of plants. Lignin strengthens plant tissues and contributes to water transport by making cell walls less permeable to water [45]. Furthermore, PODs participate in wound healing, pathogen defense, and the modulation of hormone levels, which impacts plant growth and development. In response to biotic and abiotic stresses, peroxidases facilitate the cross-linking of cell wall components, enhancing the plant's defensive barrier against pathogens [22,46]. The enzymatic activity of PODs is defined by their ability to transfer electrons from a wide variety of donor molecules, such as phenolic compounds, to hydrogen peroxide. This reaction not only helps in the degradation of H_2O_2 but also contributes to the oxidative polymerization of organic molecules, including lignin precursors. The broad substrate specificity of peroxidases allows them to participate in diverse physiological processes, making them versatile enzymes with substantial importance in plant biology.

3.2. Extraction and Purification Techniques

The extraction and purification of PODs are crucial steps in studying their structure, function, and potential biotechnological applications. Due to the widespread occurrence of PODs in various plant tissues, isolating these enzymes involves selecting appropriate sources and employing techniques that maximize yield and purity while preserving enzymatic activity. The extraction process typically begins with the disruption of plant tissues to release intracellular enzymes, followed by a series of purification steps designed to separate PODs from other proteins and impurities. Common purification methods include precipitation, dialysis, chromatography, and ultrafiltration, each tailored to exploit the unique biochemical properties of PODs such as molecular weight, charge, and hydrophobicity. A well-designed extraction and purification protocol is essential for obtaining peroxidases in sufficient quantities and purity for further biochemical characterization and practical applications.

The purification of PODs from Colombian tropical plants has yielded promising results, with several species demonstrating high enzymatic activity. Among these, the leaves of the royal palm have proven to be a particularly rich source of POD, leading to the isolation of a novel enzyme with high purity. The purification process for this POD involved several key steps. Initially, the palm leaves were homogenized, and ammonium sulfate ($(NH_4)_2SO_4$) precipitation was used to concentrate the enzyme while removing unwanted proteins [47]. Following this, colored compounds were extracted to reduce interference during the purification process. The enzyme was then subjected to successive chromatographic steps, including hydrophobic interaction chromatography on Phenyl-Sepharose, size-exclusion chromatography using Sephacryl S100 (Cytiva, Marlborough, MA, USA), and ion-exchange chromatography on DEAE-Toyopearl. This multi-step process resulted in a highly purified peroxidase with a specific activity of 6170 U/mg. Similarly, PODs

from the leaves of guinea grass have been isolated and partially purified using a biphasic polymer system consisting of polyethylene glycol (PEG) and ammonium sulfate [26]. This method allowed for an efficient separation of peroxidase from other proteins, followed by size-exclusion chromatography and ultracentrifugation to obtain a highly active and homogeneous enzyme preparation. In another approach, sweet potato peels were utilized as a POD source [20]. The purification protocol involved homogenization, removal of pigments, and subsequent chromatographic steps using Phenyl-Sepharose and DEAE-Toyopearl columns. This method produced sweet potato peroxidase (SPP) of high purity. Figure 3 shows a schematic representation of the basic stages in the purification of PODs extracted from Colombian plants. These methods underscore the versatility of purification strategies for plant PODs, highlighting the success of using both traditional and innovative techniques to achieve highly purified enzyme preparations from diverse plant sources.

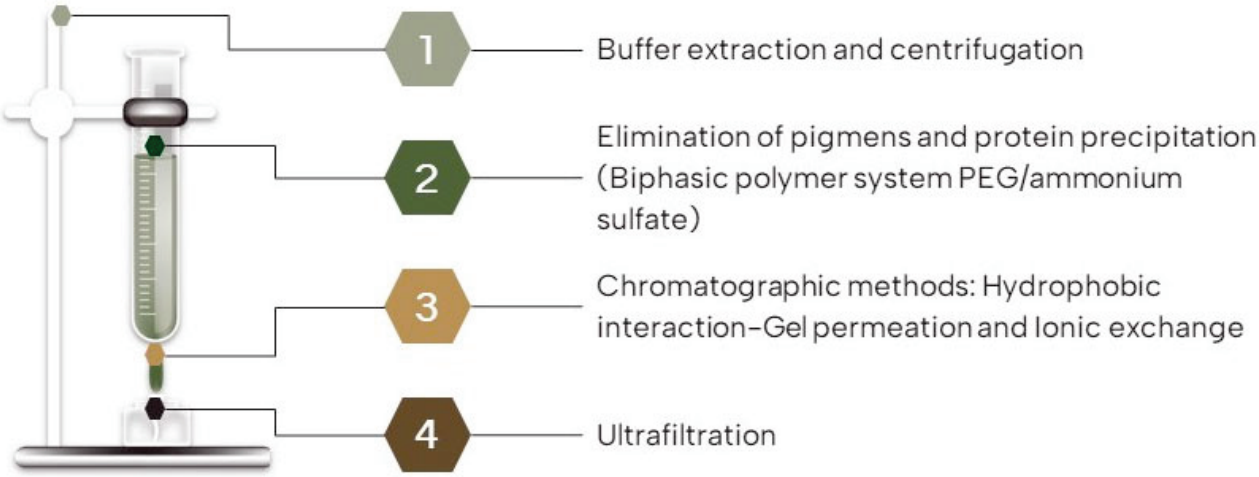

Figure 3. Stepwise protocol for POD extraction and purification from Colombian plants, illustrating innovative approaches that improve stability and scalability for biotechnological applications.

While the protocols described above have been successful in isolating highly active peroxidases from Colombian plant sources, each approach presents distinct advantages and limitations that must be considered when selecting a purification strategy. For instance, traditional precipitation methods, such as ammonium sulfate fractionation, are low-cost and simple to implement, making them suitable for small-scale laboratory extractions. However, they often result in moderate enzyme yield and can cause partial loss of enzymatic activity due to protein denaturation during salt removal steps. Chromatographic techniques, including hydrophobic interaction, ion-exchange, and size-exclusion chromatography, offer high specificity and purity, as demonstrated for royal palm and sweet potato PODs. Nevertheless, they are time-consuming, require specialized equipment, and may have limited scalability for industrial applications. Biphasic polymer systems, such as PEG/ammonium sulfate partitioning, have shown promise for Guinea grass peroxidase, providing a higher yield and preserving enzyme stability by minimizing exposure to harsh conditions. Despite these benefits, polymer-based systems can be costly and generate waste streams that require proper management. Furthermore, the choice of method directly impacts the long-term stability of the enzyme, which is critical for biosensor fabrication and industrial catalysis. Future efforts should focus on developing integrated purification workflows that balance yield, purity, cost, and environmental sustainability, potentially incorporating green extraction techniques and continuous processing to facilitate large-scale production. Table 2 illustrates the main extraction and purification techniques of Colombian plant PODs.

Table 2. Summary of extraction and purification techniques of Colombian plant peroxidases, including yield, advantages, limitations, and main biotechnological applications.

Plant Source	Extraction Technique	Yield/Specific Activity	Advantages	Drawbacks	Applications
Royal palm (*Roystonea regia*)	Homogenization → Ammonium sulfate precipitation → Hydrophobic interaction + ion-exchange chromatography	6170 U/mg [32]	High purity, excellent thermal stability	Time-consuming, high cost, not easily scalable	Electrochemical biosensors, high-temperature industrial catalysis
Guinea grass (*Panicum maximum*)	Biphasic polymer system (PEG/ammonium sulfate) → Size-exclusion chromatography	2000–3000 U/mg [26]	Good stability preservation, moderate cost	Polymer disposal issues, requires optimization	Biosensors for H_2O_2, environmental monitoring
Sweet potato (*Ipomea batatas*)	Homogenization → Pigment removal → Hydrophobic interaction + ion-exchange chromatography	1800 U/mg [20]	High substrate specificity, compatible with food industry	Moderate yield, pigment interference can complicate extraction	Food biosensors, wastewater treatment
African oil palm (*Elaeis guineensis*)	Homogenization → Ammonium sulfate precipitation → Chromatographic purification	2500 U/mg [33]	Good thermal stability, wide pH tolerance	Limited studies on scalability	Environmental remediation, CLEA synthesis
Lemongrass (*Cymbopogon citratus*)	Aqueous extraction → Ammonium sulfate precipitation → Chromatography	1200 U/mg [34]	Easy implementation, accessible raw material	Enzyme unstable at pH > 7	Biosensors for H_2O_2 and phenolic compounds
Sleepy plant (*Mimosa pudica*)	Aqueous extraction → Ammonium sulfate precipitation → DEAE-Toyopearl chromatography	460 U/mg [38]	Very low detection limit with gold electrodes	Narrow pH stability range	Biosensors for sensitive biomedical detection

3.3. Biochemical Properties

PODs are versatile enzymes with biochemical properties influenced by pH, temperature, and substrate specificity, which are critical for their functionality in various applications [48]. Typically, PODs exhibit optimal activity in slightly acidic to neutral pH ranges, generally between pH 4 and 7, though some can function in broader pH conditions depending on their source. Temperature stability is another key factor, as most PODs maintain stability and high activity at moderate temperatures (25–45 °C), but their activity may diminish, or enzymes can denature at higher temperatures. Additionally, substrate specificity is a defining characteristic of PODs, as they catalyze reactions involving hydrogen peroxide with various electron donor substrates such as phenols, aromatic amines, and certain organic compounds, though preference varies across enzyme types [49]. Understanding these general properties provides a foundation for examining the unique biochemical traits of PODs derived specifically from Colombian plants, which may offer novel or enhanced attributes.

Table 3 highlights the pH and temperature optimum, inactivation constants, and substrate specificity for PODs derived from six plant sources: royal palm, African oil palm, Guinea grass, lemongrass, sweet potato, sleepy plant, and horseradish. Each enzyme's unique characteristics underscore the diversity in PODs' biochemical profiles and offer practical information for selecting specific PODs based on environmental stability and target substrate compatibility. A key observation in the table is the significant variability in

the pH optima of these PODs. While many of the PODs demonstrate optimal activity in a broad range of pH values (for instance, African oil palm POD operates effectively from pH 4.0 to 9.0), others, such as sleepy plant POD, show a narrower optimal pH of 4.0. This range of pH adaptability indicates that these enzymes are well-suited for environments with fluctuating or extreme pH conditions, broadening the potential for POD applications across different industrial process. Horseradish peroxidase (HRP), commonly used as a benchmark enzyme, has a pH optimum between 6.0 and 6.5, restricting its usage in acidic conditions. By contrast, the broader pH stability of PODs like those from royal palm and African oil palm could offer enhanced versatility, particularly in biochemical processes where pH stability is a critical factor. Temperature stability, represented by the temperature optimum and inactivation constants, also varies across the POD sources. Notably, royal palm POD exhibits an impressive temperature optimum of 90 °C, significantly higher than that of HRP (25–30 °C). This high thermal tolerance makes it a promising candidate for applications in high-temperature environments, such as industrial catalysis or bioremediation in warmer climates. African oil palm and Guinea grass PODs, with optima at 72 °C and 66 °C, respectively, also show good thermal resilience, although slightly less than royal palm POD. Inactivation constants provide additional insight into thermal stability, with African oil palm and HRP showing relatively low inactivation rates (2.0×10^{-3} and 1.0×10^{-3} min^{-1}, respectively), indicating a slower rate of enzyme denaturation under prolonged exposure to heat. However, the higher inactivation constant of royal palm POD (1.5×10^{-2} min^{-1}) suggests that while it withstands high temperatures, its activity may decrease more quickly over time at these temperatures compared to HRP. Substrate specificity is another critical factor for enzyme selection, as it determines the types of reactions each POD can facilitate. All the PODs in the table show activity toward multiple substrates, with common electron donors like ABTS, ferulic acid, guaiacol, and o-dianisidine. Royal palm, African oil palm, and sweet potato PODs exhibit specificity for ferulic acid, which is valuable in phenolic compound oxidation processes often needed in environmental applications such as pollutant degradation. Lemongrass, Guinea grass, and HRP show specificity for guaiacol and o-dianisidine, indicating their suitability for different types of redox reactions commonly used in biosensing and diagnostic applications. HRP, well-known for its versatility, reacts efficiently with both ABTS and o-dianisidine, but its more limited stability under high temperatures makes it less suitable for high-temperature applications despite its broad substrate compatibility.

Beyond pH, temperature, and substrate specificity, understanding the kinetic behavior of peroxidases is essential for predicting their catalytic performance in different applications. Key kinetic parameters such as Km (Michaelis constant) and Vmax (maximum reaction velocity) provide insights into the affinity between the enzyme and its substrates. For instance, SPP has been reported with Km values ranging from 0.12 to 0.20 mM for substrates such as ABTS and o-phenylenediamine, indicating a high substrate affinity [20]. In comparison, GGP shows slightly higher Km values (0.30–0.45 mM), which correlates with its broader specificity but lower catalytic efficiency [26]. These parameters are critical for the design of biosensors where rapid and specific substrate turnover is desired. The structural stability of Colombian plant PODs has also been a topic of interest due to their ability to withstand extreme conditions. For example, royal palm peroxidase maintains over 80% of its activity at 90 °C, exhibiting a half-life of 50 min under these conditions [32]. Similarly, African oil palm peroxidase demonstrates stability across a wide pH range (4.0–9.0), which is advantageous for industrial processes involving variable reaction environments [19,33]. Such stability profiles are crucial for enzyme immobilization in biosensors and for repeated use in continuous industrial processes. Another important property is the redox potential (E°') of the heme active site, which determines the enzyme's ability to catalyze oxidative

reactions. Colombian PODs, such as royal palm and GGP, display redox potentials between +0.85 and +0.92 V vs. Ag/AgCl, values comparable to HRP [44,48]. A higher redox potential allows these enzymes to oxidize a broader range of phenolic and aromatic amine substrates, expanding their application in pollutant degradation and biosensing technologies. The integration of kinetic data, structural stability, and redox properties provides a holistic understanding of these enzymes, allowing for the rational selection of peroxidases for specific biotechnological applications and guiding future protein engineering efforts.

Table 3. Comparative biochemical properties of peroxidases from Colombian plants.

PODs Source	pH Optimum	Temperature Optimum (°C)	Inactivation Constant (min^{-1})	Substrate Specificity	Reference
Royal palm	7.0–9.0	90	1.5×10^{-2}	Ferulic acid ABTS	[32]
African oil palm	4.0–9.0	72	2.0×10^{-3}	Ferulic acid ABTS	[19]
Guinea grass	7.0–9.0	66	8.0×10^{-3}	Guaiacol ABTS *o*-dianisidine	[26]
Lemongrass	4.0–6.0	66	1.0×10^{-2}	Guaiacol *o*-dianisidine	[34]
Sweet potato	8.0	60	7.0×10^{-3}	Ferulic acid ABTS *o*-Phenylene diamine	[20]
Sleepy plant	4.0	55	7.0×10^{-3}	-	[38]
Horseradish	6.0–6.5	25–30	1.0×10^{-3}	*o*-dianisidine ABTS	[50]

4. Biotechnological Applications of Peroxidases

4.1. Electrochemical Biosensing

Electrochemical biosensing is a powerful approach for detecting H_2O_2 and other analytes of biomedical and environmental significance [51–53]. A central element in this method is the use of PODs sourced from plants, which can catalyze reactions involving H_2O_2, producing signals measurable Via electrochemical techniques. Plant-derived PODs are ideal for biosensors due to their efficiency in redox reactions, stability under various environmental conditions, and natural availability. They catalyze the oxidation of hydrogen peroxide, producing measurable electrochemical signals that correlate with H_2O_2 concentration. This ability is especially valuable, as H_2O_2 plays crucial roles in cellular signaling and oxidative stress in biomedical fields, and it also acts as a key pollutant indicator in environmental monitoring [49].

Electrochemical biosensors work by transducing a biochemical interaction into a readable electronic signal, typically using a working electrode modified with a biorecognition element, such as plant POD, that interacts specifically with the analyte of interest. In the case of H_2O_2, the enzyme's active site catalyzes its decomposition, triggering electron transfer between the analyte and electrode surface. This interaction yields a current or voltage change, providing a quantifiable measure of H_2O_2 levels. Notably, the specificity and sensitivity of plant PODs make them versatile for designing biosensors tailored for both clinical and environmental applications. This process is beneficial because it enables low-cost, efficient, and eco-friendly monitoring of H_2O_2, providing valuable insights into various medical conditions like inflammation, cancer, and cardiovascular diseases, which

are often linked to oxidative stress and cellular damage associated with abnormal H_2O_2 levels [54]. Beyond H_2O_2 detection, plant-derived peroxidases have shown significant potential for detecting other biologically and environmentally relevant analytes. For instance, they can be engineered to sense glucose, cholesterol, and uric acid, analytes critical for metabolic health monitoring. In glucose sensing, peroxidase enzymes facilitate the oxidation of glucose in the presence of H_2O_2, producing signals that allow for highly sensitive glucose measurement, an essential function in managing diabetes [55]. Likewise, cholesterol detection is possible through reactions catalyzed by PODs, enabling precise monitoring of lipid levels in blood and contributing to cardiovascular disease prevention. For environmental monitoring, POD-based sensors can detect phenolic compounds, nitrates, and heavy metals in water and soil samples [56]. Phenolic compounds, for instance, are industrial pollutants harmful to aquatic and soil ecosystems, and their detection is crucial for safeguarding environmental health.

Table 4 summarizes the performance of plant-derived POD enzymes immobilized on various electrode materials for electrochemical sensing of H_2O_2. Each plant POD demonstrates unique sensing characteristics based on its enzyme properties and electrode interface, impacting the detection limits and linear range suitable for H_2O_2 monitoring in biomedical and environmental applications. Among the PODs, the sleepy plant shows the lowest detection limit of 0.4 μM, achieved using gold nanoparticle-modified electrodes. This superior sensitivity is likely due to the excellent conductivity and high surface area of gold nanoparticles, which facilitate efficient electron transfer between the enzyme and the electrode surface. Gold nanoparticles also provide a stable platform for enzyme immobilization, reducing signal loss and improving sensor sensitivity. Consequently, MPP, combined with gold nanoparticles, may be highly advantageous for detecting low H_2O_2 concentrations in sensitive biomedical applications, such as monitoring oxidative stress markers in biological samples. Other plant sources, such as lemongrass and horseradish, also demonstrate relatively low detection limits (50 μM), though they utilize graphene and carbon paste electrodes, respectively. Graphene's high electron conductivity and large surface area contribute to this performance, enhancing electron transfer and enzyme stability on the electrode surface [57,58]. HRP, immobilized on a carbon paste electrode, displays a broader linear range of 0.05–10 mM, making it suitable for applications requiring a wider dynamic range, such as environmental pollutant detection or food safety monitoring. In contrast, royal palm and guinea grass PODs show higher detection limits of 87 μM and 150 μM, respectively, when immobilized on graphene or graphene-chitosan composite electrodes [59]. While graphene enhances electron transfer, the chitosan component may introduce some steric hindrance or diffusional limitations, affecting sensitivity. Despite this, chitosan's biocompatibility and film-forming properties make it an effective matrix for enzyme immobilization, potentially improving enzyme stability and reusability. The linear range of 0.1–5 mM for royal palm and 0.1–3.5 mM for GGP sensors suggests that they are best suited for environmental applications where H_2O_2 concentrations are generally higher [26]. SPP on graphene oxide has the highest detection limit (460 μM) among the sensors, which might be attributed to the material's functional groups. These groups enable strong enzyme binding, but their hydrophilicity can sometimes reduce electron transfer efficiency compared to more conductive materials like pure graphene or gold. This setup has a linear range of 0.25–5 mM, indicating that while sweet potato peroxidase sensors may be less suitable for ultra-trace H_2O_2 detection, they still hold promise for environmental or industrial monitoring, where a higher detection threshold is acceptable.

Table 4. Comparison of Plant-Derived Peroxidase-Based Electrochemical Sensors for Hydrogen Peroxide Detection.

Plant Source	Electrode Material	Detection Limit (μM)	Linear Range (mM)	Reference
Royal palm	Graphene/chitosan	87	0.1–5	[59]
Guinea grass	Graphene	150	0.1–3.5	[26]
Lemongrass	Graphene	50	0.5–4	[49]
Sweet potato	Graphene oxide	460	0.25–5	[20]
Sleepy plant	Gold nanoparticles	0.4	0.5–5	[37]
Horseradish	Carbon paste	50	0.05–10	[48]

PODs, often harnessed for H_2O_2 detection, exhibit a broad range of catalytic capabilities, positioning them as promising tools for detecting additional analytes, such as triclosan (TCS) [60] and pathogenic bacteria like *Staphylococcus aureus* [61], a prevalent antimicrobial agent. Thus, PODs can facilitate its degradation, making them valuable for monitoring TCS residues in water sources. A recent study demonstrated the amperometric detection of TCS using screen-printed carbon nanotube electrode modified with GGP. The system exhibited a redox potential of 370 mV and a linear response range from 20 μM to 80 μM and a limit of detection (LOD) of 3 μM, highlighting its potential utility in environmental analysis and food quality control applications. Furthermore, plant PODs hold potential for bacterial detection, especially targeting *Staphylococcus aureus*, a major cause of hospital-acquired infections. A novel approach employed GGP to detect *Staphylococcus aureus* in milk samples [61]. This approach involved modifying a screen-printed gold electrode with cysteine and GGP, enabling sensitive electrochemical detection through H_2O_2 reduction. The modified electrode successfully detected *S. aureus* in milk within a concentration range of 3×10^2 to 3×10^8 CFU/mL, achieving a detection limit as low as 10^2 CFU/mL and a rapid response time of around 20 min.

Compared to the commercial standard HRP, Colombian plant-derived PODs offer several unique advantages for biosensor development. For example, RPP exhibits exceptional thermal stability, maintaining activity at temperatures up to 90 °C, which is considerably higher than HRP's optimal range of 25–30 °C [32,50]. This property is particularly advantageous for sensors operating in harsh environmental or industrial conditions. Similarly, MPP demonstrates ultra-low detection limits when combined with gold nanoparticles (LOD = 0.4 μM), outperforming HRP-based systems in sensitive biomedical applications such as monitoring oxidative stress biomarkers. Additionally, the broad pH tolerance observed in AOP (pH 4.0–9.0) provides flexibility for sensors used in variable sample matrices, such as environmental monitoring or food safety testing. These characteristics highlight the potential of Colombian PODs as cost-effective, locally sourced alternatives that can surpass HRP in performance under specific application conditions.

4.2. Synthesis of Polyaniline

PANI is among the most thoroughly studied conducting polymers, renowned for its remarkable environmental stability and favorable electronic characteristics. Its versatility opens up numerous possibilities for applications, including organic lightweight batteries, light-emitting diodes, optical displays, anticorrosive coatings, and bioanalytical systems [15].

Emeraldine polyaniline exists in two forms: the non-conductive base and the conductive salt form. The salt is produced through the protonation of the imine sites in the emeraldine base using strong acids like organic sulfonic and phosphoric acids, a pro-

cess known as "doping." Although doped PANI exhibits conductivity, it suffers from poor solubility in common solvents, limiting its processability [62]. However, PANI can form polyelectrolyte complexes by interacting with soluble polymers that carry negatively charged groups, resulting in stable dispersions of nanoparticles in aqueous media, which enhances processability. In these complexes, PANI typically adopts a doped and chiral state due to interactions between its imine groups and the polymeric anions. These polyelectrolyte complexes can be synthesized through chemical or enzymatic methods. Chemical polymerization of aniline monomers occurs under strongly acidic conditions, usually with 1 M HCl or H_2SO_4, using ammonium persulfate as the oxidant. The synthesis of PANI complexes can be achieved under environmentally friendly, kinetically controlled conditions using horseradish peroxidase (HRP) as a catalyst. However, HRP exhibits low stability at pH levels below 4.5, which coincides with the pH range suitable for forming polyelectrolyte complexes with negatively charged polymers [45]. To address this issue, alternative PODs that can efficiently polymerize aniline in acidic conditions have been explored, as demonstrated in a study that use RPP for the synthesis of conducting polyelectrolyte complexes of PANI [62,63]. The polymerization of aniline was conducted at pH 2.0 and UV-vis-NIR absorption and EPR techniques confirmed the formation of an electroactive complex similar to traditionally doped PANI. Thus, the thermostable RPP is an efficient catalyst for the polymerization of aniline to obtain PANI complexes under green conditions [64].

4.3. Chemiluminescence Assays

Chemiluminescence (CL) assays are analytical techniques that harness light emission from chemical reactions to detect and quantify various molecules with exceptional sensitivity [65,66]. In these assays, a chemiluminescent substrate, such as luminol, undergoes an oxidation reaction catalyzed by an enzyme like POD in the presence of an oxidizing agent (often hydrogen peroxide), resulting in the release of light. This emitted light is captured by sensitive detectors, with the intensity directly proportional to the concentration of the target analyte. Due to their high sensitivity and low background noise, chemiluminescence assays are commonly used in clinical diagnostics, environmental monitoring, and food safety testing, where they facilitate the detection of low-abundance molecules, pathogens, or specific biomarkers. Compared to fluorescence or colorimetric assays, chemiluminescence assays offer advantages such as reduced interference from background signals and enhanced detection limits. However, these assays require careful optimization of reaction conditions (e.g., pH, temperature, and reagent concentrations) to maximize light yield and maintain enzyme stability, factors that can impact assay reproducibility and performance [65].

