agronomy

MDPI

Article

Sustainable Production of Lignocellulolytic Enzymes in Solid-State Fermentation of Agro-Industrial Waste: Application in Pumpkin (*Cucurbita maxima*) Juice Clarification

Priya Rana [1], Baskaran Stephen Inbaraj [2], Sushma Gurumayum [3,*] and Kandi Sridhar [1,*]

[1] Department of Tropical Agriculture and International Cooperation, National Pingtung University of Science and Technology, Neipu, Pingtung 91201, Taiwan; priyarana198933@gmail.com
[2] Department of Food Science, Fu Jen Catholic University, New Taipei City 24205, Taiwan; sinbaraj@yahoo.com or 138547@mail.fju.edu.tw
[3] Department of Basic Engineering and Applied Sciences, College of Agricultural Engineering and Post-Harvest Technology, Central Agricultural University, Ranipool 737135, Sikkim, India
* Correspondence: sushmagurumayum@gmail.com (S.G.); sridhar4647@gmail.com (K.S.)

Abstract: Valorization of agro-industrial waste through greener and biotechnological processes are promising approaches to minimize the generation of agro-industrial waste. Therefore, the study aimed to produce lignocellulolytic enzymes from agro-industrial waste under solid-state fermentation (SSF) conditions and study their application in the clarification of pumpkin juice. The SSF was performed with three different combinations of wheat bran + rice bran (WBRB), wheat bran + wheat straw (WBWS), and rice bran + wheat straw (RBWS) as dry solid substrates (1:1) using *Fusarium oxysporum* (MTCC 7229). The protein, carboxymethyl cellulase (CMCase), and xylanase contents ranged from 0.98–3.90 mg/g, 5.89–6.84 U/g substrate, and 10.08–13.77 U/g substrate, respectively in different agro-industrial waste as substrates (WBRB, WBWS, RBWS, and control). The increase in enzyme concentration (0.50–2.40%) added to pumpkin juice exhibited an increased juice yield (16.30–55.60%), reduced browning index (1.03–0.70), and an increase in clarity (5.31–13.77 %T), which was further confirmed by a total variance of 84.83% by principal component analysis. Thus, the low-cost lignocellulolytic enzymes can be produced from agro-industrial waste that might have applications in food and beverage industries. Hence, this approach could be used as a long-term sustainable and circular source to valorize agro-industrial waste towards the greener future and the preservation of ecosystems.

Keywords: agro-industrial waste valorization; solid-state fermentation; lignocellulolytic enzymes; juice clarification; principal components analysis

Citation: Rana, P.; Inbaraj, B.S.; Gurumayum, S.; Sridhar, K. Sustainable Production of Lignocellulolytic Enzymes in Solid-State Fermentation of Agro-Industrial Waste: Application in Pumpkin (*Cucurbita maxima*) Juice Clarification. *Agronomy* **2021**, *11*, 2379. https://doi.org/10.3390/agronomy11122379

Academic Editors: Niccolò Pampuro, Eugenio Cavallo and Lucia Vigoroso

Received: 20 October 2021
Accepted: 22 November 2021
Published: 24 November 2021

1. Introduction

Agro-industrial waste is an emerging global issue with a tremendous negative impact on social health and environmental health. Globally, agro-industrial wastes are estimated to account for more than 30% of agricultural production every year [1]. Due to the increased global population, the production of agro-industrial waste has undoubtedly increased over the years, which could also relate to the need of increased agricultural production, expansion of soil, and technological advancements in food production [2]. However, the current systems or policies failed to identify long-term sustainable methodologies for agro-industrial waste valorization. To minimize the amount of agro-industrial waste, new insights on valorization or revalorization through sustainable circular models have received much scientific attention to recuperate an upgraded raw material or value-added product. For example, FEECO International, Inc., (Green Bay, WI, USA), an industry-leading global manufacturer, is actively transforming waste materials and process by-products into value-added products [3]. Among the agro-industrial waste, lignocellulosic

residues are considered as natural, biodegradable, and eco-friendly agro-industrial waste that are available in large quantities with cost-efficient for industrial applications [4] using solid-state fermentation (SSF).

Over the years, SSF has emerged as a sustainable alternative for in situ production of endogenous enzymes through filamentous fungi due to its high product yield and natural living conditions at low water activity [5]. Although many studies attempted to develop simple and practical automated fermenter for SSF processes by optimizing physical and chemical parameters, scaling up still remains a challenge for the production of industrial enzymes [6]. Many microorganisms, including fungi, bacteria, and actinomycetes have been documented for cellulase and hemicellulase production using lignocellulosic waste by SSF [7]. In fact, many filamentous ascomycetes, such as *Aspergillus* spp., *Fusarium* spp., *Monascus* spp., and *Neurospora* spp. are well-known microorganisms for production of human food products and considered as generally recognized as safe (GRAS) microorganisms [8]. The developments in SSF using filamentous ascomycetes provided the sustainable circular approaches to use the lignocellulosic waste in production of lignocellulolytic enzymes [9,10].