HRP-catalyzed CL is a widely used method for detecting low concentrations of analytes due to its high sensitivity and low background signal [67]. HRP is commonly used to catalyze the oxidation of luminol or other substrates in the presence of hydrogen peroxide, which produces light detectable by photomultiplier tubes or other light-sensitive devices. This light output is proportional to the concentration of the target analyte, making HRP-based CL assays particularly useful in immunoassays, DNA detection, and various biochemical analyses [68]. However, despite its popularity, HRP has several limitations. One significant disadvantage is its sensitivity to environmental conditions, such as pH and temperature; HRP can quickly lose activity outside of its optimal pH range (around pH 7) or under high temperatures, which limits its utility in harsh or variable assay conditions.

POD from African oil palm, for example, has demonstrated potential for enhanced stability and reactivity in specific pH and temperature ranges, which can be advantageous for assays requiring durability under varying conditions [69]. Studies indicate that this enzyme can withstand higher temperatures compared to HRP, making it a valuable alternative for applications where thermal stability is essential. Meanwhile, POD from the royal

palm offers distinct advantages in terms of substrate affinity, particularly with substrates like 3,3′,5,5′-tetramethylbenzidine (TMB) and luminol [70]. The efficiency of these enzymes is affected by factors such as ionic strength, pH, and the presence of cofactors, which influence their conformational state and active site accessibility, thereby impacting their chemiluminescent output. Optimizing these conditions can yield stronger luminescent signals, enhancing assay sensitivity.

While HRP remains the most widely used enzyme in chemiluminescence (CL) assays, its activity declines sharply under extreme pH or elevated temperatures, limiting its robustness. In contrast, Colombian PODs such as RPP retain over 80% activity at pH 3 and 90 °C, providing a more stable catalytic platform for CL systems exposed to variable reaction environments [32,69]. This increased stability reduces assay variability and enhances reproducibility, making these enzymes suitable for low-cost diagnostic kits in regions where controlled laboratory conditions may not be feasible.

4.4. Cross-Linked Enzymatic Aggregates

CLEAs are innovative biocatalytic materials formed by aggregating enzymes through precipitation, followed by cross-linking to create stable, insoluble enzyme clusters [16]. This approach enhances enzyme stability and reusability, making CLEAs valuable for applications in industrial biocatalysis, environmental remediation, and biosensing. Unlike conventional enzyme immobilization, CLEAs do not require a solid support, which simplifies the process and reduces production costs. CLEAs are applied in fields where enzymes must withstand harsh conditions, including organic synthesis, wastewater treatment, and biotransformation of complex substrates [71].

When CLEAs are synthesized with PODs, they offer further benefits for oxidative reactions. Peroxidase-CLEAs can be used in chemiluminescence assays, biosensors for detecting hydrogen peroxide, and pollutant degradation, where they catalyze oxidation reactions with improved stability under extreme pH, temperature, or organic solvents. This stability is crucial for long-term, repeated use in bioelectrochemical sensors and diagnostic kits, enhancing their efficiency and cost-effectiveness.

HRP has been extensively used to form CLEAs due to its high catalytic activity and compatibility with a variety of substrates. However, HRP-CLEAs have limitations, including sensitivity to denaturation under extreme conditions like high temperatures or fluctuating pH levels, which can reduce enzyme activity over time [72]. These factors have driven interest in exploring alternative peroxidase sources that could provide greater resilience and cost efficiency. To address these limitations, researchers are investigating plant-derived peroxidases from species like the RPP and GGP. For example, RPP was subject to a study focus in its immobilization through CLEAs to enhance its stability and activity. The resulting RPTP-CLEAs showed remarkable activity, maintaining 40% of maximum activity even at pH 3, where free RPTP is inactive. In thermal stability tests, the RPTP-CLEAs retained high stability similar to the free enzyme, with a half-life of 50 min at 90 °C and pH 7. Unlike the free enzyme, which undergoes subunit dissociation at pH 3, RPTP-CLEAs avoided this instability, showing significant thermostabilization [73]. Additionally, RPTP-CLEAs also exhibited good stability with low hydrogen peroxide concentrations (10 mM), though stability declined at higher concentrations (300 mM), where immobilization provided limited improvement. In practical applications, the RPTP-CLEAs were effective in decolorizing methyl orange using 5 mM hydrogen peroxide for four cycles (4 h each) without noticeable activity loss, achieving around 50% substrate degradation. With 225 mM hydrogen peroxide, activity gradually decreased across cycles but allowed complete colorant degradation [72]. These findings suggest that RPTP-CLEAs can function under challenging conditions, such as pH 3 and high hydrogen peroxide

levels, where the free enzyme would typically be inactive, supporting their potential use in various industrial and environmental applications.

But not only was RPTP studied; GGP was also investigated for immobilization through CLEAs for the decolorization of indigo carmin (IC) [74]. The biocatalyst was prepared using 50% v/v ethanol and 0.88% w/v glutaraldehyde, with stirring for 1 h, achieving an immobilization yield of 93.74% and a specific activity of 36.75 U mg^{-1}. This immobilized form demonstrated 61% higher activity than the free enzyme at its optimal pH (pH 6 for both), with activity levels nearly 10 times higher at a pH of 9. GGP-CLEAs also showed significantly greater thermal stability, with improvements of 2–4 times compared to the free enzyme, and were 2–3 times more resistant to hydrogen peroxide. The GGP-CLEAs effectively removed over 80% of 0.05 mM indigo carmine at pH 5 in the presence of 0.55 mM H_2O_2 after 60 min, outperforming the free enzyme. However, operational stability tests indicated a reduction in enzyme activity of over 60% after 4 cycles, likely due to suicide inhibition [74].

These PODs offer potentially more sustainable, cost-effective, and environmentally resilient alternatives for CLEA applications. Studies suggest that PODs from these plants exhibit stability across a broader range of environmental conditions, which can be advantageous for applications requiring robust catalytic activity in varied settings. By creating CLEAs with RPP and GGP, it may be possible to develop biocatalysts that maintain high efficiency and stability in applications like biosensors, pollutant degradation, and chemiluminescent assays while reducing dependency on traditional HRP sources.

Colombian PODs also demonstrate superior performance when incorporated into CLEAs compared to traditional HRP. For instance, RPP-CLEAs maintained high activity across four decolorization cycles at pH 3, a condition under which free HRP and HRP-CLEAs would typically lose activity [73]. Similarly, GGP-CLEAs displayed 2–3 times greater resistance to hydrogen peroxide inactivation than free enzymes or HRP-CLEAs, making them more suitable for industrial wastewater treatment and other challenging oxidative processes [74]. These findings emphasize the potential of Colombian PODs to serve as resilient, sustainable alternatives for industrial biocatalysis while reducing dependency on imported HRP.

While CLEAs significantly improve enzyme stability and reusability compared to free enzymes, several limitations remain. One key challenge is operational stability, as repeated reaction cycles often result in gradual activity loss due to partial enzyme inactivation or structural degradation of the aggregates. For example, GGP-CLEAs exhibited a >60% reduction in activity after four cycles of indigo carmine degradation, likely caused by suicide inactivation in the presence of excess hydrogen peroxide [74]. Similarly, RPP-CLEAs, while stable at low H_2O_2 concentrations, showed diminished stability under high oxidative stress, limiting their long-term utility in industrial processes [73]. Another important issue is recyclability, which directly impacts process economics and environmental sustainability. Although CLEAs are theoretically reusable, repeated recovery steps may cause physical losses or partial fragmentation of the aggregates, reducing their effectiveness over time. This highlights the need for strategies to enhance mechanical robustness and improve immobilization methods. To address these limitations, advanced engineering approaches should be considered. Protein engineering through site-directed mutagenesis or directed evolution could generate POD variants with higher intrinsic resistance to oxidative stress and extreme pH or temperature conditions. Additionally, integrating PODs with nanozymes—nanomaterials with enzyme-mimicking activity—offers a promising hybrid strategy. For instance, coupling PODs with graphene oxide or metal nanoparticles could enhance electron transfer, improve stability, and provide self-regenerative catalytic activity, thus extending the functional lifespan of the biocatalyst [24,57]. These hybrid

CLEA-nanozyme systems could also enable multifunctionality, combining biocatalytic specificity with the robustness of inorganic materials. Future research should focus on scalable, green synthesis approaches for these hybrid systems to ensure environmental compatibility and economic feasibility.

5. Sustainability and the Circular Bioeconomy in the Context of Colombian Plants Peroxidases

Sustainability and the circular bioeconomy provide the conceptual framework for POD valorization in Colombia. Agricultural by-products such as palm fronds, guinea grass leaves, or sweet potato peels are abundant and frequently discarded. Their repurposing into enzyme sources reduces waste while generating added value. This approach aligns with the Sustainable Development Goals (SDGs), particularly responsible production, climate action, and innovation in green technologies [10,74–76].

The valorization of Colombian agricultural by-products for POD extraction not only contributes conceptually to the circular bioeconomy but also yields quantifiable environmental benefits. For instance, Colombia generates an estimated 3.5–4.0 million tons of agricultural residues annually, with a significant proportion coming from crops such as sugarcane, palm oil, and tubers like sweet potato [13,30]. Repurposing even 10% of these residues for biotechnological processes could prevent approximately 350,000 tons of organic waste from being landfilled or burned each year, reducing methane emissions associated with anaerobic decomposition. From a climate perspective, substituting traditional chemical processes or imported commercial enzymes with locally produced PODs offers meaningful CO_2 savings. The carbon footprint of industrial enzyme production, primarily HRP derived from horseradish, has been estimated at 8–10 kg CO_2 per kilogram of purified enzyme when factoring in cultivation, transportation, and processing [10]. By sourcing PODs from local agricultural waste streams, transportation-related emissions could be reduced by up to 40–50%, while valorizing waste biomass also sequesters carbon that would otherwise be released during open burning. Moreover, POD-based processes, such as wastewater treatment using CLEAs, have demonstrated a 30–45% reduction in chemical oxidant usage compared to conventional treatment methods [11,74]. This translates to lower secondary pollution and reduced energy requirements for chemical production. For example, implementing POD-CLEA systems at a medium-sized textile facility could reduce annual CO_2 emissions by 120–150 metric tons, primarily by decreasing the demand for synthetic oxidants like hydrogen peroxide and chlorine compounds. Quantifying these benefits provides a clearer perspective on how Colombian POD-based biotechnologies can move beyond laboratory-scale innovation to become measurable contributors to sustainability goals, including the United Nations Sustainable Development Goals (SDGs 12 and 13).

6. Conclusions

The valorization of Colombian agricultural by-products for POD extraction offers a powerful pathway to sustainable biotechnological innovation. This review highlights how plant-derived PODs can be harnessed for diverse applications, including biosensing, advanced material synthesis, environmental remediation, and health monitoring while reducing waste and supporting local economies.

Looking ahead, three main research priorities emerge:

(i) Scaling up POD production: Future studies should focus on optimizing extraction and purification methods for large-scale, cost-effective production. This includes developing continuous processing technologies, green extraction approaches, and robust immobilization strategies to meet industrial demands.

(ii) Structural characterization: Detailed studies using X-ray crystallography, cryo-EM, and computational modeling are needed to understand the structural basis of the exceptional thermal stability and substrate specificity of Colombian PODs. These insights will enable rational protein engineering and design of tailored biocatalysts.
(iii) Integration into circular bioeconomy policies: Collaboration with policymakers, industries, and local communities is essential to incorporate POD-based technologies into Colombia's circular bioeconomy framework, promoting sustainable waste valorization and contributing to national and global climate action goals.

By advancing these research areas, Colombian plant PODs can transition from laboratory-scale innovations to impactful technologies, fostering environmental sustainability, economic growth, and global leadership in green biotechnology.

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Cost-Effective Optimization of the Transfructosylation Activity of an Invertase Produced from *Aspergillus carbonarius* PC-4 Using Pineapple Crown and Determination of Its Biochemical Properties

Ryhára Dias Batista [1], Gustavo Carvalho do Nascimento [1], Nayara Bezerra Carvalho [1], Paula Candido Leite [2], Rodrigo Correa Basso [2], Sergio Andres Villalba Morales [1,2], Michelle da Cunha Abreu Xavier [1], Rafael Firmani Perna [2,*] and Alex Fernando de Almeida [1,*]

[1] Graduate Program in Food Science and Technology, Federal University of Tocantins (UFT), Palmas 77001-090, Brazil; ryharabatista@hotmail.com (R.D.B.); gustavocarvalho081294@hotmail.com (G.C.d.N.); nayara.bezerra@mail.uft.edu.br (N.B.C.); sergio.morales@mail.uft.edu.br (S.A.V.M.); michellecax@mail.uft.edu.br (M.d.C.A.X.)

[2] Graduate Program in Chemical Engineering, Institute of Science and Technology, Federal University of Alfenas (UNIFAL-MG), Poços de Caldas 37715-400, Brazil; paula.leite@sou.unifal-mg.edu.br (P.C.L.); rodrigo.basso@unifal-mg.edu.br (R.C.B.)

* Correspondence: rafael.perna@unifal-mg.edu.br (R.F.P.); alexfernando@mail.uft.edu.br (A.F.d.A.)

Abstract: Fructooligosaccharides are prebiotic sugars that are widely used in the production of functional foods, which can be produced enzymatically by the transfructosylation reaction of sucrose. This work aimed to optimize the production of an invertase with high transfructosylation activity from *Aspergillus carbonarius* PC-4 using pineapple crown as the inducer substrate and evaluate its biochemical properties. The culture medium was optimized using a Plackett–Burman experimental design and a central composite rotatable design, resulting in a maximum transfructosylation activity of 65.33 U/mL at 72 h of cultivation. The cultivation parameters were Yp/s = 1070.75 U/g and P_P = 2771.48 U/h, which showed an increase of 5.2-fold in the enzyme produced. The optimum temperature (50 °C) and pH (5.0) for the enzymatic activity were obtained by a CCR design. The enzyme showed a half-life of 60 min at 40 °C. In conclusion, the invertase produced from *A. carbonarius* PC-4 using agro-industrial waste (pineapple crown) and an inorganic nitrogen source (ammonium nitrate) exhibits high transfructosylation activity that can be used as a potential source for the production of fructooligosaccharides.

Keywords: transfructosylation activity; *Aspergillus*; invertase; agro-industrial waste; enzyme activity; submerged cultivation

1. Introduction

Invertases (β-fructofuranosidase, E.C. 3.2.1.26) are enzymes that belong to the carbohydrase class (family GH32) and catalyze the hydrolysis of the α-1,4-glycosidic bond from sucrose into glucose and fructose in equimolar proportions of inverted sugar at a concentration of approximately 10% substrate. These enzymes can be obtained from a wide variety of microorganisms, including animals, plants, bacteria, yeasts, and fungi. However, fungi stand out because they have attracted the attention of different sectors of the industry due to their biotechnological potential and because they are considered saccharolytic, that is, they ferment various carbohydrates [1,2]. The fungi that stand out in the production of invertases belong to the genera *Penicillium*, *Aerobasidium*, *Fusarium*, and, mainly, *Aspergillus* [3–5]. In addition, invertases can perform a transfructosylation reaction when exposed to a range of sucrose concentrations from 20 to 85%, thus also being

classified as fructosyltransferases (E.C. 2.4.1.9), a subclass of transferases [1,6]. Fructosyltransferases (FTases) act on the β-(1-2) bonds of sucrose and transfer the fructosyl radical to another sucrose molecule, forming fructooligosaccharides (FOSs), or transfer the fructosyl radical to an FOS by lengthening the chain by a fructose unit, releasing glucose in both situations [3,7].

Transfructosylation carried out by invertases has wide applications in several industries. In the food industry, it is used to produce fructooligosaccharides (FOSs), which act as prebiotics and improve the texture and stability of food products [8]. In the pharmaceutical industry, the resulting oligosaccharides are incorporated into prebiotic supplements and other formulations due to their benefits for intestinal health [9,10]. In biotechnology, this enzymatic reaction is exploited to synthesize new functional sugars and modify biomolecules [11]. The cosmetics industry uses products derived from transfructosylation as humectants in moisturizing formulations. Furthermore, in the agricultural industry, FOSs can be added to animal feed to promote digestive health. These applications highlight the versatility of transfructosylation in improving processes and products in several areas [9,12].

FOSs are commonly designated as 1-kestose (GF2), 1-nystose (GF3), and 1F-β-fructofuranosylnystose (GF4) [3]. They are classified as prebiotics, defined as non-digestible food ingredients that are resistant to hydrolysis by human digestion, because of the β conformation in the anomeric carbon of fructose monomers [13,14]. These biomolecules have beneficial effects on human and animal health, since they promote good absorption of minerals in the body, regulate blood glucose and cholesterol levels, promote the proliferation of beneficial bacteria in the large intestine (particularly Bifidobacteria, which regulate the intestinal microbiota), prevent the onset of colon cancer, relieve constipation, and increase immunity [15,16]. FOSs are widely used in the food industry and can be safely consumed by diabetics, since they have about one-third of the sweetening power of sucrose and are low in calories [3,7,16]. They are commonly used in dairy products, cookies, breads, confectionery products, and functional products (to promote a symbiotic effect with probiotic microorganisms), among others. Additionally, they can be used in animal feed for the same purpose as prebiotics and can also minimize the formation of tooth decay, since they are not consumed by *Streptococcus mutans* [1,17].

Enzymes can be produced by submerged cultivation or solid-state cultivation; nevertheless, most industrial enzymes are produced by submerged cultivation, since it is an efficient method for enzyme production and due to the greater ease in the control of the physicochemical parameters [18,19]. The use of agro-industrial waste for the production of microbial enzymes has been widely adopted as a substitute for pure carbon sources, since it represents a valuable and rich source of energy and other nutrients (lignocellulose, proteins, carbohydrates, lipids, etc.), and it is also a low-cost biomass, which would otherwise be discarded into the environment and cause major problems of solid waste pollution [15,20]. One of the residues that causes major environmental issues is the waste from pineapple cultivation. Brazil is the second largest producer of pineapple (*Ananas comosus*) in the world, producing around 1.56 billion pineapples per year [21]. The by-products generated from pineapple correspond to residual pulp, peels, crowns, stems, and leaves [22].

Using a statistical approach to optimize enzyme production is of the utmost importance, since it involves the analysis of different variables, such as carbon, nitrogen, salts, and physicochemical factors, in the search for optimal conditions in the production process. In this sense, Plackett and Burman designs allow for the screening of the most important parameters and the response surface for the achievement of optimal conditions [16,18,23]. Previous studies have shown that a strain of *Aspergillus* sp. isolated from canned peach syrup was able to produce high amounts of fructosyltransferase when grown in a submerged medium, using pineapple crown and raffinose as the carbon source and soy protein and ammonium nitrate as the nitrogen source. Thus, the present work aimed to optimize the production of an invertase with high transfructosylation activity produced from *A. carbonarius* PC-4 under submerged conditions using Plackett and Burman and DCCR ex-

perimental designs and to characterize the biochemical properties of the enzyme produced in the crude extract.

2. Materials and Methods

2.1. Materials

The pineapple crown was purchased from local commerce (Gurupi, Brazil). Potato dextrose agar (PDA) was purchased from Merck (Rahway, NJ, USA). The glucose oxidase–peroxidase kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Citric acid*H_2O, $ZnSO_4$*$7H_2O$, $Fe(NH_4)_2(SO_4)_2$*$6H_2O$, $CuSO_4$*$5H_2O$, $MnSO_4$*H_2O, H_3BO_3, Na_2MoO_4*$2H_2O$, sodium citrate*$5H_2O$, KH_2PO_4, NH_4NO_3, $MgSO_4$*$7H_2O$, $CaCl_2$*$2H_2O$, 0,1 mg/mL biotin solution, glucose, sucrose, and sodium acetate were purchased from Labynth® (Diadema, Brazil). All chemicals used were analytical grade.

2.2. Methods

2.2.1. Microorganism and Maintenance

A black *Aspergillus carbonarius* strain PC-4 was isolated from peach syrup and maintained in the Laboratory of Biotechnology, Food Analysis and Products (LABAP), Habite—Biotechnology Companies Incubator, Federal University of Tocantins—UFT, Gurupi, Tocantins, Brazil [17]. *A. carbonarius* PC-4 was cultivated on PDA for 3 days at 28 °C and then stored at 4 °C in a PDA slant. *A. carbonarius* PC-4 was also preserved in sterile distilled water [24]. The dishes (approximately 5 mm) containing a small portion of the culture medium and sporulated mycelium were aseptically transferred into sterile 6 mL flasks filled with 4 mL of sterile distilled water and sealed with a rubber stopper. The flasks were stored at 4 °C, and the viability of the strains was verified every six months.

2.2.2. Submerged Culture Conditions

A liquid medium was prepared using Vogel's medium (Merck, Rahway, NJ, USA) [25]. The trace element solution (solution A) was prepared containing (g/L) the following ingredients: citric acid*H_2O, 50; $ZnSO_4$*$7H_2O$, 50; $Fe(NH_4)_2(SO_4)_2$*$6H_2O$, 10; $CuSO_4$*$5H_2O$, 2.5; $MnSO_4$*H_2O, 0.05; H_3BO_3, 0.05; and Na_2MoO_4*$2H_2O$, 0.05. The salt solution (solution B) was prepared containing (g/L) the following ingredients: sodium citrate*$5H_2O$, 150; KH_2PO_4, 250; NH_4NO_3, 100; $MgSO_4$*$7H_2O$, 10; $CaCl_2$*$2H_2O$, 5 and biotin solution (0.1 mg/mL), 5 mL; and solution A, 5 mL. The solutions were maintained at 4 °C. The medium preparation consisted of a 50-fold dilution of solution B, replacing glucose with other carbon sources, and adjusting the final pH to 6.0.

The cultures were performed in Erlenmeyer flasks (100 mL) containing 20 mL of culture medium. All media were autoclaved at 121 °C for 20 min. The inocula were prepared using 3-day-old cultures. The media were inoculated with 1 mL of a conidia suspension (1×10^7 spores or cells per mL) and incubated at 28 °C, 180 rpm, for 3 and 5 days. The biomass was separated from the fermentation broth by filtration in muslin. The cell-free broth was used for fructosyltransferase activity assays. The results were assayed for fructosyltransferase activity (TFA), fructosyltransferase yield on the substrate (Yp/s), and fructosyltransferase productivity (Pp). The parameters Yp/s (U/g) and Pp (U/h) were calculated using Equations (1) and (2), respectively.

$$Y_{p/s} = P_f - \left(\frac{P_0}{S_0}\right) \quad (1)$$

$$Pp = TFA/t \quad (2)$$

where P_f is the final product, P_0 is the initial product, S_0 is the initial substrate (pineapple crown waste), and t is the time of cultivation.

2.2.3. The Transfructosylation Activity of the Invertase from *A. carbonarius* PC-4

The transfructosylation activity was determined according to Rawat et al. [26]. A total of 100 microliters of culture filtrate with 400 µL of sucrose (20% *w*/*v* in 0.1 M sodium acetate buffer pH 5.0) was heated at 50 °C for 1 h in a water bath. The reaction was stopped by boiling the mixture in a water bath at 100 °C for 10 min. The transfructosylation activity was estimated by taking 20 µL of the appropriately diluted reaction mixture and mixing it with 2 mL of the test reagent (Glucose oxidase–peroxidase kit, Sigma-Aldrich, St. Louis, MO, USA). The glucose released was measured at 505 nm. One unit of transfructosylation activity was defined as the amount of enzyme required to produce 1 mol of glucose per minute under the assay conditions.

2.2.4. Optimization of the Production of Transfructosylation Activity

Plackett–Burman Design

The Plackett–Burman experimental design [27] was used to evaluate the relative importance of carbon and nitrogen in the production of an invertase with high transfructosylation activity from *A. carbonarius* PC-4 after 72 h and 120 h of cultivation. Raffinose and pineapple crown were used as carbon sources, while ammonium nitrate and soybean protein were used as nitrogen sources (Table 1). Each variable represented three levels—high concentration (1), low concentration (−1), and intermediate concentration (0)—in eight trials (Table 1). The response variable analyzed was enzyme activity.

Table 1. Experimental variables and levels used for the production of an invertase with high transfructosylation activity from *A. carbonarius* PC-4 using the Plackett–Burman design.

Factors	Code	−1	0	1
Pineapple crown	X_1	0	5	10
Raffinose	X_2	0	5	10
Soy protein	X_3	0	1	2
Ammonium nitrate	X_4	0	1	2

Note: the values are expressed in g/L.

Central Composite Rotatable Design

After the identification of the carbon and nitrogen sources affecting enzyme production in the Plackett–Burman design, a response surface methodology of a central composite rotatable design (CCRD) was used to discover the optimal combination of the independent variables for invertase production. The two main independent variables chosen for this experiment were pineapple crown (X_1) and ammonium nitrate (X_2) with 4 trials under the axial conditions and 3 repetitions at the central point, totaling 11 trials (Table 2). The response variable analyzed was enzyme activity.

Table 2. Experimental variables and levels using a CCRD for the production of an invertase with high transfructosylation activity from *A. carbonarius* PC-4.

Factors	Code	−1.41	−1	0	1	1.41
Pineapple crown	X_1	6	10	20	30	34
Ammonium nitrate	X_2	0.5	3	9	15	17.5

Note: the values are expressed in g/L.

All experiments were conducted as previously described, and invertase production was considered to be the independent variable or response (Y). The second-order polynomial coefficients were obtained and evaluated statistically by analysis of variance (ANOVA). The model's accuracy in predicting the maximum transfructosylation activity was confirmed by a triplicate experiment in the optimized conditions. The predicted and experimental values of transfructosylation activity were confirmed using the optimal values for the significant variables generated by CCRD.

2.2.5. The Biochemical Properties of the Invertase

Effect of pH and Temperature on Invertase Activity

The optimal conditions for transfructosylation activity in different ranges of pH and temperature were determined in a test tube immersed in a water bath, containing 20% (*w*/*v*) sucrose, in McIlvaine buffer, with a reaction time of up to 60 min. This experiment was carried out using the response surface methodology of CCRD, in which pH was defined as X_1 and temperature as X_2 (Table 3). Each independent variable was studied at five coded levels (Table 3). The response variable analyzed was enzyme activity.

Table 3. Experimental variables and levels using the CCRD for the influence of pH and temperature on the activity of the invertase with high transfructosylation activity from *A. carbonarius* PC-4.

Factors	Code	−1.41	−1	0	1	1.41
pH	X_1	2.17	3	5	7	7.83
Temperature (°C)	X_2	14.64	25	50	75	85.36

All experiments were conducted as previously described, and invertase production was considered to be the independent variable or response (Y). The second-order polynomial coefficients were obtained and evaluated statistically by analysis of variance (ANOVA). The model's accuracy in predicting the maximum transfructosylation activity was confirmed by a triplicate experiment in the optimized conditions. The predicted and experimental values of transfructosylation activity were confirmed using the optimal values for the significant variables generated by CCRD.

The Effects of pH and Temperature on the Stability of the Transfructosylation Activity

The effect of pH on the enzyme stability was tested using the McIlvaine buffer with pH values ranging from 3.0 to 8.0. The enzyme preparation was diluted in each buffer (1:2, *v*/*v*) and incubated for 7 h and 24 h at 15 °C, without substrate.

Thermal stability assays were conducted by incubating the crude extract, without substrate, at the temperatures of 40 °C, 50 °C, and 60 °C for up to 150 min, at the previously determined pH value.

Aliquots were withdrawn at specific time intervals and maintained in an ice bath for 5 min. The residual activity was determined as previously described, using the McIlvaine buffer with 20% of sucrose (*w*/*v*) for 60 min and at 50 °C. Results were expressed as relative activity, considering the initial activity (t = 0) to be 100%.

2.2.6. Statistical Analysis

The experimental design was evaluated using the software "Protimiza Experimental Design" (https://experimental-design.protimiza.com.br, accessed on 9 August 2024) [28]. The polynomial models with 90% and 95% confidence levels were evaluated by analysis of variance (ANOVA), and Student's *t*-test and Fischer's test (F) were used to determine the statistical significance of the regression coefficients and to obtain the second-order model equation, respectively. In turn, the quality of the fit polynomial model was assessed by the coefficient of determination (R^2).

3. Results and Discussion

3.1. Screening of Carbon and Nitrogen Sources by the Plackett–Burman Design

A. carbonarius PC-4 has been previously identified as the producer of an invertase with high transfructosylation activity under submerged conditions using pure and complex carbon and nitrogen sources [17]. Under these conditions, the maximum enzyme production was obtained using Vogel's salt medium supplemented with raffinose and pineapple crown as carbon sources and ammonium nitrate and soybean protein as nitrogen sources (44.40 U/mL) after 168 h of cultivation. Sequential optimization approaches were applied in this study, initially by screening the nutritional factors that affected the production of the

invertase with transfructosylation activity using the Plackett–Burman design after three and five days of cultivation (Table 4). The results of enzyme production were given in units per mL (U/mL), production yield ($Y_{P/S}$), and productivity (P_P). In this sense, a large variation was observed, from 0.02 to 46.26 U/mL after 72 h of cultivation, and from 0 to 52.20 U/mL after 120 h of cultivation. The mean effects of the factors examined for enzymatic activity are presented in the Pareto plot (Figure 1).

Table 4. CCRD for the transfructosylation activity of the invertase produced from *A. carbonarius* PC-4.

Run	Pineapple Crown (X_1, g/L)	Ammonium Nitrate (X_2, g/L)	TFA (U/mL)	*Yp/s* (U/g)	*Pp* (U/h)
1	10	3	35.18 ± 1.50	2526.17	7.02
2	30	3	16.73 ± 0.35	409.49	3.41
3	10	15	29.88 ± 5.42	1711.80	4.75
4	30	15	40.83 ± 7.61	941.26	7.84
5	6	9	24.92 ± 3.23	2891.45	4.82
6	34	9	16.73 ± 3.58	309.41	2.92
7	20	0.5	32.76 ± 0.69	1443.03	8.02
8	20	17.5	65.86 ± 0.12	2958.73	16.44
9	20	9	68.98 ± 3.92	2916.05	16.20
10	20	9	69.44 ± 3.00	2657.67	14.76
11	20	9	52.48 ± 3.35	2233.18	12.41

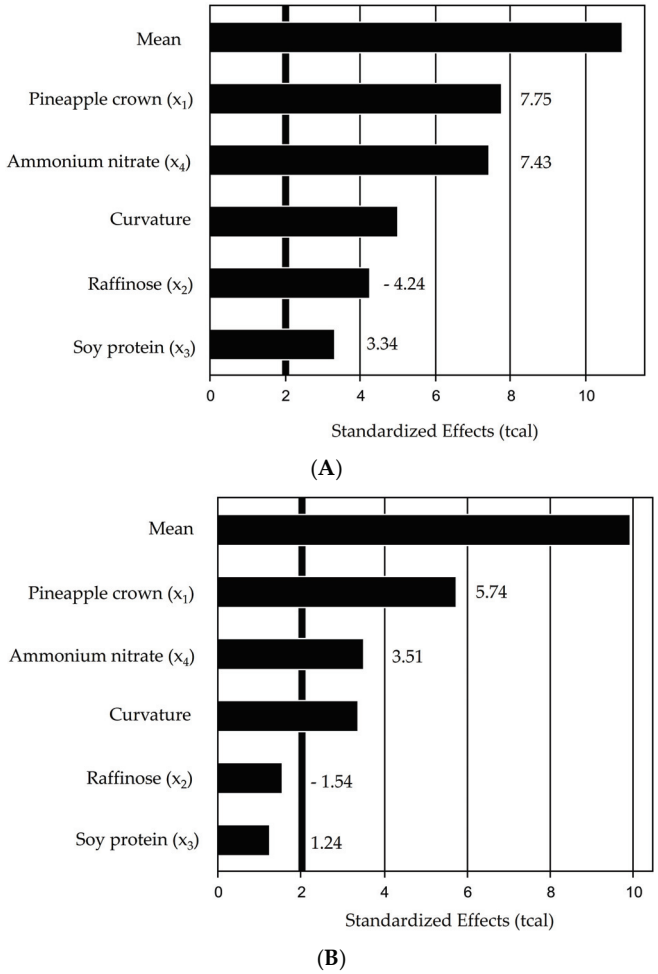


Figure 1. Pareto chart of the effects of independent variables on the response variable of the transfructosylation activity of invertase, (**A**) 72 h and (**B**) 120 h.

The analysis of the cultivation parameters of the transfructosylation activity of the invertase produced using *A. carbonarius* PC-4 with the Plackett–Burman design showed that the enzyme production (46.26 U/mL) was not reduced after three days of cultivation. As a comparison, the initial cultivations carried out using the parametric technique of culture optimization with carbon and nitrogen sources showed maximal production after 168 h of cultivation [17]. According to the results obtained using the Plackett–Burman design, the variables pineapple crown, ammonium nitrate, and soy protein had a significant positive effect ($p < 0.10$), while raffinose had a significant negative effect, at the 72 h time point. At the 120 h time point, only the variables pineapple crown and ammonium nitrate had a significant positive effect ($p < 0.10$) (Figure 1). These results suggest that higher concentrations of these components lead to increased enzymatic activity for the variables with a positive effect. Conversely, for the variable with a negative effect, lower concentrations result in higher enzymatic activity. Furthermore, a medium of the following composition is expected to lead to similar results: 20 g/L pineapple crown, 4 g/L ammonium nitrate, and 4 g/L soy protein. Under these conditions, $Y_{P/S}$ was 3700.44 U/g of the substrate and P_P was 10.28 U/h. The results showed a 1.59-fold increase in enzyme productivity while maintaining the production yield. In this study, a decrease in the duration of the cultivation to 72 h was achieved, which represents an important reduction in energy costs to produce the invertase from *A. carbonarius* PC-4 under submerged conditions using agro-industrial waste and a mineral nitrogen source.

Organic and inorganic nitrogen sources are essential for enzyme synthesis. Inorganic nitrogen can be utilized quickly, while organic sources provide various cell growth factors and amino acids that are essential for cell metabolism and enzyme production [29]. Ademakinwa et al. [18] observed that ammonium ions can influence fructosyltransferase production from *Aureobasidium pullulans* using the Plackett–Burman design. Ammonium nitrate has been used as a nitrogen source for the production of xylanase from *Aspergillus candidus* [30], amylase from *Aspergillus niger* [31], and lipase from *Candida viswanathii* [32].

Agro-industrial waste is economically feasible for enzyme production, with a reduction in operational costs, and it is environmentally friendly. Pineapple waste contains a considerable amount of soluble sugars, such as sucrose, which makes it suitable for use as a substrate in microbial fermentation [33,34]. Therefore, agro-industrial waste, such as pineapple crown, are potentially suitable substrates for the production of value-added biomolecules when used in the composition of microbial culture media [17,28]. This substrate has been used to produce β-glucosidase and xylanase from *Trichoderma viride* [22,35], cellulases from *Trichoderma ressei* [36], and invertase from *A. niger* [34].

3.2. Medium Composition Optimization by Central Composite Rotational Design (CCRD)

For the optimization experiment using CCRD and RSM methodologies, and according to previous results from the Plackett–Burman design, at the 72 h time point, despite the soy protein having a significant positive effect on the enzymatic activity, only ammonium nitrate was selected as the nitrogen source due to its more pronounced positive effect. Pineapple crown was chosen as the sole carbon source, because raffinose had a negative effect on the enzymatic activity. The response data based on the independent variables were obtained from the experiments and are depicted in Table 4.

The factors analyzed showed that the concentration of ammonium nitrate had a positive linear effect and a negative quadratic effect, indicating a tendency for maximum FTase production near the central point, while the concentration of the pineapple crown had a negative quadratic effect, indicating that an increase or decrease in the value of this variable would lead to a reduction in FTase production [37] (Table 5).

Table 5. Estimates of the coefficient of independent variables on invertase with transfructosylation activity production using the CCRD to determine the transfructosylation activity of invertase.

Factors	Coefficient	Error	t	*p*-Value
Média	63.63	4.66	13.65	0.000
X1	−2.38	2.85	−0.84	0.441
$X1^2$	−22.51	3.40	−6.63	0.001
X2	8.20	2.85	2.87	0.035
$X2^2$	−8.26	3.40	−2.43	0.059
X1.X2	7.35	4.04	1.82	0.128

A second-order equation was fitted to the data by multiple regression analysis, which generated the reparametrized model that describes the measured responses for the significant independent variables (pineapple crown and ammonium nitrate). The mathematical model was expressed with the following equation:

$$\text{TFA } (U/mL) = 63.63 - 22.51\ X_1^2 + 8.20\ X_2 - 8.26\ X_2^2 \quad (3)$$

The experimental model was validated by the analysis of variance (ANOVA) with a coefficient of determination R^2 = 0.8533, an F value calculated from the regression to be 13.6 (greater than the tabulated F value of 5.27), and an *F* lack of adjustment value of 0.9 (not significant), making the model valid in the 90% confidence interval (Table 6).

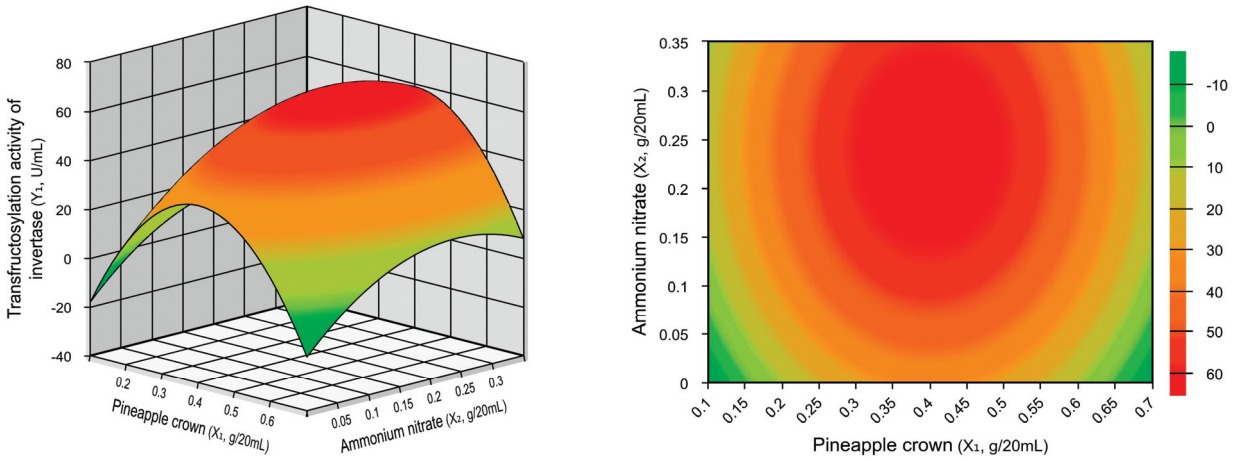
Table 6. Analysis of variance (ANOVA) for transfructosylation activity of the invertase.

Variation Source	Sum of Squares	Degree of Freedom	Mean Square	F_{cal}	*p*-Value
Regression	3415.5	3	1138.5	13.6	0.00265
Residue	587.4	7	83.9		
Lack of fit	400.7	5	80.1	0.9	0.61566
Pure error	186.7	2	93.4		

$F_{tab\ regression/residue}$ (3; 7; 0.10): 3.07; $F_{tab\ lack\ of\ fit/pure\ error}$ (5; 2; 0.10): 9.29. R^2: 85.33%.

A response surface plot (Figure 2) was generated from the reparametrized model equation, indicating that the maximum transfructosylation activity of the invertase was achieved at the midpoint of the experimental design (pineapple crown, 2.0%, w/v, and ammonium nitrate, 1.0%, w/v) after 72 h of cultivation. The increase in pineapple crown causes a decrease in the enzymatic activity. This fact was explained by Amim et al. [38], according to whom the gradual increase in substrate concentration will cause the enzyme production to increase at a directly proportional rate until the medium becomes saturated with the substrate. After reaching the saturation point, the addition of extra substrate will no longer make a difference.

Park et al. [39] stated that increasing the amount of nitrate has no significant effect on the enzymatic activity of fructosyltransferase. This fact can be observed, using other nitrogen sources, as it was in the study conducted by Ademakinwa et al. [23], who performed a response surface experiment to evaluate the variables sucrose, NH_4Cl, and yeast extract in the production of FTase from *Aureobasidium pullulans*. By increasing the sucrose concentration and maintaining the NH_4Cl concentration, there was an increase in the enzymatic activity of fructosyltransferase; on the other hand, the increase in yeast extract had a negative effect on the activity of fructosyltransferase. The same behavior was observed when the concentration of yeast extract was kept constant and the concentration of NH_4Cl varied, resulting in a decline in the enzymatic activity.



(**A**) (**B**)

Figure 2. Invertase production with transfructosylation activity influenced by the interaction between pineapple crown (X1) and ammonium nitrate (X2). (**A**) Response surface and (**B**) contour curve.

In this study, a 5-fold increase in enzyme production was observed compared to the initial production of 13.38 U/mL during 72 h of cultivation [17], and there was also an increase in yield from 1070.75 U/g to 2771.48 U/g and a 5.19-fold increase in productivity. Although many researchers have investigated various effects on fructosyltransferase production [17,23,37], as far as we know, no optimization study has used the combination of ammonium nitrate and pineapple crown, which successfully increased enzyme production.

3.3. Validation

The results predicted by the model suggested that the maximum transfructosylation activity of the invertase produced from *A. carbonarius* PC-4 would be 65.33 ± 4.62 U/mL with the supplementation of 0.40 g of pineapple crown and 0.21 g of ammonium nitrate. The obtained results yielded an average FTase production of 66.46 ± 2.67 U/mL (Table 7). According to the predicted and experimental results, the model was validated based on the existence of the optimum point. There was a 1.77-fold increase compared to the best result for enzymatic activity (3 days of cultivation) of the Plackett and Burman design and 3.82-fold compared to the result obtained by Nascimento et al. [5] (17.36 U/mL).

Table 7. Validation of the culture conditions for the production of the invertase with transfructosylation activity from *A. carbonarius* PC-4.

Variable	Experimental Conditions	Predicted Value	Experimental Value
Pineapple crown Ammonium nitrate	20 g/L 10.5 g/L	65.33 ± 4.62 U/mL	66.46 ± 2.67 U/mL

3.4. The Physical and Chemical Properties of the Transfructosylation Activity

3.4.1. Influence of Temperature and pH on Enzyme Activity

The effect of pH and temperature on the enzymatic activity was analyzed using a central composite rotational design (Table 8). The relative activity values were presented in relation to the maximum activity value, considered to be 100%. Thus, it was observed that pH and temperature values at different levels strongly influenced the transfructosylation activity of the enzyme produced from *A. carbonarius* PC-4. According to Ghazi et al. [40], there was no significant fructosyltransferase activity at pH values below 3.5 or above 9.5, and temperatures higher than 65 °C inactivated the fructosyltransferase.

Table 8. Relative activity at different pH values and temperatures of transfructosylation activity in the invertase using CCRD.

Run	pH X_1	Temperature (°C) X_2	TFA (U/mL)	Relative Activity (%)
1	3	25	0.00	0.00
2	7	25	2.01	3.49
3	3	75	0.00	0.00
4	7	75	0.86	1.47
5	2.17	50	0.01	0.01
6	7.83	50	1.36	2.35
7	5	14.64	2.09	3.62
8	5	85.36	0.09	0.15
9	5	50	54.55	89.48
10	5	50	57.65	94.62
11	5	50	51.59	100

The factors analyzed showed that the pH and temperature are significant variables in quadratic terms, and both have negative effects, indicating that an increase in the value of these variables above the studied limits would lead to a decrease in invertase production (Table 9). A second-order equation was fitted to the data by multiple regression analysis, generating a reparametrized model that describes the measured responses for the significant independent variables (pH and temperature). The mathematical model was expressed with the following equation:

$$TFA\ (U/mL) = 54.60 - 27\ X_1^2 - 26.80\ X_2^2 \quad (4)$$

Table 9. Estimates of the coefficients of independent variables on invertase production with transfructosylation activity using the CCRD for transfructosylation activity.

Factors	Coefficient	Error	t	*p*-Value
Mean	54.60	1.12	48.64	0.000
X_1	0.60	0.69	0.87	0.4243
X_1^2	**−27.00**	**0.82**	**−33.00**	**0.0000**
X_2	−0.50	0.69	−0.73	0.4997
X_2^2	**−26.80**	**0.82**	**−32.75**	**0.0000**
$X_1.X_2$	−0.29	0.97	−0.30	0.7778

Factors are in bold when $p < 0.05$.

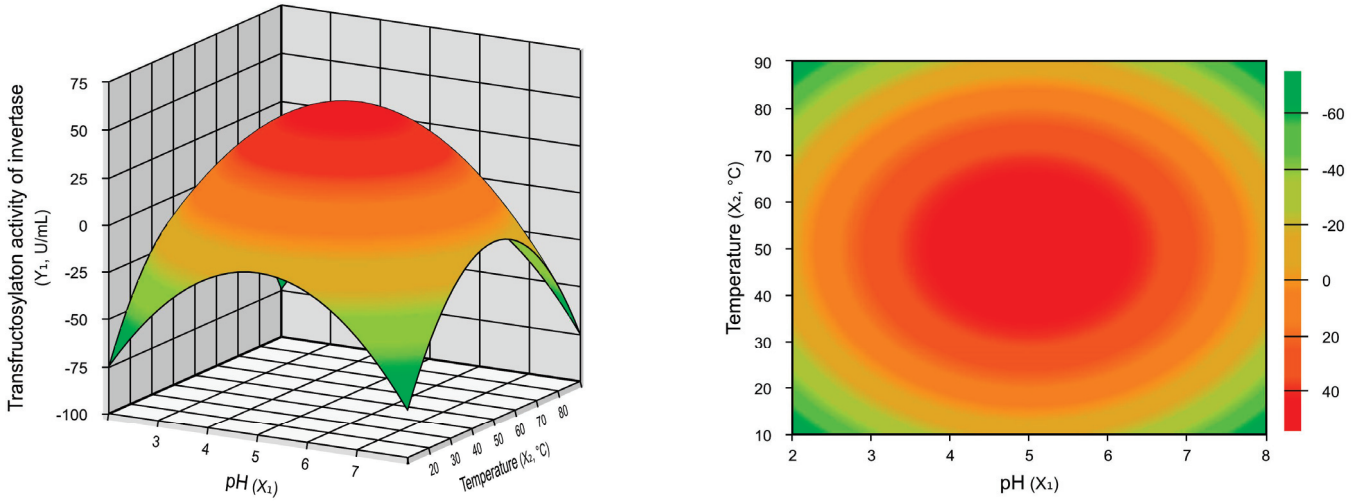
The experimental model was validated by analysis of variance (ANOVA) with a coefficient of determination R^2 = 99.62%, an F value calculated from the regression as 1048.5 (greater than the tabulated F value of 4.46), and an F value for lack of adjustment (0) that was not significant, making the model valid in the 95% confidence interval (Table 10).

Table 10. Analysis of variance (ANOVA) for transfructosylation activity of the invertase.

Variation Source	Sum of Squares	DF	Mean Square	Fcal	*p*-Value
Regression	6314.5	2	3157.3	1048.5	0.00000
Residue	24.1	8	3.0		
Lack of fit	5.7	6	1.0	0.0	0.99683
Pure error	18.4	2	9.2		
Total	6338.6	10			

$F_{tab\ regression/residue}$ (2; 8; 0.05): 4.46; $F_{tab\ lack\ of\ fit/pure\ error}$ (6; 2; 0.05): 19.33. R^2: 99.62%.

The maximum transfructosylation activity was reached at the central point of the experimental design (pH of 5 and temperature of 50 °C) (Figure 3), where the ionization state and the established temperature stabilize the conformation of the enzyme, as well as the adequate binding of the substrate to the active site. Similar results were observed by Nemukula et al. [41], who characterized the fructosyltransferase from *Aspergillus aculeatus* and found that it has optimal activity at a pH of 6 and temperature of 60 °C. Regarding temperature, Aguiar-Oliveira and Maugeri [42] conducted a study on fructosyltransferase and stated that the highest transfructosylation rates occur at temperatures between 45 °C and 65 °C. Kashyap et al. [43] found that the relative activity of the fructosyltransferase from *A. aculeatus* increased as the pH increased to 4.5 at the temperature of 55 °C and subsequently declined and became constant.



(**A**) (**B**)

Figure 3. Transfructosylation activity in interaction between pH (X_1) and temperature (X_2). (**A**) Response surface (**B**) contour curves.

For the purified enzyme, L'Hocine et al. [44] demonstrated that the best pH and temperature of fructosyltransferase from *A. niger* are 5.8 and 50 °C, respectively. Zeng et al. [45] reported that different microbial species can produce different molecular structures and active sites of the enzyme, leading to different optimal initial reaction pH values.

3.4.2. Validation

To confirm the model's accuracy in predicting the maximum transfructosylation activity, an additional experiment was performed in triplicate under the optimized conditions. The model suggested that the maximum activity is 54.60 ± 1.12 U/mL, which can be achieved when the process conditions include a pH = 5.0 and a temperature of = 50 °C. The results obtained yielded a maximum transfructosylation activity of 53.85 U/mL. According to the predicted and experimental results, the model was validated based on the existence of the optimum point.

3.5. Thermal Stability and pH Stability

The FTase produced from *A. carbonarius* PC-4 was stable at 40 °C, where 50% of its activity was verified after 60 min of incubation and remained at around 30% after 155 min. On the other hand, at the temperatures of 50 °C and 60 °C, the half-life of the enzyme was reached after 15 min of incubation. The enzyme was not stable at 60 °C, and only 17% of its activity was verified after 60 min of incubation, while at 50 °C, the FTase retained 35% of its activity. After 155 min, both temperatures led to activity below 10% (Figure 4A).

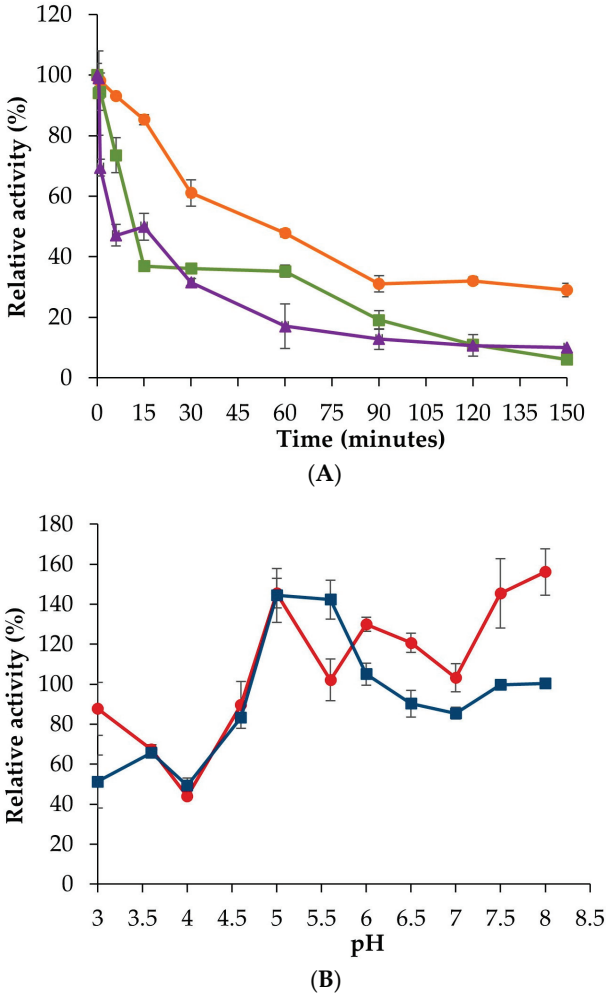


Figure 4. Thermal stability of the invertase at (**A**) 40 °C (●) orange line, 50 °C (■) green line, and 60 °C (▲) purple line for different periods and pH stabilities after (**B**) 7 h (●) red line and 24 h (■) blue line at 15 °C.

Similar results were achieved by Yang et al. [46], who found that fructosyltransferase purified from *A. niger* was more stable at 40 °C for 60 min; at 50 °C, it retained 60% of its activity, and at 60 °C, the enzyme was not stable, with a rapid decline in its activity. The fructosyltransferase from *A. aculeatus* was stable at the temperatures of 25 to 50 °C; nevertheless, it decreased sharply when incubated at 60 °C and was completely inactivated when subjected to the temperature range from 65 °C to 70 °C for 60 min [40]. The invertase with transfructosylation activity was more stable in the temperature range from 25 to 30 °C for 60 min [37].

In this work, the transfructosylation activity of the invertase from *A. carbonarius* PC-4 was more stable at pH 5, reaching a maximum of 144.40% of its activity at 24 h of incubation and 156.134% of its activity when incubated at pH 8 for 7 h. This increase may be related to the ionization state of the enzyme, which, when incubated at pH 5 for 24 h and at pH 8 for 7 h, was better at stabilizing the protein conformation [47]. On the other hand, this fact may be related to the enzyme being present in the crude extract and being influenced by factors such as metal ions, the presence of other enzymes, cofactors, and others.

For the pH range of 3–5, only at pH 4 was the relative activity below 50%. This fact may be related to the pH influencing the kinetic parameters of the enzymatic reaction and being able to change the stability of the enzyme–substrate complex [48]. For the pH ranges 5–5.5 and 6.5–8, the activity was maintained above 90% (Figure 4B). According to Yang et al. [46], a fructosyltransferase from *A. aculeatus* reached the ideal stability at pH 6.0 and

retained more than 90% of its activity in the pH range of 4.0–9.0 after 24 h of incubation. Furthermore, it also retained approximately 80% of activity in pH values below 2.0 or above 11.0, indicating that FTase is stable in a wide pH range, favoring its production, storage, and industrial applications.

4. Conclusions

This study demonstrates the cost-effective optimization of an invertase from *A. carbonarius* PC-4 with high transfructosylation activity using pineapple crown as a carbon inducer source and ammonium nitrate as an inorganic nitrogen source. The experimental design of this study allowed a 1.77-fold increase in the transfructosylation activity after 72 h of cultivation. However, this combination is not found in the literature and may serve as a model for further studies. A partial characterization of the enzyme in the crude extract showed that the reaction conditions of the enzyme for the maximum transfructosylation activity occurred at pH 5.0 and a temperature of 50 °C. The enzyme showed thermal stability at 40 °C after 1 h of incubation and was stable over the wide pH range of 3.0–8.0. These parameters are important for the development of a successful bioprocess, which may favor its production, storage, and industrial applications. Low-cost agro-industrial waste, in conjunction with ammonium nitrate, has the potential to be used in the production of invertases with the aim of optimizing their transfructosylation activity for application in the production of fructooligosaccharides.

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Optimization of Cellulase Production from Agri-Industrial Residues by *Aspergillus terreus* NIH2624

Elen Ayumi Kadoguchi [1], Josman Velasco [2], Silvio Silvério da Silva [1], Avinash P. Ingle [3], Fernando Segato [1] and Anuj Kumar Chandel [1,*]

[1] Department of Biotechnology, Engineering School of Lorena, University of São Paulo (EEL-USP), Lorena 12602-810, SP, Brazil
[2] Departamento de Ciencias Biologicas, Universidad de los Andes, Bogotá 111711, Colombia; j.velascomendoza@uniandes.edu.co
[3] Biotechnology Centre, Department of Agricultural Botany, Dr. Panjabrao Deshmukh Agricultural University, Akola 444 104, India
* Correspondence: anuj10@usp.br or anuj.kumar.chandel@gmail.com

Abstract: The objective of this study was to assess the cellulase production of four fungi: *Aspergillus terreus* NIH2624, *Aspergillus clavatus* NRRL1, *Aspergillus versicolor* CBS583.65 and *Aspergillus phoenicis* ATCC3157, under submerged cultivation conditions. When these fungi were cultured in shake flasks using Mandels and Weber's minimal medium with 1% sugarcane bagasse as a carbon source and 1.8 g/L of rice bran extract as a nitrogen source, *A. terreus* showed maximum cellulase production (filter paper activity (FPase) 3.35 U/mL; carboxymethyl cellulase activity (CMCase) 1.69 U/mL). Consequently, *A. terreus* was selected for the optimization study for cellulase production. Among the different tested carbon sources, *A. terreus* showed higher CMCase activity when it was cultivated on delignified sugarcane bagasse (1.64 U/mL) and higher FPase activity on sugarcane straw (7.95 U/mL). Regarding the nitrogen sources, the maximum FPase activity was observed when using rice bran (FPase, 8.90 U/mL) and soybean meal (FPase, 9.63 U/mL). The optimized fermentation medium (minimal medium with delignified sugarcane bagasse and rice bran as carbon and nitrogen sources, respectively) resulted in an enzymatic cocktail mainly composed of xylanases, with a maximum activity of 1701.85 U/mL for beechwood xylan, 77.12 U/mL for endoglucanase and 21.02 U/mL for cellobiohydrolase. Additionally, the enzymatic cocktail showed efficient activities for β-glucosidase, β-xylanase, arabinofuranosidase and lytic polysaccharide monoxygenases (LPMOs). This cellulase enzyme solution has the potential to efficiently hydrolyze lignocellulosic biomass, producing second-generation sugars in biorefineries.

Keywords: *Aspergillus* sp.; agro-industrial by-products; cellulases; LPMOs; fermentation

1. Introduction

The limited availability of gasoline and its consequent impacts on the environment are important concerns that highlight the urgent demand for sustainable gasoline alternatives. One of the main challenges in the renewable industry is the conversion of lignocellulosic biomass into fermentable sugars at a low cost [1]. Among the strategies proposed to reduce the cost of cellulase production, the use of agro-industrial residues from feedstock shows great promise due to their wide availability and low cost [2]. Agricultural residues such as sugarcane bagasse, sugarcane straw, corn cob, rice straw, etc. have been recognized as viable substrates to produce fungal enzymes [3]. These substrates are abundantly available throughout the year in many agricultural countries, including Brazil, the USA, China and India. Therefore, such lignocellulosic biomasses can be utilized to develop sustainable cellulase production processes in fermentation industries, holding significant economic importance [4].

Lignocellulosic biomass usually primarily consists of cellulose, hemicellulose and lignin. However, the interaction between these three components in the plant cell wall renders the biomass rigid, complex and recalcitrant. Consequently, an effective biomass pre-treatment becomes extremely necessary to enable the enzyme access to biomass during saccharification. The sugars derived from enzymatic hydrolysis are referred to as second-generation (2G) and serve as building blocks in lignocellulose biorefineries to produce renewable fuel, biomaterials and other high value-added chemicals [5].

An ideal cellulolytic enzyme cocktail should contain a good amount of exoglucanases, endoglucanases, β-glucosidase and other auxiliary enzymes for efficient enzyme hydrolysis of the lignocellulosic biomass [4]. Cellulase enzymes are not only of great interest in biorefineries but also in the textile, beverage, cleaning product and pulp and paper manufacturing industries. Moreover, cellulases are being used in the degradation and smoothing of fabric fibers, aging of jeans, production of fruit juice, production of detergent and in winemaking, the animal feed industry and in the pulp and paper industries [6].

It is evident that cellulases are enzymes that exhibit different specificities for cellulose degradation, converting into soluble sugars that are then assimilated by microorganisms for fuels and specialty chemical products. The complete enzymatic degradation of cellulose requires the coordinated action of hydrolytic and oxidative enzymes. Hydrolytic enzymes include endoglucanases (responsible for the hydrolysis of the internal regions of the cellulose), exoglucanases (acting at the ends of the microcrystalline cellulose molecule, releasing cellobiose units) and β-glucosidases (which hydrolyze cellobiose and some glucose-soluble oligosaccharides). Oxidative enzymes, such as lytic polysaccharide monoxygenases (LPMOs), act in synergy with cellulases to potentiate cellulose degradation, being activated by enzymes that act as electron donors, such as cellobiose dehydrogenase (CDH) [7,8].

According to Florencio et al. [9], among the microorganisms capable of producing cellulolytic enzymes that efficiently degrade plant biomass, filamentous fungi stand out, especially *Trichoderma reesei* and *Aspergillus niger* strains. In this study, we chose to an *Aspergillus* species, as these fungi are well-known to produce a complete cellulase cocktail, including cellobiohydrolases, endoglucanases, β-glucosidases, β-xylosidases, endoxylanases, xyloglucanases and α-arabinofuranosidases. *Aspergillus* spp. are robust fungi that thrive on a variety of low-cost carbon and nitrogen sources, yielding high titers of plant biomass-degrading enzymes. It is evident from the literature that *A. niger* produces more effective hemicellulases and β-glucosidases than *Trichoderma* spp., with strong resistance to phenolic compounds from lignin and deactivators. Florecio et al. [9] studied the proteomic analysis of the *A. niger* strain and *T. reesei* and found that the former showed a secretome with a greater number of identified proteins and greater titers of cellulase enzymes.

The production of cellulase enzymes by *Aspergillus* sp. and *Trichoderma* sp. has been well-documented; however, there are only a few reports available on the production of cellulase by *A. terreus*. To the best of our knowledge, this study is first to report a comparison of enzyme production potentials in four different species viz. *Aspergillus clavatus, A. versicolor, A. terreus* and *A. phoenicis* growing on agro-industrial residues as carbon and nitrogen sources in a submerged fermentation process.

2. Materials and Methods

2.1. Microorganisms

Four different filamentous fungi viz. *A. clavatus* NRRL1, *A. terreus* NIH2624, *A. versicolor* CBS583.65 and *A. phoenicis* ATCC3157 were kindly provided by the Synthetic and Molecular Biology Laboratory at the Department of Biotechnology-EEL/USP, Lorena, São Paulo, Brazil for the production of cellulase.

2.2. Inoculum Preparation

Spores from all four *Aspergillus* strains were transferred to a pre-inoculum with 50 mL of growth medium (2% D-glucose, 2% malt extract, 1% peptone, pH: 5.5) in an orbital shaker (30 °C and 200 rpm) for 48 h. Subsequently, 1 mL of pre-inoculum was transferred to

an Erlenmeyer flask containing 100 mL of the minimal medium (2 g/L of KH_2PO_4, 1.4 g/L of $(NH_4)_2SO_4$, 0.3 g/L of $MgSO_4.7H_2O$, 0.3 g/L of $CaCl_2$, 0.3 g/L of urea, 2 ml/L of trace elements (5 mg/L of $FeSO_4.7H_2O$, 1.56 mg/L of $MnSO_4.H_2O$, 1.4 mg/L of $ZnSO_4.7H_2O$, 2 mg/L of $CoCl_2$)) with 1% sodium carboxymethylcellulose (CMC) and a pH of 5.5, as described by Mandels and Weber [10], and kept at 30 °C for 96 h under agitation at 200 rpm. Samples were withdrawn after every 24 h with 5 min of rest for decanting, and 2 mL of the supernatant was removed for the cellulase assay.

2.3. Carbon and Nitrogen Sources in the Production Medium

For cellulase production, two different parameters were used: the carbon source and the nitrogen source (defined and non-defined). The carbon sources consisted of sugarcane bagasse, sugarcane straw, corn cob and pequi mesocarp, as these are agro-industrial residues and have different concentrations of cellulose, lignin and hemicellulose. The defined nitrogen sources were based on the medium described by Mandels and Weber [10], where each compound (urea, peptone and ammonium sulfate) was tested separately. The non-defined nitrogen sources consisted of rice straw, soybean bran and rice bran.

2.4. Alkaline Pre-Treatment of Carbon Sources

The native biomass samples were alkaline pre-treated using a 4% NaOH solution (w/v) in an autoclave at 121 °C for 40 min. Briefly, 10% lignocellulosic biomass (sugarcane bagasse, sugarcane straw, corn cob and pequi mesocarp) was added to the 4% NaOH solution. After the end of the pre-treatment, the biomass was filtered under a vacuum pump until most of the liquid was removed. Subsequently, the pre-treated material was washed with distilled water until the yellow color disappeared to ensure the removal of the remaining lignin present and to reach a neutral pH. After this procedure, the biomass was subjected to drying at room temperature and grinding in a knife mill to obtain a size smaller than a 20 mesh (0.841 mm). All samples were stored in a cold room at 4 °C.

2.5. Fermentation Assay under Optimized Conditions

Fermentation conditions, such as the pH (5.5), temperature (30 °C), agitation frequency (200 rpm) and culture medium (except for the carbon and nitrogen sources), were the same in all analyzed fermentations. To prepare the medium to select the best carbon source, the carboxymethyl cellulose (CMC) was replaced with 1% of a single carbon source (sugarcane bagasse or sugarcane straw or corn cob or pequi mesocarp) and the rest of the components followed the minimal medium protocol described above.

To select the best nitrogen source, synthetic nitrogen source substrates were added to the medium with a defined equimolar fraction as well as to the minimal medium already described, obtaining a total weight of 2.25 g/L for urea and ammonium sulfate and 2.7 g/L for peptone. The non-synthetic nitrogen sources were used at a concentration of 1.8 g/L of the total weight. Sugarcane bagasse was used as a major carbon source in this test due to its better performance, as shown in previous assays. For both carbon and nitrogen selection, fermentation was carried out using the same procedure used in the study of potential cellulolytic enzyme-producing fungi but, this time, varying only the carbon and nitrogen sources.

The *A. terreus* fungus was transferred to a pre-inoculum in 50 mL of medium in an orbital shaker (30 °C, 200 rpm) for 48 h. Subsequently, 2 mL were transferred to an Erlenmeyer flask containing 200 mL of minimal medium with 1% sugarcane bagasse and 1.8 g/L of rice bran, then kept at 30 °C for 96 h while agitated at 200 rpm. After fermentation, the contents of the Erlenmeyer flask were centrifuged, and the liquid fraction was used later for protein quantification and to determine enzyme concentration and activity.

2.6. Carboxymethyl Cellulase (CMCase) Activity

The CMC test indicates the presence of endoglucanases in the enzyme solution. In this step, 2 mL of the sample, obtained after the cultivation of fungi from the best carbon and

nitrogen sources, was centrifuged at 10,000 rpm and 4 °C. Then, 1 mL of the supernatant was taken for analysis. The CMCase activity was determined by adding 0.5 mL of the crude enzyme extract into 0.5 mL of the 1% sodium carboxymethylcellulose solution in an acetate buffer (0.05 M, pH 5.0) followed by incubation in a water bath at 50 °C for 60 min. Periodically, the substrate-enzyme system was shaken to keep the cellulose in suspension. Then, 1 mL of a dinitrosalicylic acid (DNS) reagent was added, and the mixture was subjected to a water bath for 10 min at 100 °C. It was then cooled in an ice bath for 5 min, and 3.5 mL of distilled water was added. Finally, the absorbance was read in a spectrophotometer at 540 nm after calibrating the device with a blank composed of DNS reacted with the substrate without crude enzyme, which was replaced by distilled water. The enzyme activity tests of the enzyme solutions obtained from all four fungal strains were performed in triplicate.

Subsequently, CMCase activity tests were also performed on Greiner microplates to obtain enzymatic cocktail production profiles. In this case, 50 µL of the 0.5% CMC solution, 40 µL of a citrate buffer solution (pH 6) and 10 µL of the enzymatic extract were added in microtubes. The same was done for the blank, however, without the addition of the enzyme extract. Then the samples were placed in a dry bath at 50 °C for 30 min. After that, 100 µL of the DNS reagent was added to the samples and 10 µL of the enzymatic extract was added to the blank. The samples were submitted to a dry bath for 5 min at 100 °C and later added to Greiner microplates and analyzed in a plate reader at 540 nm.

The CMCase activity was determined in terms of glucose released (µmoles of reducing sugar produced per minute, using per ml of enzyme extract (U/mL) from the carboxymethylcellulose (substrate; CMC) at a pH of 6.0 and temperature of 50 °C) following the statistical analysis proposed by Triola et al. [11].

2.7. FPase Activity

FPase activity, i.e., filter paper activity, is determined based on the degradation of a strip of Whatman No. 1 filter paper (1.0 × 6.0 cm) by a mixture of exoglucanases and endoglucanases. In the tube containing the reaction assay, 1 mL of sodium citrate buffer solution (pH 4.8 to 50 mM), 0.5 mL of enzymatic extract and a strip of filter paper were added. In another tube, the reaction control was carried out by adding 1 mL of the same buffer solution and 0.5 mL of enzymatic extract; 1.5 mL of buffer solution and a strip of filter paper were added to the third tube, which was compared to the control. The samples were incubated in a water bath at 50 °C for 60 min, and the reaction was stopped with the addition of 3 mL of DNS reagent. The tubes were placed in boiling water for 5 min, and then 20 mL of distilled water was added for subsequent measurement of the absorbance in a spectrophotometer at 540 nm. The FPase activity was determined in terms of glucose released (µmoles of reducing sugar produced per minute, using per ml of enzyme extract (U/mL) from the Whatman filter paper (substrate) at a pH of 4.8 and temperature of 50 °C).

2.8. Biomass Characterization

Native and pre-treated sugarcane bagasse were characterized in terms of structural polymeric fractions (cellulose, hemicellulose, lignin), ash and extractives based on the analytical procedures established by the National Renewable Energy Laboratory (NREL), CO, USA [12].

2.9. Protein Quantification

Protein quantification was performed before and after concentrating the enzymatic extract using the Bio-Rad DC II kit, a colorimetric assay for protein concentration after detergent solubilization [13]. To prepare the blank, in duplicate, 20 µL of the minimal medium were added to microplates with rice bran. Then, 10 µL of reagent A (2% solution of Na_2CO_3 in 0.1 M of NaOH) and 80 µL of reagent B (0.5% solution of $CuSO_4.5H_2O$ and 1% sodium citrate) were added. The plates were left to rest for 15 min, and then the absorbance was measured in the plate reader at 750 nm. As for the samples, in triplicate, the same

procedure mentioned above was performed, replacing the minimal medium with 20 µL of the enzymatic extract.

2.10. Concentration of the Crude Enzyme Extract

The enzymatic extract, obtained from the optimized fermentation, was filtered through filter paper and concentrated up to 3 or 10 times by ultrafiltration in an Amicon stirred cell apparatus (Merck-Millipore, São Paulo, Brazil) with a 10 KDa cut-off membrane.

2.11. Xylanase Assay Using Beechwood and Birchwood Xylan

The xylanase activity of the enzymatic solution was performed using beechwood and birchwood as substrates. The substrates beechwood and birchwood were each added to microtubes followed by 50 µL of xylan 1%, 40 µL of citrate buffer (pH 4.8) and 10 µL of the enzyme extract. The same was done for the blank, replacing the enzymatic extract with the buffer. After that, the samples were incubated in a dry bath at 50 °C for 30 min. Then 100 µL of DNS reagent was added to the samples while 100 µL of DNS and 10 µL of enzyme extract were added to the blank. The samples were placed in a dry bath at 100 °C for 5 min and then diluted to a ratio of 1:4 (*v*/*v*) using distilled water before being transferred to Greiner microplates and analyzed in a plate reader at 540 nm. The activity value was calculated by considering 1 international unit (IU) equivalent to 1 µmol of xylose released per minute, per ml of enzyme (U/mL) using beechwood and birchwood as substrates at a pH of 4.8 and temperature of 50 °C.

2.12. Assay with Phosphoric Acid Swollen Cellulose (PASC)

For the PASC's preparation, 1 g of Avicel was added first to 25 mL of phosphoric acid (85% w/v) in an ice bath and stirred for 1 h. Then 400 mL of distilled water was added and stirred for another 1 h. After that, the solution was filtered with filter paper and washed extensively with 2 L of distilled water. The solution was then neutralized with 700 mL of a 1% $NaHCO_3$ solution and washed again with 1.5 L of distilled water. The PASC was removed with the aid of a spatula and resuspended in a buffer until use.

Samples were made in triplicate by adding 250 µL of PASC (0.5%) to 200 µL of citrate buffer (0.5 M, pH 4.8) with 50 µL of enzyme extract. For the blanks, a sample without substrate and another without the enzymes were made as controls. The citrate buffer was used to replace the substrate and enzymes, respectively, in these two cases. The assay was performed in a shaker bath at 50 °C for 60 min at 1000 rpm. Afterwards, the samples (reaction mixtures) were heated to 100 °C for 5 min to stop the reaction. Thereafter, the samples were cooled and centrifuged at 12,500 rpm for 5 min. After centrifugation, 100 µL of the supernatant was mixed with 100 µL of DNS reagent and incubated in a water bath at 100 °C for 5 min. The absorbance was then read by a spectrophotometer using a plate reader at 540 nm.

For the analysis of lytic polysaccharide monoxygenases (LPMOs), the same procedure as described above was used, with the addition of 1 mM of ascorbic acid in the final concentration. Therefore, 250 µL of PASC, 150 µL of citrate buffer, 50 µL of enzyme extract and 50 µL of ascorbic acid were used for the samples. For these reactions, a reaction containing all the reagents except the enzyme extract was used as a blank.

2.13. Assays with Para-Nitrophenyl (pNP)-Labeled Substrates

The enzymatic extract was used for a multi-enzyme assay. For this, pNP-glucose, pNP-cellobiose, pNP-xylose and pNP-arabinofuranose were taken as substrates to test for the presence of β-glucosidase, cellobiohydrolase, β-xylosidase and arabinofuranosidase, respectively.

For this, 40 µL of the substrate (1 mM), 10 µL of the enzyme extract and 50 µL of the citrate buffer (0.5 M, pH 4.8) were added to the samples. As for the blank, which was made in duplicate, 40 µL of the substrate and 60 µL of the citrate buffer were used. The reaction was incubated at 50 °C for 30 min, then 100 µL of 1 M sodium carbonate

was added and the solution was transferred to microplates for absorbance readings at 405 nm by a UV-visible spectrophotometer. After the 30 min incubation, there was a clear linearity in enzyme activity. The same method was used for each substrate and activity calculations were performed considering 1 international unit (IU) as equivalent to 1 nmol of pNP released per minute per ml of enzyme (U/mL) using the specific substrates at a pH of 4.8 and temperature of 50 °C.

3. Results and Discussion

Pre-treated sugarcane bagasse was used in this study as a primary carbon source for the four *Aspergillus* strains (i.e., *A. clavatus* NRRL1, *A. phoenicis* 275 ATCC3157, *A. versicolor* CBS583.65 and *A. terreus* NIH2624). The characterization of native sugarcane bagasse and alkaline pre-treated sugarcane bagasse is demonstrated in Table 1, showing the amount of total lignin (soluble and insoluble), glucose, xylan, arabinose and ash.

Table 1. Chemical composition (% dry wt.) of native and alkaline pre-treated (4% NaOH, 10% bagasse concentration, 120 °C, 30 min) sugarcane bagasse (SCB).

SCB (native)						
Sample	**Total lignin**	**Glucan**	**Xylan**	**Arabinosyl**	**Ashes**	**Total**
A	30.88	39.92	22.33	9.73	3.46	106.31
B	33.42	40.62	20.64	10.79	3.66	109.15
C	31.62	41.02	24.52	9.12	3.52	109.81
Media	31.62	40.62	22.33	9.73	3.52	109.15
Pre-Treated SCB						
Sample	**Total lignin**	**Glucan**	**Xylan**	**Arabinosyl**	**Ashes**	**Total**
A	6.78	66.65	27.8	6.24	1.53	109
B	7.35	62.25	26.72	6.88	0.79	103.99
C	8.38	54.88	23.77	6.08	0.69	93.8
Media	7.58	60.77	25.78	6.16	0.74	101.03

The characterization data of biomass, i.e., native sugarcane bagasse and bagasse pre-treated with sodium hydroxide (NaOH), showed that the concentration of total lignin and arabinose was found to be less in pre-treated sugarcane bagasse compared to native sugarcane bagasse. The cellulosic fraction rationally increased in the substrate as lignin was removed from the pre-treated biomass. The higher concentration of glucose in the pre-treated bagasse is due to lignin removal. Similarly, the concentration of xylan was also found to be higher in pre-treated biomass but cannot be considered significant (variance less than 10%). Table 1 also confirms the presence of a higher composition of glucose in the pre-treated sugarcane bagasse because of the removal of lignin and a fraction of hemicellulose.

The results recorded in the present study showed a resemblance with observations recorded in previous studies [14–16] where an alkaline treatment with NaOH was found to be the most efficient method to delignify agricultural residues, increasing the internal surface area and decreasing the degree of polymerization and crystallinity, in addition to separating the structural bonds between lignin and carbohydrates by breaking the lignin structure. The disadvantage of this technique is that it also degrades a fraction of the hemicellulose [15]. Other types of pre-treatments, on the other hand, have little effect on reducing lignin content and do not show a profound impact on cell wall compactness, eventually reducing the accessibility of microorganisms to cellulose entities during cultivation.

The enzymatic activity (CMCase and FPase) of all four test *Aspergillus* strains (i.e., *A. clavatus, A. versicolor, A. terreus* and *A. phoenicis*) was evaluated and is shown in Figure 1.

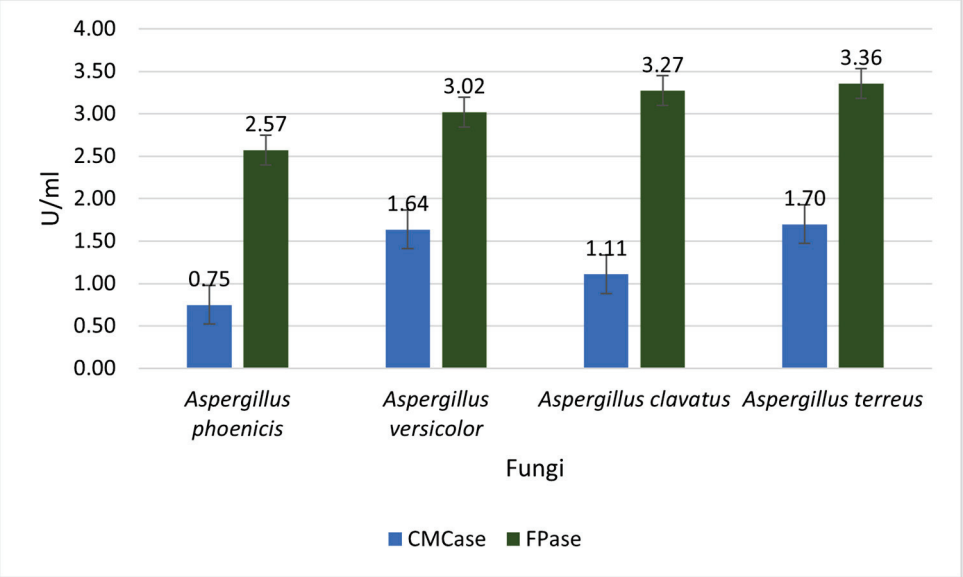

Figure 1. Cellulase production potential of filamentous fungi: *A. clavatus* NRRL1, *A. phoenicis* ATCC3157, *A. versicolor* CBS583.65 and *A. terreus* NIH2624.

The analysis demonstrated that *A. terreus* NIH2624 showed the maximum enzymatic activity (CMCase activity of 1.70 U/mL and FPase activity of 3.36 U/mL) compared to other strains (Figure 1). Therefore, *A. terreus* NIH2624 was selected for the further optimization of various carbon and nitrogen sources for cellulase production. The FPase activity (3.35 U/mL) reported in the present study was found to be significantly higher compared to previous studies, whereas CMCase activity (1.69 U/mL) was found to be lower. For example, Hui et al. [17] obtained an FPase value of 1.40 U/mL. Shahriarinour et al. [18] obtained an FPase of 0.69 U/mL and a CMCase of 7.41 U/mL for *A. terreus*, using peptone as a source of nitrogen in the minimum medium and using cellulose as carbon source. Sohail et al. [19] found lower values of FPase (0.15 U/mL) and CMCase (0.295 U/mL) for *A. terreus* activity with a mineral salt medium while employing solid-state fermentation. The CMCase value (1.32 U/mL) of this study is like the value found by Silva et al. [20] that used the mutant strain of *T. reesei* RP698.

After the selection of *A. terreus* NIH2624 as the fungus of study, four different carbon sources, i.e., corn cob, pequi mesocarp, sugarcane straw and sugarcane bagasse, were evaluated for cellulase production. The cellulase production profile of *A. terreus* NIH2624 growing on these carbon sources is presented in Figure 2.

Figure 2 shows the maximum CMCase (1.64 U/mL) and FPase (7.95 U/mL) production from sugarcane bagasse and sugarcane straw, respectively. Because sugarcane straw showed low CMCase production (0.29 U/mL), this biomass was not used further as a main carbon feedstock for cellulase production and, as the sugarcane bagasse showed a considerably higher production of cellulase, it was further selected as a primary carbon source in fermentation reactions.

The cellulase production by *A. terreus* NIH2624 can be compared with existing reports documented in the scientific literature. For instance, Corrêa [21] evaluated other *A. terreus* strains and found that *A. terreus* BLU24 produced 0.30 U/mL of CMCase and 0.50 U/mL of FPase, whereas Sohail et al. [19] found that *A. terreus* MS105 produced 0.726 IU/L/h of CMCase activity when grown on sugarcane bagasse. After the selection of *A. terreus* NIH2624 and the carbon source (sugarcane bagasse), the nitrogen sources were tested separately.

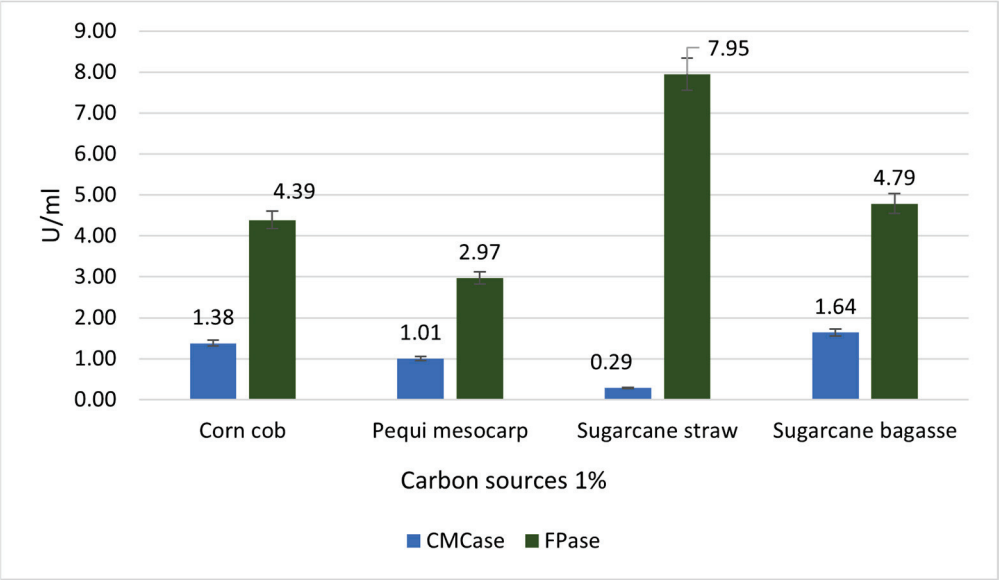

Figure 2. Cellulase production profile of *A. terreus* NIH2624 when grown on four different carbon sources (corn cob, pequi mesocarp, sugarcane straw and sugarcane bagasse).

As shown in Figure 3, higher CMCase activity was observed when using rice bran (2.35 U/mL) and higher FPase activity was observed when using soybean bran (9.63 U/mL) while using pre-treated sugarcane bagasse as a carbon source. Considering that an enzyme cocktail must contain, mainly, endoglucanase and exoglucanase, rice bran was chosen as the best source of nitrogen.

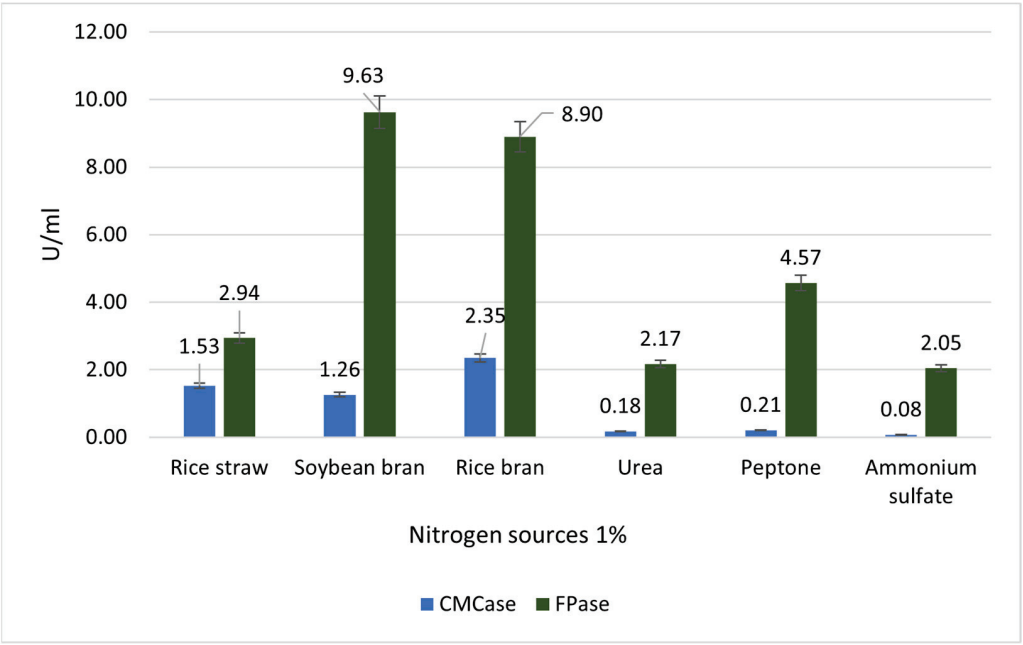

Figure 3. Effect of various nitrogen sources on cellulase production by *A. terreus* NIH2624 when grown on sugarcane bagasse as a primary carbon source.

The FPase values of this study are promising when compared to the literature. Latifian et al. [22] found FPase values of 1.163 U/mL for *T. reesei* QM9414 cellulases. In another study using rice bran and husk, a strain of *Rhizopus oryzae* showed an activity of 0.92 U/mL and 0.43 U/mL for CMCase and FPase, respectively, while the *T. reesei* strain showed an activity of 0.97 U/mL and 0.32 U/mL for CMCase and FPase, respectively [23]. *Aureoba-*

sidium pullulans LB83 showed maximum cellulase activity (FPase of 2.27 U/mL; CMCase of 7.42 U/mL) on sugarcane bagasse and, while using soybean meal as a non-defined nitrogen source also showed maximum cellulase activity (FPase of 2.45 U/mL; CMCase of 6.86 U/mL) after 60 h [24].

To have a better understanding of the enzymatic cocktail profile, fermentation was carried out employing optimized conditions (i.e., growing *A. terreus* on a production medium containing alkaline pre-treated sugarcane bagasse as a carbon source and dilute acid pre-treated rice bran as an organic nitrogen source) (Table 2). The enzymatic extract obtained was concentrated from 0.353 μg/μL of protein to 0.575 μg/μL and used to evaluate the presence of different enzyme families through a substrate panel to analyze the auxiliary and plant cell wall-degrading activities.

Table 2. Plant cell wall-degrading enzymes produced by *A. terrus* NIH2624 growing on a production medium consisting of alkaline pre-treated sugarcane bagasse (carbon source) and rice bran (nitrogen source) and employing standard conditions.

Substrate	**U/mL (μmol/(min·mL))**	**Enzymes**
CMC	77.12 ± 2.8	Endoglucanase
Xylan from beechwood	1701.85 ± 98.8	Xylanases
Xylan from birchwood	1559.27 ± 31	Xylanases
Phosphoric acid swollen cellulose	21.03 ± 1.4	Endoglucanase, Cellobiohydrolase
Substrate	**U/mL (nmol/(min·mL))**	**Enzymes**
para-nitrophenyl-glucose	23.58 ± 0.1	β-glucosidase
para-nitrophenyl-cellobiose	8.46 ± 0.2	Cellobiohydrolase
para-nitrophenyl-xylose	4.45 ± 0.4	β-xylosidase
para-nitrophenyl-arabinofuranose	10.73 ± 0.4	Arabinofuranosidase

It can be noted that the concentrated enzyme cocktail has large amounts of xylanases (1701.85 U/mL and 1559.26 U/mL for beechwood xylan and birchwood xylan, respectively). These xylanase values are much higher when compared to the literature. A secretomic analysis study of *T. ressei* RUT-C30 and *A. niger* A12, which aimed to produce cellulolytic enzymes and hydrolyze sugarcane bagasse, that was carried out by Florencio et al. [9] showed xylanase activities of 7.9 IU/mL and 7.8 IU/mL for *T. reesei* RUT-C30 and *A. niger* A12, respectively. In addition, compared to the study of Florencio et al. [9], the endoglucanase activities shown in the present work were higher than the ones Florencio et al. [9] found of 1.6 IU/mL and 0.6 IU/mL for *T. reesei* RUT-C30 and *A. niger* A12, respectively. Zhao et al. [25] found activities for endoglucanase (EG), cellobiohydrolase (CBH) and β-glucosidase of 430.45 mg/h/mL, 10.09 mg/h/mL and 0.25 μmol/min/mL, respectively. Compared to the values obtained in the present work (EG: 832.93 mg/h/mL; CBH: 227.09 mg/h/mL; β-glucosidase: 0.02μmol/min/mL), the activities of EG and CBH were 1.9 and 22.5 times higher, respectively. Thus, *A. terreus* is notable for its ability to produce cellulolytic enzymes.

The PASC test was also performed by adding ascorbic acid to analyze the presence of LPMOs, important enzymes that have revolutionized our understanding of the biological degradation of plant cell walls. The PASC test can indicate the presence of endoglucanase and cellobiohydrolase [26]. The addition of ascorbic acid to the test and the observation of increased activity strongly indicates the presence of LPMOs since LPMOs are activated in the presence of an electron donor, which is, in this case, ascorbic acid. Comparing the activity with and without ascorbic acid, we noted an increase of 17.84% in the activity (Figure 4), which suggests the presence of LPMOs in the obtained enzymatic cocktail.

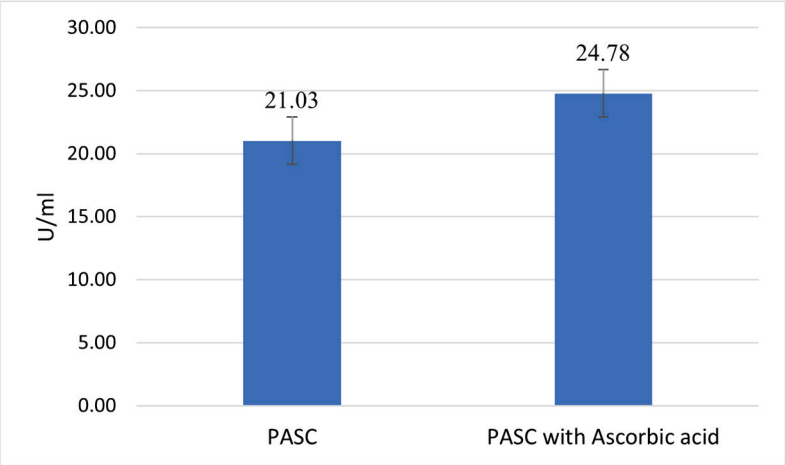

Figure 4. Increase in the saccharification power of the *A. terreus* NIH2624 cocktail on phosphoric acid swollen cellulose (PASC) by the addition of an electron donor (ascorbic acid) for lytic polysaccharide monooxygenases (LPMOs).

The boosting effect of LPMOs acting on cellulose in synergy with cellulases is well known but little explored for *A. terreus* enzymes. The *A. terreus* genome harbors 12 genes that encode LPMOs, making it an important source for this type of enzyme [27]. The diversity of proteins produced by *A. terreus* was also evidenced in SDS-PAGE gels, where it was possible to observe band patterns of different sizes and intensities. The bands with the greatest intensities correspond to approximate sizes of 100 and 25 KDa, which are frequently sizes associated with cellobiohydrolases, beta-glucosidases (~100 KDa) [28], xylanases, endoglucanases and LPMOs (~25 kDa) [29].

4. Conclusions

A. terreus NIH2624 showed a higher production of cellulase and hemicellulose enzymes compared to the other tested fungi. When this microorganism was grown in the production medium containing sugarcane bagasse (as a carbon source) and rice bran (as a nitrogen source), it showed improved production of endoglucanases, exoglucanases and xylanases. Furthermore, the enzyme cocktail from *A. terreus* NIH2624 showed mainly xylanase activity, with a potential to complement cellulolytic enzyme cocktails of other known fungi, such as *T. reesei* and other *Aspergillus* species. *A. terreus* NIH2624 showed higher endoglucanase and CBH activities compared to the enzyme activity reported with other filamentous fungi. In addition to these enzymes, activities for cellobiohydrolases, β-xylosidases, arabinofuranosidases and LPMOs were also obtained. The use of this fungus shows promising results for holistic cellulolytic enzyme cocktail production in lignocellulose biorefineries, supporting the effective hydrolysis of biomasses to produce second-generation sugars.

Author Contributions: E.A.K. collected samples, performed experiments, and wrote the first draft of the manuscript; J.V. performed the enzyme assays, analyzed the data and jointly wrote the manuscript; S.S.d.S. analyzed data and reviewed the manuscript; A.P.I. analyzed the data and edited the original draft; F.S. provided microbial strains and performed the LPMOS analysis; A.K.C. planned the experiments, supervised the research, and reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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Enzymatic Pretreatment of Slaughterhouse Wastewater: Application of Whole Lipolytic Cells of *Rhizopus oryzae* Produced from Residual Vegetable Oil

Willian S. M. Reis [1], Heitor B. S. Bento [2,*], Ana K. F. Carvalho [3,*] and Ernandes B. Pereira [4]

[1] Department of Biotechnology, Engineering School of Lorena, University of Sao Paulo, Lorena 12602-810, SP, Brazil; willian.matias@usp.br
[2] Department of Engineering of Bioprocesses and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara 14800-903, SP, Brazil
[3] Department of Basic and Environmental Sciences, Engineering School of Lorena, University of Sao Paulo, Lorena 12602-810, SP, Brazil
[4] Faculty of Pharmaceutical Sciences, Federal University of Alfenas, Alfenas 37130-001, MG, Brazil; ernandes.pereira@unifal-mg.edu.br
* Correspondence: heitor.bento@unesp.br (H.B.S.B.); anacarvalho@usp.br (A.K.F.C.)

Abstract: This study assessed the application of whole lipolytic cells in the pretreatment of slaughterhouse wastewater to reduce its lipid content. The fungal biomass of *Rhizopus oryzae* was evaluated in the hydrolysis of slaughterhouse wastewater containing high lipid concentrations, focusing on the biomass's concentration and the effect of using an emulsifier and surfactant. The use of the whole-cells lipase of *Rhizopus oryzae* grown in a residual vegetable oil medium proved effective in the hydrolysis of slaughterhouse wastewater, generating concentrations of free fatty acids (FFA) ranging from 40.36 to 90.14 mM. The action of lipase in the hydrolysis of slaughterhouse residues indicated its effectiveness in pretreating lipid-rich liquid residues, potentially boosting the microbiota of this anaerobic treatment. The results showed that lipase activity without surfactant exhibited a similar performance to that of Triton X-100 in the hydrolysis of liquid residues. However, the combination of lipase and surfactant could represent a promising strategy to optimize free fatty acid production from slaughterhouse residues, strengthening anaerobic treatment processes and potentially enhancing the overall efficiency of waste management systems.

Keywords: whole cell; lipase; hydrolysis

1. Introduction

The growth of the world's population, coupled with improving economies in developing countries, is rapidly driving global food demand [1]. The global supply of meat is expected to increase to meet rising demand, reaching 377 million tons by 2031, with Brazil as one of the primary leaders in the global export of poultry and beef. This growth is driven by a favorable exchange rate and an abundance of grains for feed. Brazil is predicted to maintain and expand its position as the leading exporter of poultry and beef throughout the projected period, until 2031 [2]. The growth in meat production is driving the increase in slaughterhouse waste, raising concerns about waste generation and pollution.

Slaughterhouses are places where animals are slaughtered to meet the growing demand for meat for human consumption. The waste generated in these places is mostly byproducts from animal processing [3,4]. The meat processing industry is a major consumer of fresh water (2.5 to 40 m^3 of water per metric ton of produced meat), generating large volumes of effluents. Their composition includes fats, proteins, and fibers from the slaughtering process. These residues contain contaminants, primarily blood, stomach, and intestinal mucus, along with high levels of organic products, microorganisms, and cleaning agents [5,6].

The wastewater discharged after proper treatment must comply with local regulations for the safe disposal of effluents. In the case of organically rich wastewater, and especially effluents from slaughterhouses, biological treatment is generally preferred over other treatment options like electrocoagulation, membrane separation, and advanced oxidation [7]. However, waste from dairies, slaughterhouses, and meat processing, while rich in nutrients and organic matter, also contains high levels of fats and oils, which are challenging for biological treatments. A prior separation of fats and oils is essential to optimize this treatment [8].

Anaerobic digestion is a biological treatment method employed for wastewater from slaughterhouses. It involves a community of microorganisms performing different tasks to convert organic matter into biogas. Initially, organic macromolecules (carbohydrates, lipids, proteins) undergo hydrolysis into monomers. These monomers are subsequently converted into volatile fatty acids during the acidogenesis phase. Following acidogenesis, acetogenesis occurs, where volatile fatty acids are transformed into acetic acid, carbon dioxide, and hydrogen gas (H_2). Finally, in methanogenesis, acetic acid and a portion of H_2 are converted into methane and carbon dioxide [9–11].

The primary reason for the removal of oils and fats is that an excess of these elements can accumulate on the sludge surface, reducing the transfer rates of soluble substrates to biomass and oxygen due to aerobic microorganisms. This inhibits sludge activity and the development of filamentous microorganisms, hindering sludge settlement and leading to biomass losses due to reactor overflows. Additionally, long-term challenges such as blockage occurrence and the generation of unpleasant odors caused by fats and oils in wastewater are evident [8,12,13]. Therefore, the pretreatment process is necessary to hydrolyze fats and oils, enhancing efficiency of the subsequent biological treatment of wastewater.

The inhibitory effects of lipids during the biodigestion process are also associated with interference in the electron transport chain, by compromising nutrient uptake, inhibiting specific enzymatic activities, or generating peroxidation products. Since inhibition is reversible, it is partially driven by the adsorption of lipid onto biomass. Methanogenic activity can resume once the lipids accumulated in the biomass are progressively metabolized through pretreatments [14,15].

An alternative treatment method involves utilizing a biochemical pathway, and particularly lipases, due to their clean application and low environmental impact. Lipases can be applied to a large variety of biotechnological applications, including the treatment of effluents with high fat contents. The biological anaerobic treatment of this kind of effluent has some problems, which are caused by high fat contents [16]. Thus, a pretreatment with enzymes has been proposed as an alternative or a complement to conventional biological processes. Another alternative is the use of biosurfactants that can make fats and oils more available to microbial biomasses [17].

The application of lipases in the treatment of wastewater with a high lipid content faces challenges such as high production costs, purification issues, and stability concerns under ideal conditions. Specific operational conditions and factors like a high organic load, temperature, pH, and the presence of metallic ions can limit the effectiveness of lipases in treating industrial effluents. Although enzyme immobilization may enhance their stability, its scalability for wastewater treatment is still underexplored [16]. Low-cost enzymatic products for wastewater treatment are crucial because high-cost commercial lipase products would render the process economically unfeasible. As an alternative, there is the production of whole-cell biomass from filamentous fungi with high lipolytic activity, which can be used for wastewater treatment, eliminating the need for the recovery and purification of these enzymes [18].

Given the scarcity of studies involving the application of commercial lipases and/or lipolytic cells in the pretreatment of slaughterhouse wastewater, the current study aims to produce whole cells with high hydrolytic activity and evaluate their application in the pretreatment of poultry and swine slaughterhouse wastewater (*SSW*) to hydrolyze the

present oils and fats. The fungus *Rhizopus oryzae* has been highlighted as one of the most promising fungal species in the production of whole cells with mycelium-bound lipase, as well as for its efficient application in the hydrolysis reactions of substrates containing different compositions of fatty acids. This underscores the potential of this fungus to be applied as a biocatalyst in the pretreatment of slaughterhouse wastewater.

2. Materials and Methods

2.1. Microorganism

The strain used was *Rhizopus oryzae* CCT3759, obtained from the André Tosello Tropical Research and Technology Foundation (Campinas, SP, Brazil). In order to obtain and maintain culture spores, fungal cells had been previously inoculated on Sabouraud agar medium under aseptic conditions. The cultures were incubated at 30 °C for 72 h or until they reached their highest sporulation status.

2.2. Materials

Sabouraud agar (HIMEDIA®, Kennett, MI, USA); soy peptone (HIMEDIA®, Kennett, MI, USA); magnesium sulfate heptahydrate (Vetec®, Duque de Caxias, Brazil); olive oil (Carbonell®, Córdoba, Spain); gum arabic powder (Dinâmica®, Indaiatuba, Brazil); disodium phosphate dibasic (Dinâmica®, Indaiatuba, Brazil); sodium hydroxide (Vetec®, Duque de Caxias, Brazil); ethyl alcohol; Triton™ X-100 (70% v/v) (Vetec®, Duque de Caxias, Brazil); acetone (Synth®, Diadema, Brazil); phenolphthalein (Synth®, Diadema, Brazil). Slaughterhouse wastewater (SWW) was obtained from a local slaughterhouse farm focused on poultry and pork production (Alfenas, Minas Gerais, Brazil). *SWW* samples were collected before and after, from a flotation tank located at the treatment plant itself. Slaughterhouse waste samples were then frozen and maintained at −20 °C prior to utilization.

2.3. Physicochemical Characterization of SWW

For the characterization of the raw effluent, its pH, total solids (TSs), fixed solids (TFSs), volatile solids (TVSs), free fatty acids (FFAs), acidity index, and saponification were analyzed. All analyses were conducted following procedures outlined in the Standard Methods for the Examination of Water and Wastewater [19]. Fatty acid composition of both synthetic and real effluents was determined as fatty acid methyl ester (FAME) by gas chromatography according to the American Oil Chemists' Society (AOCS) official method [20].

2.4. Enzymatic Activity

Mycelium-bound lipase activity was assessed in terms of its dry biomass concentration (g L^{-1}) and hydrolytic activity (U g^{-1}), using the method of olive oil emulsion hydrolysis. Enzyme activity (U g^{-1}) is defined as the amount of dry biomass required to release 1 µmol of free fatty acids per minute under experimental conditions (0.1 g of biomass at a 37 °C reaction temperature, 100 mM sodium phosphate buffer, and at a pH of 7.0 for a 5 min reaction) [18].

2.5. Culture Medium and Conditions

The culture medium consisted of 30 g L^{-1} of residual frying oil after vacuum filtration, 70 g L^{-1} of soy peptone, 1 g L^{-1} of $NaNO_3$, 1 g L^{-1} of KH_2PO_4, and 0.5 g L^{-1} of $MgSO_4 \cdot 7H_2O$, all previously autoclaved (121 °C for 15 min) [18]. After autoclaving, Erlenmeyer flasks were cooled and olive oil (3% w/v) was added in a sterile manner. The cultures were established in 250 mL Erlenmeyer flasks containing 100 mL of autoclaved medium and inoculated with a suspension of 1×10^6 spores at 30 °C, with orbital agitation at 180 rpm. The spore concentration was determined by counting the cells in a Neubauer chamber using an Olympus® binocular microscope (Hicksville, NY, USA). At the end of the cultivation, the produced biomass was separated from its medium by vacuum filtration,

washed with water and acetone and quantified for its hydrolytic activity [21]. Subsequently, the fungal biomass was stored at 4 °C before use.

2.6. Enzymatic Pretreatment of Slaughterhouse Wastewater in Stirred Tank Reactors

In 250 mL glass-lined reactors, 100 mL of SWW was used as substrate for a hydrolysis reaction that used 2.0 g of dry biomass. The tests were carried out at 40 °C and 600 rpm of mechanical agitation, which was carried out using a suspended agitator motor with a steel helical impeller for up to 24 h. A 50:50 (v/v) mixture of acetone and ethanol was added to the aliquots (0.5 g), which were removed periodically, and the FFA concentration was quantified by titration with 20 mM of sodium hydroxide (NaOH) solution using phenolphthalein as the indicator. The concentration of free fatty acids (FFAs) (mM) was calculated using Equation (1).

$$\text{FFA (mM)} = \frac{(V_a - V_b) * C_{NaOH} * 1000}{m} \quad (1)$$

where V_a is the volume of NaOH in the sample (mL); V_b is the volume of NaOH in the control (mL); C_{NaOH} is the molar concentration of NaOH (20 mM); and m is the sample mass (0.5 g).

Initial reaction rates were analyzed by the formation of FFAs (mM) in the initial 12 h of the reaction. Results were plotted using the software Origin Pro version 5.0 so as to obtain a linear equation for the initial hydrolysis reaction rates of the *SWW*.

2.7. Variation in Biomass Mass during the Enzymatic Pretreatment of Slaughterhouse Wastewater

Different initial masses of fungal biomass were evaluated as biocatalysts in the enzymatic pretreatment of *SWW*. The assessed masses were 0.5, 1.0, and 2.0 g of dry biomass, corresponding to an average hydrolytic activity of 100, 200, and 400 U, respectively. The produced lipase was evaluated for its ability to generate free fatty acids (FFAs) by hydrolyzing the triglycerides present in the *SWW* pretreatment.

2.8. Evaluation of pH Adjustment in the Enzymatic Pretreatment of SWW

The need for a pH adjustment was assessed to determine the influence of the natural pH of slaughterhouse wastewater on the performance of the produced lipase. The pH was adjusted using a concentrated HCl solution to reach a pH of 6.0, known as the optimal pH for the activity of the lipase bound to the mycelium of the fungus *Rhizopus oryzae* CCT3759.

2.9. Influence of Surfactants and Emulsifiers on the Enzymatic Pretreatment of SWW

The influence of a surfactant and an emulsifier was evaluated in the enzymatic pretreatment in association with lipase-catalyzed hydrolysis from *Rhizopus oryzae* biomasses. Both the surfactant and the emulsifier were assessed at a concentration of 3% (w/v), with Triton X-100 evaluated as the surfactant and arabic gum as the emulsifier. Both were applied individually and in combination.

3. Results and Discussion

3.1. Characterization of Slaughterhouse Wastewater

The meat industry utilizes significant amounts of water in its industrial processes, generating considerable volumes of effluents characterized by high organic loads and concentrations of suspended solids. The characteristics of these effluents vary depending on the type of industrial process adopted [3,5]. In this study, effluent from slaughterhouses (birds and pigs), collected from two sectors of the slaughterhouse treatment plant, was used. One sample was taken from raw slaughterhouse wastewater (*RSWW*), prior to it entering the main flotation tank, and another sample from within the flotation tank (*SWWF*). The characterization of both samples is described in Table 1.

Table 1. Physical–chemical characterization of raw slaughterhouse wastewater (*RSWW*) and slaughterhouse wastewater obtained from the flotation tank (*SWWF*).

Parameter	*RSWW*	*SWWF*
pH	6.15 ± 0.06	7.59 ± 0.35
Total solids (mg L^{-1})	18,609.17 ± 661.98	13,530 ± 1300.34
Total fixed solids (mg L^{-1})	3665.83 ± 195.26	2545.33 ± 139.91
Total volatile solids (mg L^{-1})	14,943.33 ± 698.49	10,984.67 ± 57.18
Free fatty acids (%)	0.75 ± 0.031	0.51 ± 0.023
Acidity index (mgKOH g^{-1})	1.64 ± 0.689	1.11 ± 0.051
Saponification (mgKOH g^{-1})	5.38 ± 0.39	4.32 ± 0.77
Fatty acids composition (%)		
Caprylic acid—C8:0	4.30	-
Myristic acid—C14:0	4.49	-
Palmitic acid—C16:0	30.00	30.71
Stearic acid—C18:0	0.34	-
Oleic acid—C18:1	31.30	35.92
Linoleic acid—C18:2	29.56	33.37
Average molecular weight of FFAs (g mol^{-1})	273.79	265.68

The results in Table 1 highlight that the evaluated *RSWW* presented higher concentrations of solids, as previously reported in the literature [7,22]. A lower concentration of solids is evident in the effluent collected directly from the flotation tank (*SWWF*), showing a decrease in total solids of 27.29% (from 18,609.17 to 13,530 mg L^{-1}), total fixed solids of 30.57% (from 3665.83 to 2545.33 mg L^{-1}), and total volatile solids of 26.49% (from 14,943.33 to 10,984.67 mg L^{-1}). Similarly, the measurements of the free fatty acids, acidity index, and saponification also decreased due to their treatment in the flotation tank. The primary flotation treatment system is well known for separating suspended and/or fatty particles from the effluent, directly influencing its physicochemical parameters that are related to oils and fats [23].

Regarding the distribution of fatty acids within the triacylglycerols present in the lipids of *RSWW*, the raw effluent contained saturated fatty acids that were not detected in the *SWWF*, such as caprylic acid (4.30%), myristic acid (4.49%), and stearic acid (0.34%); whereas the most concentrated saturated fatty acid, palmitic acid (30%), was also quantified in the *SWWF* with similar concentrations (30.71%). Despite the low percentage of fatty acids present in the flotation tank wastewater, higher concentrations of unsaturated fatty acids were found in this sample; 35.92% and 33.57% for oleic and linoleic acids, respectively. The higher proportions of oleic (31.30–35.92%) and palmitic (30–30.71%) acids were expected, as these acids are reported to be the most abundant in food processing effluents, such as slaughterhouse effluent [13].

3.2. The Influence of Biocatalyst Mass on the Hydrolysis of Slaughterhouse Wastewater

In order to evaluate the influence of the mass of catalytic biomass added on the hydrolysis process, that is, how much hydrolytic activity will be generated, different masses of biomass were evaluated, using 0.5, 1.0, and 2.0 g of dry mycelium. The obtained results are shown in Figure 1.

The higher hydrolytic activity provided was effective for the RSSW (Figure 1A), with a maximum FFA formation of 85.34 ± 2.59 mM. However, a better performance was observed in the system with 1.0 g of biomass, showing a profile similar to the 2.0 g system up to 10 h of operation. However, after this time, the FFA formation stabilized for 1.0 g of the biocatalyst, reaching only 68.12 ± 0.36 mM, whereas the reaction with 2.0 g of biomass increased from 60.84 to 85.34 mM.

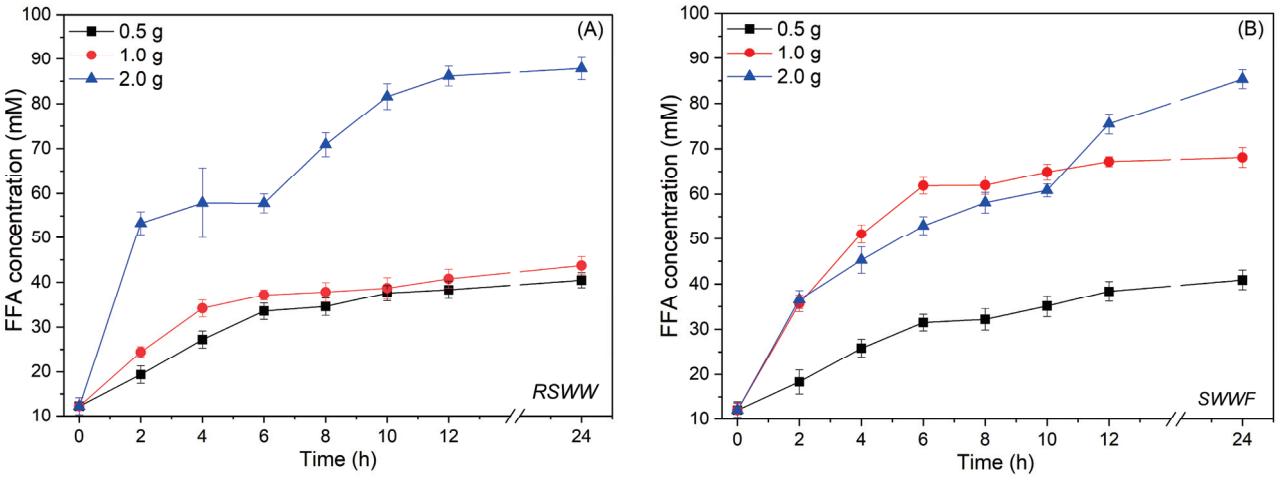

Figure 1. Evaluation of the initial mass of the biocatalyst in the formation of FFA from the hydrolysis of *RSWW* (**A**) and *SWWF* (**B**).

In the hydrolysis process of *SWWF* (Figure 1B), a higher initial supply of hydrolytic activity favored a higher formation of FFAs within the first 2 h of the reaction, with an FFA value of 53.33 ± 2.53 mM, which reached 87.85 ± 0.28 mM. For the masses 0.5 g and 1.0 g, similar hydrolysis profiles were observed, with maximum FFA formation values of 40.46 ± 0.12 mM and 43.71 ± 1.59 mM, respectively. From 8 h of reaction time onwards, the system containing 2.0 g of biomass managed to produce twice the results of the other systems (0.5 g and 1.0 g) by the end of the 24 h operation. This result was expected, due to the known influence that the mass of a biocatalyst has on the efficiency of lipase in the hydrolysis reaction of an effluent [24,25]. In a study by Valladão, Freire, and Cammarota (2007) [26], the influence of enzymatic load was also evaluated in the pretreatment of a slaughterhouse effluent using a solid enzymatic pool (SEP) for the subsequent application of an anaerobic treatment. Within the adopted conditions, it was observed that an increase in the SEP led to a higher formation of fatty acids; however, a lower SEP concentration favored organic matter removal.

3.3. Influence of the Initial pH of Slaughterhouse Wastewater on the Hydrolysis Process

A pH adjustment of the *SWW* was evaluated to determine its influence on the enzyme's performance, as the pH of the reaction medium directly affects the stability of the lipase's molecular structure and, consequently, its catalytic power and, furthermore, studies generally adjust the pH to the optimum pH for the enzyme that is to be used. The pH of the SWW was adjusted to pH 6.0, which is the optimal pH of *Rhizopus oryzae* CCT3759 whole-cell lipase [13,26,27].

Based on the results of the influence of pH shown in Figure 2, the hydrolysis profile presented by *RSWW* with an adjusted pH was slightly higher than that presented by the original pH (6.15) for up to 12 h of the reaction, but, after the first 12 h, the hydrolysis of *RSWW* without an adjustment of pH had a maximum FFA formation of 101.62 ± 0.75 mM, while the pH-adjusted system had a maximum conversion of 87.85 ± 0.48 mM. For *SWWF*, the hydrolyses without (pH 7.59) and with a pH adjustment were also similar, reaching values close to optimal FFA formation at the end of 24 h of reaction; values of 85.11 and 85.34 mM, respectively. The small differences in the hydrolysis process of the *SWW*s may have been due to the close pH values of the *SWW*s to the optimum pH of the enzyme, thus the lipase could act in a similar way in both study conditions; therefore, as the pH adjustment did not provide a considerable increase in the formation of FFAs, the other tests continued without pH adjustment.

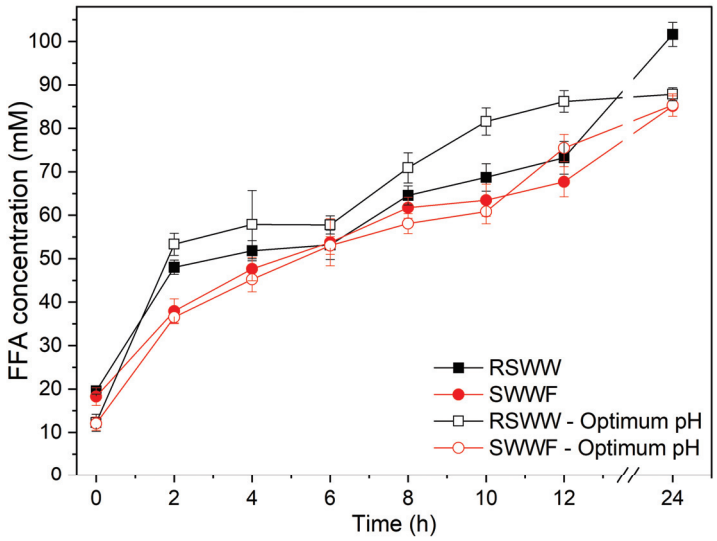

Figure 2. Influence of a pH adjustment on the enzymatic pretreatment.

3.4. Influence of Surfactants and Emulsifiers on the Enzymatic Pretreatment

Surfactants and emulsifiers can emulsify triglycerides, facilitating the action of lipases at the water/oil interface and thus accelerating the biodegradation process, eliminating the need for additional pretreatment processes to remove fats, which results in lower operating costs in the treatment of fatty effluents [28].

The surfactant Triton X-100 and the emulsifier arabic gum were evaluated in the enzymatic hydrolysis process of *RSWW* and *SWWF*. The formation of FFAs was evaluated during 24 h of reaction, as were the initial reaction rates of each system, and the results obtained are given in Figure 3 (*RSSW*) and Figure 4 (*SWWF*).

The results of the use of surfactant/emulsifier in the hydrolysis of *RSWW* (Figure 3A) show a higher production of FFAs with the use of Triton over 24 h of reaction. On the other hand, arabic gum was the system with the lowest FFA production among the systems evaluated, reaching 84.34 ± 0.50 mM. The hydrolysis generated only by the action of lipase linked to the mycelium of *Rhizopus oryzae* was superior to that achieved by arabic gum + lipase during the first 12 h of reaction, with a production of 88.97 ± 0.19 mM, which came close to the 24 h reaction value of FFA obtained by the arabic gum + lipase system, which was a maximum volume of FFAs of 90.14 ± 0.48 mM.

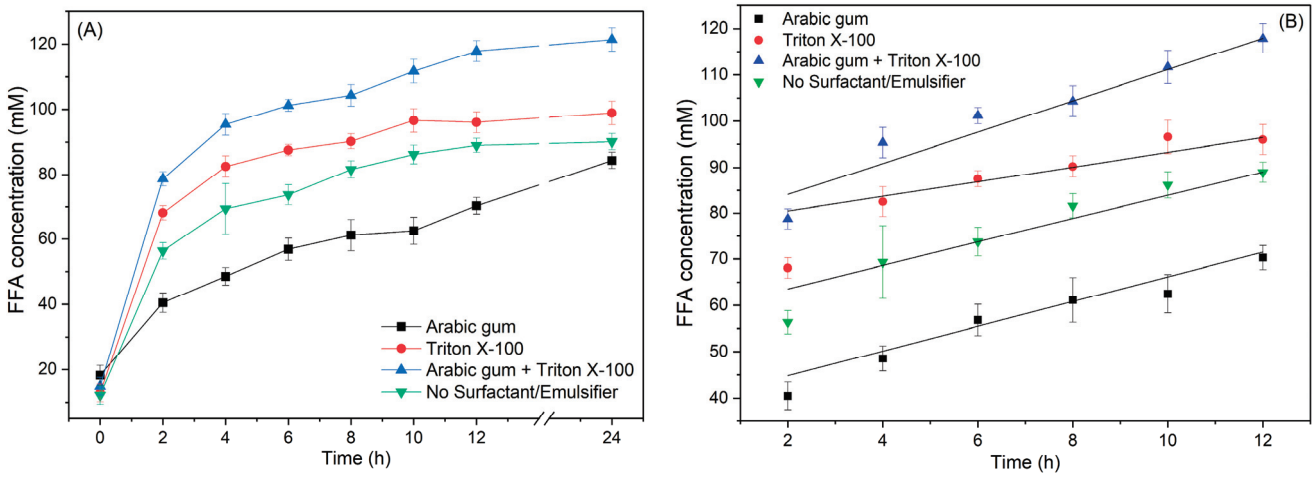

Figure 3. Influence of the use of surfactant and emulsifier on the *RSWW* pretreatment. (**A**) FFA concentration over 24 h; (**B**) FFA concentration in the first 12 h for initial hydrolysis rate analysis.

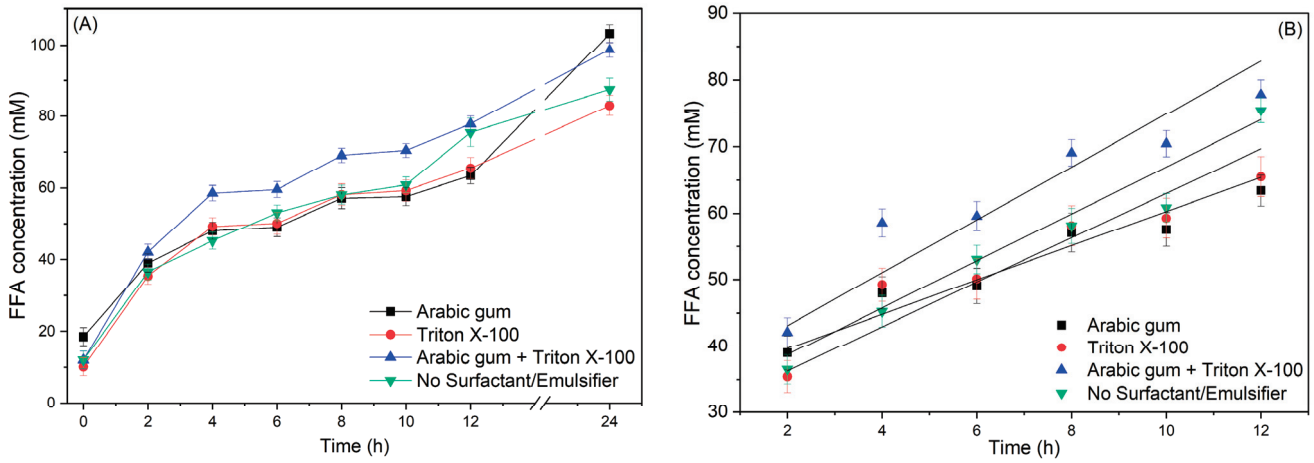

Figure 4. Influence of the use of surfactant and emulsifier on the *SWWF* pretreatment. (**A**) FFA concentration over 24 h; (**B**) FFA concentration in the first 12 h for initial hydrolysis rate analysis.

The combined action of Triton X-100 + arabic gum resulted in the highest FFA formation when compared to the use of the biosurfactant and emulsifier individually, with a maximum production of 121.38 ± 1.60 mM. This outcome may indicate that both components promoted a better stabilization of the formed emulsions, consequently enhancing the lipase-catalyzed reaction. The data also suggest that, at least within the initial 12 h of the reaction, Triton X-100 might have had a better role in stabilizing the water/lipid interface. This is supported by the fact that, when used individually, Triton X-100 led to higher FFA formation compared to the results of arabic gum alone.

The initial rates promoted in the *RSWW* (Figure 3B and Table 2) also indicate the positive effect of the combined use of Triton X-100 and arabic gum, as this showed the highest initial reaction rate (3.38 mM/h). It is worth noting that the reaction rates exhibited by the biosurfactant were the lowest obtained, with an FFA formation rate of 1.60 mM/h, possibly due to the smaller difference in FFA production over the reaction time (from 68.08 to 98.91 mM).

Table 2. Initial reaction rates of the hydrolysis of *RSWW* catalyzed by whole-cell *Rhizopus oryzae* CCT3759.

SWW—Pre-Flotator	Maximum FFA Concentration (mM)	v (mM/h)
Arabic gum	107.11 ± 0.50	2.66
Triton X-100	84.34 ± 1.60	1.60
Arabic gum + Triton X-100	121.38 ± 1.63	3.38
No surfactant/emulsifier	90.14 ± 0.48	2.55

When evaluating the action of the surfactant and emulsifier in the hydrolysis process of the *SWW* collected from the flotation unit (Figure 4A), a different reaction profile is observed. The systems containing Triton X-100, arabic gum, and the system without surfactant/emulsifier showed similar hydrolysis profiles during the initial 10 h of the reaction, differing only after 12 h of reaction, with a maximum FFA production of 82.95 ± 0.15, 103.17 ± 0.40, and 87.43 ± 2.59 mM, respectively. This result might be due to the lower complexity of the fatty acids in the *SWWF* (Table 1) compared to the fatty acids in the *RSWW*, which exhibited a higher proportion of various saturated fatty acids (C 8:0, C 14:0, C16:0, C18:0), while the *SWWF* presented only one type of saturated fatty acid (C 18:0). Despite showing higher FFA production values in the first 12 h of the reaction, the reaction system containing Triton X-100 and arabic gum also remained close in performance to the results obtained in the other assays.

Observing the results presented in Figure 4 and Table 3, it is evident that similar initial reaction rates were obtained for the use of Triton X-100, Triton X-100 and arabic gum, and without the use of surfactant and emulsifiers, which show initial reaction rates of 3.34, 3.38, and 3.53 mM/h. It is noteworthy that higher reaction rates were achieved in the hydrolysis of *SWWF* than in *RSWW*, which could be attributed to the difference in the fatty acid composition of both wastewater types. The *SWWF*, besides having lower proportions of saturated fatty acids, also exhibits a higher proportion of unsaturated fatty acids. Studies on the lipase linked to *Rhizopus oryzae* CCT3759's mycelium demonstrate its higher selectivity for substrates with higher proportions of oleic and linoleic acid [29].

Table 3. Initial reaction rates of the hydrolysis of *SWWF* catalyzed by whole-cell *Rhizopus oryzae* CCT3759.

SWW—Flotator	Maximum FFA Concentration (mM)	v (mM/h)
Arabic gum	103.17 ± 0.40	2.57
Triton X-100	82.95 ± 0.15	3.34
Arabic gum + Triton X-100	98.59 ± 0.15	3.38
No surfactant/emulsifier	87.43 ± 2.59	3.53

The obtained results align with studies reported in the literature. In Damasceno et al.'s [28] study, the combined use of a biosurfactant produced from *Pseudomonas aeruginosa* PA1 and an enzymatic pool produced by solid-state fermentation with *Penicillium simplicissimum*, in the anaerobic treatment of a high-fat content effluent from a poultry slaughterhouse, resulted in higher lipid hydrolysis and subsequently led to higher specific methane production in the anaerobic treatment.

In studies by Alves et al. [18]; Braz et al. [27]; and Ferreira et al. [30], integral cells of filamentous fungi were evaluated for pretreating wastewater from the dairy industry. The mycelium of *Penicillium citrinum* and the fungus *Mucor circinelloides* were used as enzymatic catalysts in a hybrid treatment process during anaerobic treatment. It is also worth highlighting that filamentous fungi have a versatile ability to grow on a wide range of substrates and are able to produce lipolytic cells from several low-cost substrates, such as residual vegetable oil. The combined use of lipolytic integral cells not only increased the biodegradability of dairy waste but also promoted methane generation during treatment. These results highlight the potential of the biomass produced to act in tandem with the biodigestion process of oil- and fat-rich wastewater, such as that from slaughterhouses.

4. Conclusions

Whole cells of *Rhizopus oryzae*, with high hydrolytic activity, were effective in the hydrolysis process of slaughterhouse wastewater, resulting in FFA formations ranging between 40.36 and 90.14 mM. Adjusting the pH of wastewater to the optimal pH for lipase activity did not lead to a higher efficiency in FFA formation, as the original pH of the wastewater is close to the optimal pH for lipase. The performance of the lipase in the hydrolysis of *RSWW* and *SWWF* indicates its effectiveness in both residues, contributing to FFA production and potentially enhancing the anaerobic treatment microbiota. However, the use of a surfactant might be more suitable when combined with lipase in *RSWW* hydrolysis to increase FFA production, since our results indicated that the lipase's performance without the addition of surfactant was similar to its performance with Triton X-100 in *SWWF* hydrolysis. In conclusion, whole cells of *Rhizopus oryzae* demonstrate significant potential for application as an enzymatic pretreatment for slaughterhouse wastewater, aiming to enhance the biodegradability of the residue during the anaerobic digestion stage. Future studies will assess the optimization of the enzymatic pretreatment's efficiency at hydrolyzing slaughterhouse wastewater during the biodigestion process, as well as evaluate the toxicity parameters of slaughterhouse wastewater before and after the enzymatic

pretreatment. The operational stability of lipase and its reaction conditions will be vital parameters to be assessed for the feasibility of applying lipolytic cells of *R. oryzae* to the enzymatic pretreatment of wastewater with high levels of oils and fats.

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Xylanase Production by *Cellulomonas phragmiteti* Using Lignocellulosic Waste Materials

Kata Buda [1], Tünde Fekete [1], Ornella M. Ontañon [2], Eleonora Campos [2] and Csaba Fehér [1,*]

[1] Biorefinery Research Group, Department of Applied Biotechnology and Food Science, Faculty of Chemical Technology and Biotechnology, Budapest University of Technology and Economics, Szent Gellért tér 4, H-1111 Budapest, Hungary; kata.buda.bk@gmail.com (K.B.); fek.tundike@gmail.com (T.F.)
[2] Instituto de Agrobiotecnología y Biología Molecular (IABIMO), Instituto Nacional de Tecnología Agropecuaria (INTA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), De los Reseros y N. Repetto s/n, Hurlingham, Buenos Aires B1686IGC, Argentina; ontanon.ornella@inta.gob.ar (O.M.O.); campos.eleonora@inta.gob.ar (E.C.)
* Correspondence: feher.csaba@vbk.bme.hu; Tel.: +36-1-463-2843

Abstract: Lignocellulosic biomass holds promise as a renewable feedstock for various applications, but its efficient conversion requires cost-effective degradation strategies. The main objective of this study was to investigate the effect of the growth conditions of *Cellulomonas phragmiteti* in the production of (hemi)cellulosic supernatants. To meet this aim, different lignocellulosic residues were used as carbon sources for growth using defined mineral or nutritive culture media. Cell-free culture supernatants with xylanolytic activity were produced in all the conditions evaluated, but the highest xylanase activity (15.3 U/mL) was achieved in Luria–Bertani (LB) medium containing 1% waste paper. Under these conditions, almost negligible β-glucosidase, cellobiohydrolase, β-xylosidase, and α-arabinofuranosidase activity was detected. The xylanolytic supernatant showed tolerance to salt and displayed maximal catalytic efficiency at pH 6 and 45 °C, along with good activity in the ranges of 45–55 °C and pH 5–8. As it showed good stability at 45 °C, the supernatant was employed for the hydrolysis of birchwood xylan (50 g/L) under optimal conditions, releasing 10.7 g/L xylose in 72 h. Thus, *C. phragmiteti* was found to produce a xylanolytic enzymatic supernatant efficiently by utilizing the cheap and abundant lignocellulosic residue of waste paper, and the produced supernatant has promising attributes for industrial applications.

Keywords: bacterial enzymes; hemicellulases; enzyme fermentation; waste valorization; xylanase characterization; enzyme stability

1. Introduction

Lignocellulosic biomass serves as a vital and abundant resource for the production of renewable energy and bioproducts [1]. Industrial and agricultural residues and forestry by-products usually remain as non-utilized lignocellulosic wastes; however, they contain substantial amounts of carbohydrates and other valuable substances. In order to valorize these waste materials into energy and bioproducts in an environmentally friendly way, the efficient biodegradation of their carbohydrates is crucial [2]. However, due to the complex polymeric structure of lignocellulose, accessing the sugar components that built it up is still a major challenge [3]. Lignocellulose represents a complex highly recalcitrant structure comprising cellulose surrounded by hemicellulose and lignin. The complete deconstruction of lignocellulose requires the simultaneous action of many different enzymes, such as cellulases, hemicellulases, and lignin-decomposing enzymes [4,5]. Among hemicellulases, xylanases hold particular significance due to the abundance of xylan hemicellulose in many lignocellulosic residues having great potential for biotechnological valorization. Xylanases are hydrolytic enzymes that break xylan into smaller units [6]. These enzymes have wide applications in the food industry, animal feed production, and paper manufacturing, and

in recent years, they have garnered increasing attention for their role in the production of biofuels and biochemicals [7,8].

Bacterial xylanase production using lignocellulosic waste shows great promise for cost-effective enzyme production. Various microbial strains, including *Clostridium* sp., *Cellulomonas* sp., *Bacillus* sp., *Thermomonospora* sp., and *Streptomyces* sp., exhibit the capability to produce xylanases that can be used in lignocellulose degradation [9]. *Bacillus* species such as *B. halodurans* and *B. pumilus* have been widely studied as efficient xylanolytic enzyme producers [10,11]. *Cellulomonas* strains are also promising sources of xylanolytic enzymes [12]; however, less attention has been dedicated to them so far. *Cellulomonas fimi* and *Cellulomonas flavigena* have been reported to have extracellular xylanase activity [9,13]. Fourteen genes potentially encoding xylanases were found in the *C. flavigena* genome sequence [14]. Ontañon et al. investigated *C. fimi* and *Cellulomonas* sp. B6 and their use for biomass deconstruction. Different lignocellulosic residues (e.g., sweet corn cob, waste paper, and wheat bran) were tested. Both strains showed high xylanolytic activity in culture supernatants, although *Cellulomonas* sp. B6 was more efficient [15].

The production of xylanases from lignocellulosic waste materials offers a dual advantage. On one hand, it utilizes inexpensive substrates, thereby reducing the production costs of xylanases significantly; on the other hand, it contributes to the eco-friendly management of these waste materials [16,17]. Lignocellulosic wastes such as agricultural residues, forestry by-products, and industrial remnants can serve as low-cost and efficient carbon sources for the production of xylanases and other high-value enzymes [18]. Xylanases produced from lignocellulosic wastes can have versatile applications. They can be employed in different tasks in various industries, including biofuel production, bioremediation, and food processing, making them a high-value bioproduct that can be produced from low-cost substrates [19].

In this context, this article explores the production of bacterial xylanases by *Cellulomonas phragmiteti* using lignocellulosic residues as substrates and characterizes the produced enzyme preparation (e.g., pH and temperature optima, NaCl tolerance, thermostability).

2. Materials and Methods

2.1. Microorganism

Cellulomonas phragmiteti NCAIM B.02303 was kindly donated by the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary). *Cellulomonas phragmiteti* is a Gram-positive, facultatively anaerobic bacterium with motile rod-shaped cells. Its colonies on King's B agar plates became smooth, yellow to pale orange, and circular within 3 days of cultivation at 25 °C [20]. *C. phragmiteti* grows well at 15–37 °C (optimum: 25 °C) and pH 7.0–9.0 (optimum: pH 8.0), and in the presence of 2.0–7.0 *w*/*v*% NaCl (optimum: 5.0 *w*/*v*%) [20]. *C. phragmiteti* was maintained at 4 °C on Luria–Bertani (LB) [21] agar in Petri dishes containing the following: 0.5 *w*/*w*% yeast extract, 1 *w*/*w*% NaCl, 1 *w*/*w*% trypton and 1.5 *w*/*w*% agar.

2.2. Lignocellulosic Materials

Wheat bran (WB), brewer's spent grain (BSG), rye bran (RB), rice straw (RS), distillers dried grain with solubles (DDGS), and waste paper (WP) were examined as carbon sources for xylanase enzyme production by *C. phragmiteti*. Xylan from birchwood was used in enzymatic hydrolysis tests, and it was purchased from Sigma-Aldrich (St. Louis, MI, USA). The WB was provided by Gyermelyi Ltd. (Gyermely, Hungary); the RB was kindly donated by the Research Group of Cereal Science and Food Quality, Budapest University of Technology and Economics (Budapest, Hungary); the RS was provided from Suranaree University of Technology (Nakhon Ratchasima, Thailand); the DDGS was provided from Hungarian University of Agriculture and Life Science (Budapest, Hungary); and the WP was provided from a local paper and stationery shop (Budapest, Hungary). The WP consisted of unprinted, pressed, recycled, corrugated cardboard paper sheets, which were cut into small pieces (smaller than 0.5 cm) (Figure S2), mixed with distilled water to set

10 w/w% dry matter content, homogenized with a hand blender for 10 min, and autoclaved (121 °C) for 30 min before being used to prepare culture media for cell cultivation or xylanase fermentation. WB, BSG, RB, and DDGS were mixed with distilled water to set 10 w/w% dry matter and then autoclaved (121 °C) for 30 min before being used in preparing culture media for cell cultivation or xylanase fermentation. RS was cut into small pieces (0.5 cm) and then prepared in the same way as WB, BSG, RB, and DDGS before being used in preparing culture media for cell cultivation or xylanase fermentation. The structural carbohydrates (glucan, xylan, and arabinan) and acid-insoluble solids (Klason lignin) of WP were determined according to the method of the National Renewable Energy Laboratory (DEN, USA) [22]. The structural carbohydrate analysis of WP was carried out in triplicate (Table 1).

Table 1. Relative structural carbohydrate and acid-insoluble solid contents of waste paper. Average values and standard deviations (indicated in parentheses) were calculated from triplicates.

	Percentage of Dry Matter
Glucan	56 (0.7)
Xylan	15 (0.24)
Arabinan	0 (0.0)
Acid-insoluble solid (Klason lignin)	10.5 (0.07)

2.3. Enzyme Production in Shake Flask

C. phragmiteti was grown on culture media containing LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or minimal medium (MM) (1.67 g/L K_2HPO_4, 0.87 g/L KH_2PO_4, 0.05 g/L NaCl, 0.1 g/L $MgSO_4 \times 7H_2O$, 0.04 g/L $CaCl_2$, 0.004 g/L $FeCl_3$, 0.005 g/L $Na_2MoO_4 \times 2H_2O$, 0.01 g/L biotin, 0,02 g/L nicotinic acid, 0.01 g/L pantothenic acid, 1 g/L NH4Cl, 1 g/L yeast extract), all supplemented with 1 w/w% of different lignocellulosic carbon sources (WP, WB, BSG, RB, RS, and DDGS) which were pretreated according to the method described in Section 2.2. Culture media (LB or MM media with lignocellulosic carbon sources) were sterilized in autoclave at 121 °C for 20 min. Starter cultures were obtained by inoculating colonies (from agar plates) in 10 mL LB medium and incubating them at 30 °C and 220 rpm for 72 h in 100 mL flasks closed by cotton plugs. Cultures for enzyme production (fermentations) were inoculated from the starter cultures to set an initial cell concentration that corresponded to an optical density (OD) of 0.05. Fermentation experiments were performed in 25 mL culture media poured into 100 mL shake flasks at 30 °C and 220 rpm for 72 h. Fermentations were performed in triplicate. After 72 h, the fermentation media were vacuum-filtered to separate the solids (cells and biomass) from the fermentation supernatant, which was stored at −18 °C until further use.

2.4. Enzymatic Activity Measurements

The xylanase and carboxymethyl cellulase (CMC-ase) activities of the fermentation supernatants were assayed in test tubes by using a beechwood xylan (Sigma-Aldrich, St. Louis, MI, USA) suspension (1 w/w%) or wheat arabinoxylan (arabinoxylan for reducing sugar assays, low-viscosity arabinoxylan, medium-viscosity arabinoxylan, and high-viscosity arabinoxylan (Megazyme, Bray, Ireland)) solutions (1 w/w%) and CMC solution (2 w/w%) as substrates, respectively [23]. For these assays, 0.75 mL of appropriately diluted cell-free fermentation broths were added to 0.75 mL of each substrate prepared in citrate buffer (0.1 M, pH 6). The assays were carried out at 45 °C with magnetic stirring for 10 min. Reducing sugars released during the assay were measured by the dinitrosalicylic acid (DNS) method [24] using glucose or xylose standard curves.

The β-glucosidase, cellobiohydrolase, β-xylosidase, and α-arabinofuranosidase activities were assayed using 5 mM p-nitrophenyl-β-D-glucopyranoside (pNPG), p-nitrophenyl-β-D-cellobiosid (pNPC), p-nitrophenyl-β-D-xylopyranoside (pNPX), and p-nitrophenyl-α-L-arabinofuranoside substrates, respectively, according to previously established proto-

cols [25]. Briefly, the reactions were performed in test tubes; 1 mL p-nitrophenyl reagent was added to 0.1 mL appropriately diluted cell-free fermentation broth. The reactions were performed at 45 °C with magnetic stirring for 10 min. The reactions were stopped with 2 mL sodium carbonate (1 M), and absorbance was determined at a wavelength of 400 nm using a spectrophotometer. A p-nitrophenol (pNP) curve was used as a standard.

All enzymatic assays were conducted in triplicate, and control enzymes without a substrate and a substrate without an enzyme were included. In all cases, one international unit (U) was defined as the amount of enzyme required to release 1 μmol of product per minute under the assay conditions.

2.5. Investigation of the Time Dependence of Xylanase Production

Xylanase production by *C. phragmiteti* as a function of the fermentation time was also investigated. Enzyme production was performed on WP as a carbon source as described in Section 2.3. Xylanase activity production was monitored by daily sampling (0, 24, 48, 72, 96 h). Xylanase activity measurements were performed at each sampling time as described in Section 2.4.

2.6. Total Protein Content Assay

The total protein contents of the fermentation supernatants were quantified by the Bradford assay. The Bradford reagent contained 10 mg Coomassie blue, 5 mL 96 *w*/*w*% ethanol, and 10 mL 85 *w*/*w*% phosphoric acid, and the solution was completed to 100 mL with distilled water. The assay was performed in test tubes. Next, 5 mL of reagent was added to a 0.1 mL sample and carefully homogenized. Two minutes later, the absorbance was determined at a wavelength of 595 nm using a spectrophotometer. A bovine serum albumin curve was used as a standard.

2.7. Effect of NaCl Concentration on Xylanase Activity and Xylanase Production

The effect of NaCl concentration on xylanase activity was investigated using cell-free fermentation broth derived from fermentations containing 1 *w*/*w*% WP as a carbon source. Xylanase enzyme activity was measured according to the method previously described in Section 2.4., with small modifications. Different amounts of NaCl (25 g/L, 50 g/L, 100 g/L, 150 g/L, and 200 g/L) were added to the reaction mixtures. The NaCl stock solutions were made using citrate buffer (0.1 M, pH 6). The effect of NaCl concentration on xylanase production by *C. phragmiteti* was investigated by adjusting 100 g/L NaCl concentration during xylanase fermentation on 1 *w*/*w*% WP.

2.8. Determination of the pH and Temperature Optima of the Produced Xylanolytic Supernatant

The pH and temperature optima of the xylanase activity produced by *C. phragmiteti* on WP were investigated. Regarding pH, the experiments were carried out within a pH range of 3–11, at 45 °C. At pH 3, pH 4, pH 5, and pH 6, citrate buffer (0.1 M) was used. At pH 6, pH 7, and pH 8, phosphate buffer (0.1 M) was used, and at pH 8, pH 9, pH 10, and pH 11, carbonate buffer (0.1 M) was used. The substrate suspension (1 *w*/*w*% beechwood xylan) was made using the same buffers as the reaction mixture. Regarding temperature, 25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, and 85 °C were applied during the enzyme activity measurements in citrate buffer (0.1 M, pH 6).

2.9. Enzyme Stability Measurement

Enzyme stability tests were carried out at two different temperatures (45 °C and 55 °C) at four different pH values: pH 5, pH 6, pH 7, and pH 8. Enzymatic stability was measured in 0.05 M phosphate buffers. The produced xylanase-containing supernatant was diluted (tenfold) with buffer, and the diluted enzyme solutions were incubated at elevated temperatures (45 °C or 55 °C; 220 rpm for 72 h). During the test, samples were taken at 0, 3, 24, 48, and 72 h to perform xylanase activity measurements.

2.10. Enzymatic Hydrolysis of Xylan

An enzymatic hydrolysis experiment was performed on xylan from birchwood by using the supernatant derived from the fermentation using 1 *w*/*w*% WP as a carbon source. Five *w*/*w*% dry matter of xylan and 10 U/mL of the produced xylanases were set in the enzymatic hydrolysis. The hydrolysis reaction was carried out at 45 °C, pH 6, and 150 rpm for 72 h. Daily sampling was conducted from the reaction mixture. The supernatants were separated by vacuum filtration through a nylon filter (0.2 µm pore size), and they were analysed by high-performance liquid chromatography (HPLC) for xylose. HPLC analysis was performed by using a BioRad (Hercules, CA, USA) Aminex HPX-87H (300 × 7.8 mm) column equipped with a Micro-Guard Cation H+ Refill Cartridge (300 × 4.6 mm) pre-column at 65 °C and a refractive index detector. The eluent was 5 mmol/L sulphuric acid at a flow rate of 0.5 mL/min. The injection volume was 40 µL. To determine the total solubilized xylose content (including the xylose liberated in monomer and oligomer forms), an oligomer hydrolysis step was also carried out with the 72 h samples. They were mixed with sulphuric acid solution (8 *w*/*w*%) at a volume ratio of 1:1 and treated at 120 °C in an autoclave for 15 min to decompose oligosaccharides prior to HPLC analysis.

3. Results and Discussion

3.1. Effect of Different Media and Lignocellulosic Culture Substrates on the Production of a Xylanolytic Enzymatic Supernatant

The use of lignocellulosic residues and by-products as substrates for microbial growth has the advantage of being an inexpensive strategy for inducing the production of enzymes [26]. Thus, *C. phragmiteti* was cultivated on LB and MM media supplemented with different lignocellulosic residues as carbon sources, which were WP, WB, BSG, RB, RS, and DDGS. After 72 h cultivation, the xylanase activities were measured from each cell-free fermentation broth. The results of the xylanase activity measurements are shown in Figure 1.

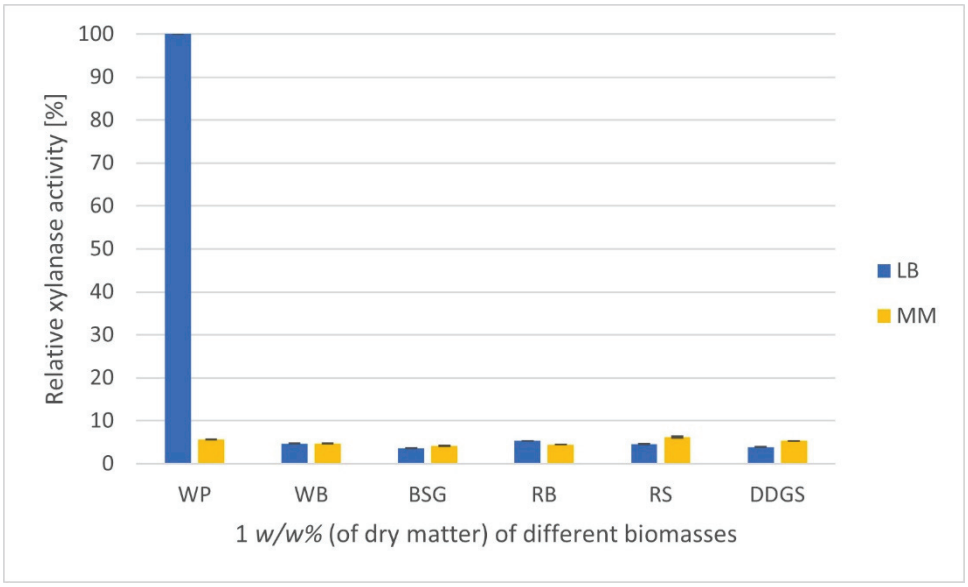

Figure 1. Relative xylanase activities [%] produced by *C. phragmiteti* on different carbon sources (1 *w*/*w*% of dry matter) in LB and MM media. WP—waste paper; WB—wheat bran; BSG—brewer's spent grain; RB—rye bran; RS—rice straw; DDGS—distillers dried grains with solubles. The average values of relative xylanase activity are presented with error bars representing the standard deviations of triplicate cultures in the case of WP and WB and duplicate cultures in the case of RB, RS, BSG, and DDGS.

The highest xylanase activity (referred to as 100% relative xylanase activity) was obtained using WP in LB medium. The relative xylanase activities were between 3.6–5.4% and 4.1–6.2% for the other biomasses (WB, BSG, RB, RS, and DDGS) in LB and MM media, respectively. The xylans of RB, RS, WB, DDGS (corn), and BSG are complex xylans highly decorated with side chains that frequently contain arabinofuranosyl units [27–30]. The complex structure of these xylans hinders the action of xylanases in the absence of accessory side chain-hydrolysing activities [10], which might have contributed to their limited potential for inducing xylanase enzyme production by *C. phragmiteti*. In contrast, the waste paper used in our study contained xylan (15% in terms of dry matter) without arabinose substitutions. In addition, during the process of paper production, the complex structure of its lignocellulose is disintegrated to decrease the lignin content, which could make the remaining cellulose and hemicellulose fractions of paper materials more accessible for hydrolytic enzymes. These factors could play a role in making waste paper an efficient substrate to induce the xylanase production of *C. phragmiteti*. Thus, LB medium containing WP was chosen for the following fermentations for xylanase activity production by *C. phragmiteti*. The use of *Cellulomonas* sp. B6 [31], *C. fimi* [32], *C. flavigena* [14], and *C. biazotea* [33] for the production of xylanases has also been reported. *Cellulomonas* sp. B6 and *C. fimi* showed high xylanase production on wheat bran and pre-treated waste paper, respectively [15], while xylanase production was mainly induced by sugarcane bagasse in the cases of *C. flavigena* and *C. biazotea* [14,33].

3.2. Effect of the Amount of Waste Paper on Xylanase Production

As WP was found to be the most appropriate substrate, the effect of WP concentration on the xylanolytic activity was investigated. The experiments were performed by using 0.5 *w*/*w*%, 1 *w*/*w*%, 1.5 *w*/*w*%, 2 *w*/*w*%, 2.5 *w*/*w*%, and 3 *w*/*w*% WP in LB medium. The highest xylanase activity (15.3 U/mL) was measured when 1 *w*/*w*% dry matter of WP was used for fermentation (Figure 2). Also, high xylanase activities were detected using 0.5 *w*/*w*% and 1.5% *w*/*w*% of WP. Between 1.5 *w*/*w*% and 2 *w*/*w*% of WP, a large decrease was observed in the produced xylanase activity (from 15 U/mL to 2.2 U/mL). Low xylanase activity values were measured in the case of 2 *w*/*w*% (2.2 U/mL), 2.5 *w*/*w*% (1.3 U/mL), and 3 *w*/*w*% (0.3 U/mL) of dry matter of WP (Figure 2). The supernatant derived from the fermentation using 1 *w*/*w*% WP showed the highest xylanase activity, so it was chosen for our further investigations.

C. fimi and *Cellulomonas* sp. B6 were also cultivated on pretreated waste paper (1 *w*/*w*% dry matter) by Ontañon et al. during an investigation of their xylanases enzyme production [15]. The highest xylanase activities achieved were 1.3 U/mL and 1.9 U/mL in the case of *C. fimi* and *Cellulomonas* sp. B6, respectively [15]. Thus, WP seems to be much more suitable for inducing the production of xylanolytic enzymes in *C. phragmiteti* than in the other two strains.

The decrease in the activity of the enzyme extract when the substrate concentration is above the optimal value has been related to the increase in viscosity and nutrient content in the medium. This inhibits microbial growth, lowering enzyme production. Some authors have reported that a lower substrate concentration is more effective for enzyme induction than higher concentrations [34,35]. This could also be an economic advantage in the large-scale production of hemicellulases at low substrate loads.

The xylanase activity of the supernatant derived from fermentation on 1 *w*/*w*% WP was measured by using different substrates, namely xylan from beechwood (BX) and wheat arabinoxylans with different viscosities (WAX-RS—arabinoxylan for reducing sugar assays; WAX-L—low-viscosity arabinoxylan; WAX-M—medium-viscosity arabinoxylan; WAX-H—high-viscosity arabinoxylan) (Figure 3). Relative activity values are displayed compared to that measured with beechwood xylan (100%). Higher xylanase activities were measured using WAX-RS and WAX-L (129% and 131%) compared to BX. Verma et al. indicated that enzymatic activity can vary significantly with the nature of the xylan used [36]. For example, the choice of the type of xylan is critical for obtaining positive clones during a

functional screening. This phenomenon may be related to the good solubility and low viscosity of the listed arabinoxylan substrates, in contrast with xylan from beechwood, which is an insoluble substrate. In addition, BX contains glucuronic acid substitutions instead of arabinose. Regarding WAX-M and WAX-H, slightly lower xylanase activities were detected (102% and 108%) compared to WAX-RS and WAX-L. This is probably because the viscosity of the substrates can affect the enzyme activity [37]. Thus, the higher viscosity values of WAX-M and WAX-H could have caused the lower xylanase activities [35].

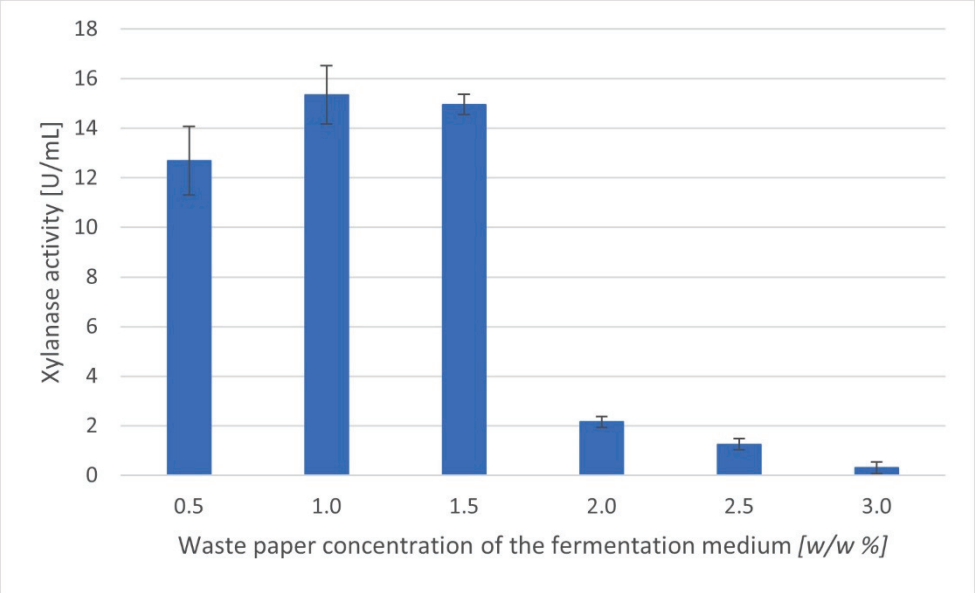

Figure 2. Xylanase activities [U/mL] obtained in LB medium containing different amounts of waste paper (*w*/*w*% of dry matter). The average values of xylanase activity are presented with error bars representing the standard deviations of triplicate cultures.

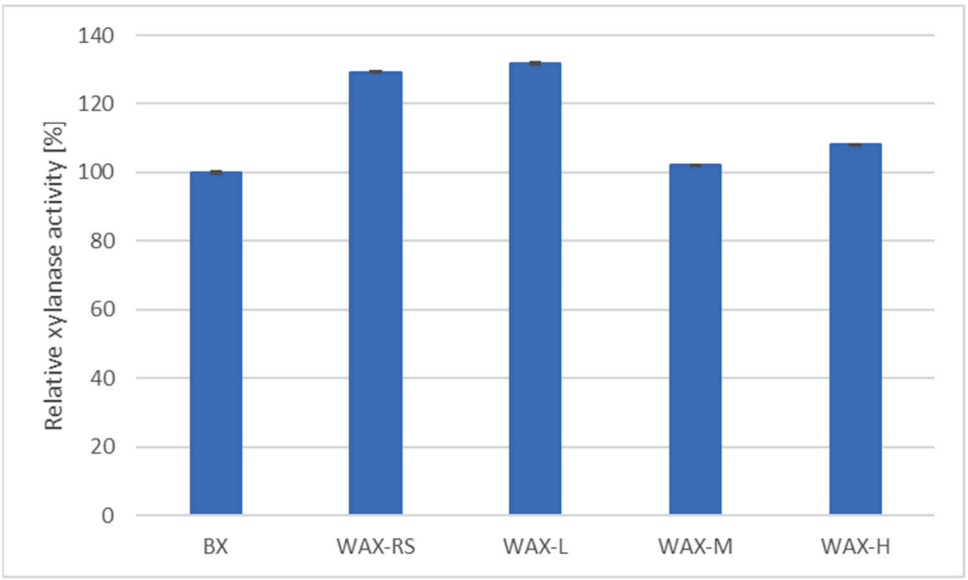

Figure 3. Relative xylanase activities [%] obtained from fermentation by using *C. phragmiteti* in LB medium containing 1 *w*/*w*% of dry matter waste paper. Xylanase activity measurements were performed using different xylan substrates: BX—xylan from beechwood, WAX-RS—wheat arabinoxylan for reducing sugar assays, WAX-L—low-viscosity wheat arabinoxylan, WAX-M—medium-viscosity wheat arabinoxylan, WAX-H—high-viscosity wheat arabinoxylan. Average values of xylanase activity are presented with error bars representing standard deviations of triplicates.

Side enzymatic activities (Table 2) were also measured from cell-free supernatants produced by *C. phragmiteti* on 1 *w*/*w*% WP, such as β-glucosidase, β-xylosidase, cellobiohydrolase, CMC-ase, and α-arabinofuranosidase. β-xylosidase had the highest side activity (0.61 U/mL) (Table 2). β-xylosidase activity was around six times higher compared to that of β-glucosidase (0.11 U/mL), cellobiohydrolase (0.12 U/mL), and CMC-ase (0.15 U/mL). Arabinofuranosidase had the lowest side activity (0.007 U/mL). These results prove that the most dominant enzyme, in terms of activity, in the extracellular extracts of *C. phragmiteti* is xylanase, and other lignocellulolytic enzyme activities are also present. Similarly, Akermann et al., Mayorga-Reyes et al., and Ontañon et al. reported a mainly xylanolytic activity in extracts of *Cellulomonas uda*, *C. flavigena*, *Cellulomonas* sp. B6, and *C. fimi* B-402 obtained by growth on lignocellulosic biomass [14,15,38]. These authors related this activity to the large amount of xylanases secreted by the bacteria under appropriate culture conditions.

Table 2. β-glucosidase, β-xylosidase, cellobiohydrolase, carboxymethyl-cellulase, and α-arabinofuranosidase enzyme activities (U/mL) of a fermentation supernatant obtained by *C. phragmiteti* on 1 *w*/*w*% waste paper in LB. The average values and standard deviations (indicated in parentheses) were calculated from triplicates.

Enzyme Activity	U/mL
β-glucosidase	0.11 (0.010)
β-xylosidase	0.61 (0.013)
Cellobiohydrolase	0.12 (0.005)
Carboxymethyl-cellulase	0.15 (0.032)
α-arabinofuranosidase	0.007 (0.002)

In comparison, *C. biazotea* showed higher cellulolytic activity than xylanolytic activity by growing on CMC and sugarcane bagasse in a study by Saratale et al. [33]. CMC and sugar cane bagasse induced cellulase (endoglucanase, exoglucanase, and cellobiase) production [33]. In our study, *C. phragmiteti* growing on WP showed much lower cellulase (CMC-ase) (Table 2) activity than the reported activity of *C. biazotea* on sugarcane bagasse and CMC. Thus, future investigations could focus on investigating whether other carbon sources would induce preferable cellulase production in *C. phragmiteti*.

3.3. Investigation of the Time Curve of Xylanase Activity Production

An investigation of the produced xylanase activity as a function of fermentation time was carried out for 96 h (Figure S1). Relative activity values at all sampling times are displayed compared to the values measured after 96 h (100%). Xylanase activity was barely observed after one day (1.5%). The production of xylanases started after 24 h. The relative activity of xylanase increased from 1.5% to 67.5% and 91.8% after 48 and 72 h, respectively. The production rate of xylanase activity greatly slowed down between 72 and 96 h; so, 72 h fermentation time was selected. Amaya-Delgado et.al. studied the induction of xylanase activity by sugar cane bagasse in *C. flavigena*, and they found that the maximal xylanase activity was reached after 100 h, but the most significant increase in the xylanase activity occurred between 60 and 82 h [39], which is in line with our results.

3.4. Effect of NaCl Concentration on Xylanase Activity

According to Rusznyák, *C. phragmiteti* is a salt-tolerant bacterium [20], so xylanase activity production might also be affected by the NaCl content of the medium, and the produced xylanolytic supernatants might have a salt-tolerating attribute. In order to investigate these hypotheses, xylanase activity was examined by setting different concentrations of NaCl in the test tubes during the measurement of xylanase enzyme activity, and xylanase enzyme production was measured in the presence of a high concentration of NaCl (100 g/L) in the fermentation medium.

The effect of NaCl concentration on the measured xylanase activity is represented in Figure 4. The xylanase activity slightly decreased by increasing the concentration of

NaCl. Using 25 g/L (0.43 M) NaCl, 13 U/mL xylanase activity was measured, which is slightly lower compared to that measured without NaCl (15.3 U/mL). Similar values were obtained by using 50 g/L (0.86 M) and 100 g/L (1.71 M) of NaCl; these values were 12.4 U/mL and 12.2 U/mL, respectively. The xylanase activity further decreased to 11.6 U/mL and 10.3 U/mL by using 150 g/L (2.56 M) and 200 g/L (3.42 M) of NaCl, respectively. The lowest activity was 10.3 U/mL (achieved by using 200 g/L NaCl), which is approximately 32.7% decrease compared to the highest (15.3 U/mL) activity measured without NaCl. The presence of high salt concentrations can negatively influence xylanase activity; however, our results suggests that the xylanases produced by *C. phragmiteti* are quite resistant against high NaCl concentrations, which might be useful in specific applications. An important challenge of industrial biotechnology is the high consumption of fresh water. A practical solution is the replacement of fresh water with abundant seawater in water-intensive industrial settings such as lignocellulosic biorefineries. In this context, a halophilic xylanase has the potential for application in the enzymatic decomposition of plant biomass in seawater [40].

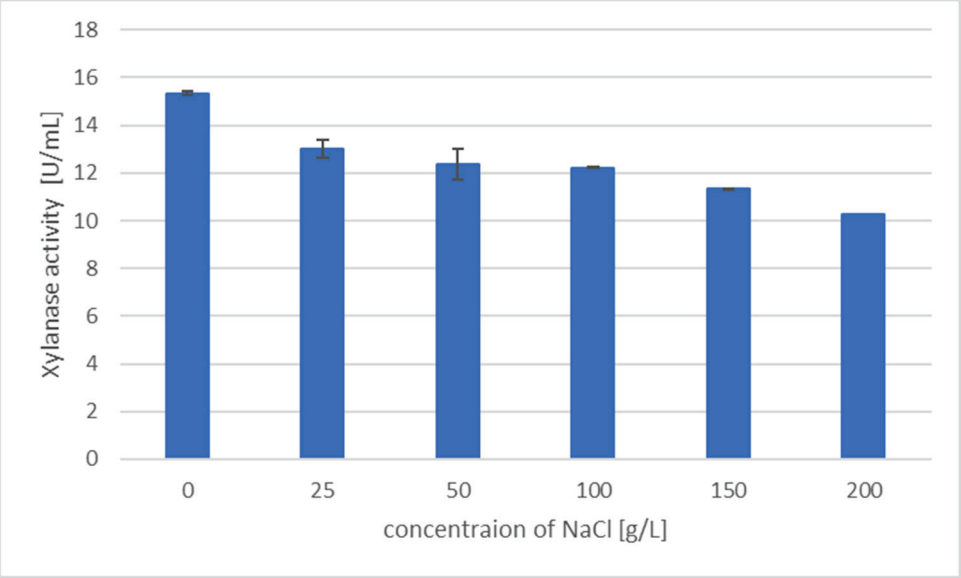

Figure 4. Xylanase activity values (U/mL) using different concentrations of NaCl (25 g/L, 50 g/L, 100 g/L, 150 g/L, and 200 g/L) during the xylanase assay (performed using cell-free fermentation broth of *C. phragmiteti* cultivation in LB medium containing 1 *w*/*w*% of waste paper).

Fermentation was also performed in the presence of high concentrations of NaCl to investigate the influence of salt on the enzyme production of *C. phragmiteti*. The xylanolytic activity dropped to 0.77 U/mL in the medium containing 100 g/L of NaCl despite the fact that *C. phragmiteti* can grow in a salty environment [20]. Rusznyák et al. stated that 5–7 *w*/*v*% NaCl concentration is optimal for its growth. Our measurements prove that xylanase production by *C. phragmiteti* is greatly inhibited by high salt concentrations. However, the xylanolytic supernatant seems to have salt-tolerant attributes. Since, in this study, only the use of 100 g/L NaCl was investigated during enzyme production, further experiments are needed to explore how the concentration of NaCl influences the xylanase production of *C. phragmiteti*.

3.5. Investigation of the pH and Temperature Optima of the Produced Xylanases

The determination of the operating range and optimal conditions of an enzyme of interest is crucial to specify its possible fields of application. Thus, the effect of pH and temperature on the xylanase activity produced by *C. phragmiteti* on 1 *w*/*w*% WP were investigated (Figure 5).

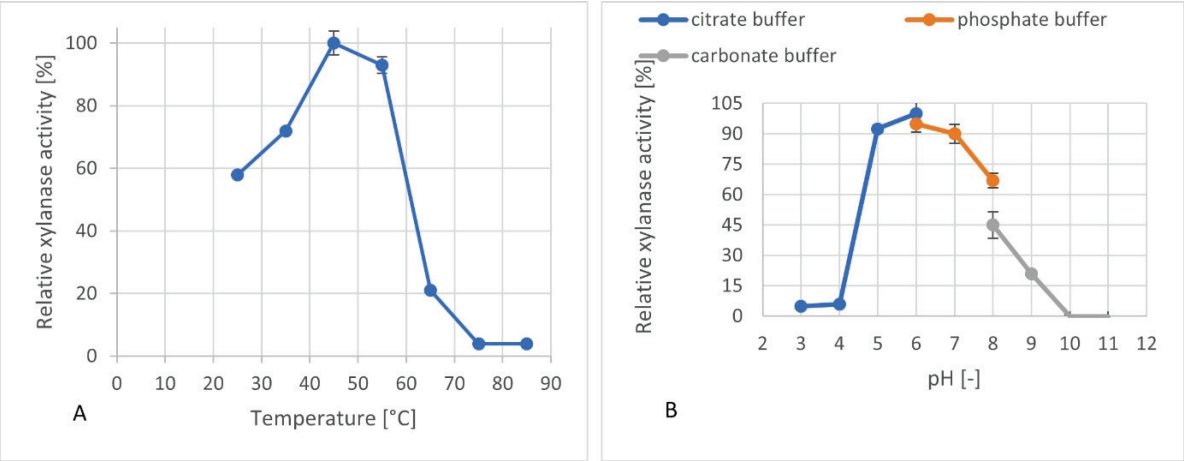

Figure 5. Relative xylanase activities at different temperatures (**A**) and pH values (**B**) measured in fermentation supernatant of *C. phragmiteti* cultivated in LB medium containing 1 *w*/*w*% of waste paper. Average values of relative xylanase activity are presented with error bars representing standard deviations of triplicates.

The results in Figure 5A,B represent the relative xylanase activity compared to the maximal activity, which was reached at 45 °C and pH 6 in citrate buffer. At 55 °C, an activity nearly as high as at 45 °C was detected (93%). At 25 °C, the relative xylanase activity was 58%. The xylanase activity experienced extreme decreases at higher temperatures (65 °C, 75 °C, 85 °C). Almost zero xylanase activity was measured at over 70 °C (Figure 5A).

The effect of pH on the xylanase activity was investigated by changing it between pH 3 and pH 11 and by using three types of buffers: citrate, phosphate, and carbonate (Figure 5B). At the pH values where a buffer change was necessary, xylanase activity was measured by using both buffers in order to monitor the effect of the type of buffer on the measured xylanase activity.

The maximal activity was detected at pH 6 (0.1 M) using citrate buffer (100%). The appropriate pH range for the produced xylanolytic supernatant was found to be pH 5–pH 8, which is a relatively wide range. A considerable effect regarding the type of buffers was observed between the phosphate and carbonate buffers at pH 8; higher xylanase activity was reached using the phosphate buffer (67%) compared to the use of the carbonate buffer (45%). At pH 10 and pH 11, xylanase activity was not measurable. Lisov et al. investigated the temperature and pH optima of xylanase enzymes derived from *C. flavigena* [13]. The optimum activity occurred at pH 7–7.5 and 40–50 °C, which is quite similar to the results observed for the xylanolytic supernatant of *C. phragmiteti*, whose optimal conditions were 45 °C and pH 6 (Figure 5).

3.6. Enzyme Stability Test

The stability of the xylanolytic supernatant of *C. phragmiteti* was evaluated during incubation at elevated temperatures and under different pH conditions. According to the previous results, the highest xylanase activities were detected between pH 5 and pH 8 and at 45 °C and 55 °C. Thus, the supernatant derived from fermentation on WP in LB medium by *C. phragmiteti* was incubated under these conditions for 72 h, and its residual activity was measured at different times. The results obtained at 45 °C are represented in Figure 6.

Relative activity values at all pHs are displayed compared to that of measured at pH 6 (100%) without previous incubation (0 h). The highest xylanase activity was detected at 0 h at each pH value. The most marked loss of activity, between 22 and 26%, was observed for all the evaluated pHs in the first 3 h. Then, the activity remained almost constant. After 72 h of incubation, 57%, 62%, 56%, and 53% of the original xylanase activity were remained at pH 5, pH 6, pH 7, and pH 8, respectively. Thus, all pH values applied in the experiments resulted in similar decreases in xylanase activity at 45 °C.

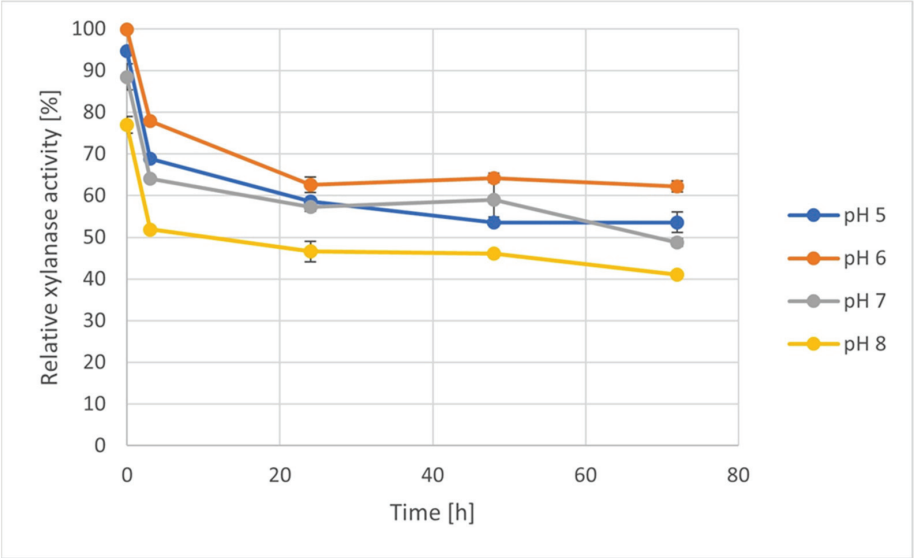

Figure 6. Results of the xylanase enzyme stability test at 45 °C and pH 5, 6, 7, 8 using the fermentation supernatant of *C. phragmiteti* cultivated in LB medium containing 1 *w*/*w*% of waste paper. The average values of relative xylanase activity are presented with error bars representing the standard deviations of triplicates.

The xylanase activity decreased faster and to a greater extent at 55 °C compared to 45 °C (Figure 7). In the first 3 h, the activity decreased by 44%, 35%, 36%, 43%, and 68% at pH 5, pH 6, pH 7, and pH 8, respectively. After 72 h, only 4%, 8%, 15%, and 3% of the original xylanase activities remained at pH 5, pH 6, pH 7, and pH 8, respectively. Thus, xylanases produced by *C. phragmiteti* on 1 *w*/*w*% WP seem to be more stable at 45 °C compared to 55 °C. In comparison, a greater thermostability was reported for the xylanolytic extracts of *Cellulomonas* sp. B6, which retained almost 80% activity after 48 h at 45 °C, although their specific activity was significantly lower than the extract from *C. phragmiteti* [31]. Our results suggest that the extracts of *C. phragmiteti* can be used in long-term reactions at 45 °C with good catalytic efficiency [41].

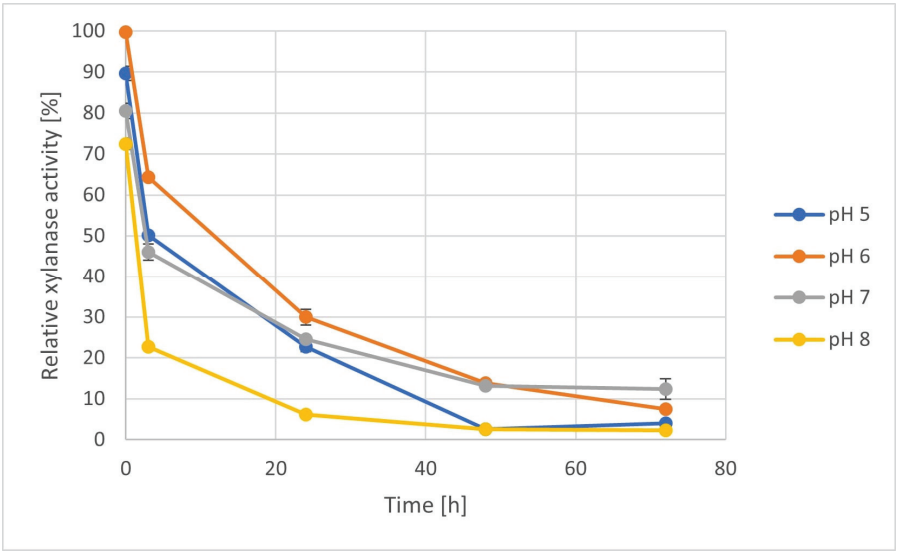

Figure 7. Results of the enzyme stability test at 55 °C and pH 5, 6, 7, and 8 by using the fermentation supernatant of *C. phragmiteti* cultivated in LB medium containing 1 *w*/*w*% of waste paper. The average values of relative xylanase activity are presented with error bars representing the standard deviations of triplicates.

3.7. Enzymatic Hydrolysis of Xylan

The xylanolytic supernatant of *C. phragmiteti* grown on WP was employed to hydrolyse birchwood xylan in a 3-day reaction at 45 °C, taking into account the stability results reported in the previous section. The xylose released from the reaction was quantified daily. Increasing values were measured after each day: 4.7 g/L, 7.2 g/L, and 10.7 g/L xylose was liberated after 24, 48, and 72 h, respectively. The 72 h supernatant was also subjected to sulphuric acid treatment in order to decompose oligosaccharides and determine the amount of total solubilized xylose (monomers and oligomers). The total solubilized xylose content was similar to the released monomer xylose concentration, indicating that the solubilized fraction of the xylan substrate was completely hydrolysed until reaching monomer form by the xyalnolytic supernatant of *C. phragmiteti*.

4. Conclusions

In this study, *C. phragmiteti* was found to be a promising candidate for the production high-value xylanase enzymes from the abundant and cheap lignocellulosic residues of WP. Producing enzymes using low-cost lignocellulosic wastes could reduce the price of the enzymes, which is usually a critical point in biotechnology, and could also contribute to mitigating waste handling issues. The most suitable culture medium and WP concentration to enhance xylanase production by *C. phragmiteti* was determined, and the obtained xylanases were characterized in terms of substrate specificity, pH and temperature optima, thermal stability, and salt tolerance. Water-intensive industrial areas might take advantage of the xylanase produced by *C. phragmiteti* because it can tolerate high salt concentrations. Thus, seawater might be used instead of fresh water in bioprocesses applying this enzyme. In contrast, using high concentrations of salt in the production of xylanases via *C. phragmiteti* should be avoided. Xylanases produced by *C. phragmiteti* show high activity at a wide pH range (from pH 5 to 8) and between 45 and 55 °C, which could provide them with a versatile portfolio of industrial applications, including biofuel, feed, and food production. These xylanases also have great thermal stability, preserving more than half of their activity after 3 days of incubation at 45 °C, making them great candidates for bioprocessing techniques that require long residence times (e.g., second-generation biofuel production) and allowing for them to be recycled multiple times. Thus, cultivating the novel bacterium *C. phragmiteti* on waste paper offers a great alternative for xylanase production, as *C. phragmiteti* shows promising attributes that could be useful in many industrial applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12020258/s1, Figure S1. Relative xylanase activities [%] obtained from fermentation by using *C. phragmiteti* in LB medium containing 1 *w*/*w*% of dry matter waste paper as a function of fermentation time. Average values of relative xylanase activity are presented with error bars representing the standard deviations of triplicates. Figure S2. Appearance of the waste paper used in the experiments.

Author Contributions: K.B. conducted experiments, analysed data, and wrote the manuscript. T.F. conducted experiments and analysed data. O.M.O. and E.C. analysed data and made corrections to the manuscript. C.F. conceived and designed the study, was responsible for funding acquisition, and corrected the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available from the corresponding author upon request. The data are not publicly available due to privacy restrictions.

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MDPI AG
Grosspeteranlage 5
4052 Basel
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