Generally, high prices of refined substrates, such as cellulose, hemicellulose, and others are the limiting factors in the production of enzymes. In addition, refined substrates are directly used as food and/or feed applications, which are cope-up with the rising demand for these enzymes. Therefore, in order to produce cost-efficient hydrolases, low-cost and alternative substrates, such as wood residues (sawdust and paper mill discards) and agricultural residues (straw, peelings, cobs, stalks, nutshells, non-food seeds, and bagasse) are used to produce hydrolytic enzymes [11]. Moreover, production of these enzymes by agro-industrial waste using SSF is a long-term sustainable waste management approach for the development of green industrial processes and to achieve environmental sustainability.

Recently, there have been many studies published in valorisation of agro-industrial waste and enzyme production by SSF [12–14], but these studies highly concentrated to parameters optimization in order to increase the enzyme production. However, we would recommend production and industrial application of enzymes that may be considered as an opportunity to feed the inclined world population in an economically sustainable way and the preservation of natural harmony in ecosystems.

Lignocellulolytic enzymes have shown potential applications in various industries, including food, pharma, and chemical industries. For instance, cellulase and xylanase are highly suitable lignocellulolytic enzymes used in extraction, clarification, and maceration of fruit juices [15]. Clarification of fruit and vegetable juice is one of the major processing in modern beverage industries. For example, high viscosity and turbidity juices are generally clarified by membrane filtration. However, high viscosity juices (e.g., pumpkin juice) is commonly macerated with water to produce a thick, creamy texture, and appearance, which requires the use of enzymes in order to obtain a pulpy juice with lower viscosity. Hence, we hypothesized that the low-cost lignocellulolytic enzymes can be produced by renewable lignocelluloses from agro-industrial waste for application in pumpkin juice clarification.

Previously, many studies failed to address the efficiency of enzyme extracts in SSF [12–14]. For instance, a recent study by Llimós, et al. [16] investigated biotransformation of brewer's spent grain to produce lignocellulolytic enzymes and polyhydroxyalkanoates in a two-stage valorization. Moreover, most of these studies in this area are limited to the production with only a few studies focusing on application of the produced enzymes. Generally, agro-industrial waste (wheat bran, rice bran, and wheat straw) are mostly burned agricultural residues in the world, thus creating environmental issues. To utilize these wastes in food and beverage industries through SSF, we selected above-mentioned agro-waste as dry solid substrates.

Therefore, the objectives of the study were to evaluate the production of lignocellulolytic enzymes (carboxymethyl cellulase and xylanase) from agro-industrial waste by

Fusarium oxysporum MTCC 7229 under solid-state fermentation and study their application in clarification of pumpkin (*Cucurbita maxima*) juice. The findings of this study may provide the valorisation of agro-industrial waste as an alternative and cheap substrate for production of cost-effective lignocellulolytic enzymes used in food and beverage industries.

2. Materials and Methods

2.1. Agro-Industrial Waste

Agro-industrial waste, such as wheat bran, rice bran, and wheat straw were collected from agricultural fields. The samples were thoroughly washed with tap water and sun-dried for 10 days. All the samples were individually grounded to a fine powder using a house-hold electric grinder (Samu Industries Pvt. Ltd., Gujarat, India), sieved (particle size of 1 mm), and transported to the laboratory in resealable biaxially-oriented polypropylene (BOPP) bags (400 × 300 mm) at room temperature for further experimental analysis. All the samples were sterilized (Rockwell Industrial Plants Ltd., Punjab, India) and used for experimental analysis.

2.2. Fungi

Fusarium oxysporum (MTCC 7229) was obtained from the Central Scientific Instruments Organization (Chandigarh, India). The strains were then cultivated on potato dextrose agar slants (Sisco Research Laboratories Pvt. Ltd., Maharashtra, India) and incubated at 28 °C for 7 days. Strains were preserved at 4 °C and cultured monthly. The spores were harvested using autoclaved water and Tween 20 at 10:1. Then, the spore concentration was adjusted to 10^6 spores/g of dry matter of solid substrate and used as an inoculum. The microscopic characteristics of fungus were observed under the compound microscope at 40× using lactophenol cotton blue staining, while macroscopic characteristics were performed by observing colour, form, elevation, and texture.

2.3. Solid-State Fermentation

The SSF was conducted using cotton-plugged 250 mL Erlenmeyer flasks containing three different combinations of wheat bran + rice bran (WBRB), wheat bran + wheat straw (WBWS), and rice bran + wheat straw (RBWS) as dry solid substrates at a ratio of 1:1 (5 g each) and wetted appropriately in a 15 mL of basal medium containing $(NH_4)_2SO_4$ (1.40 g/L), KH_2PO_4 (1.40 g/L), $MgSO_4.7H_2O$ (0.30 g/L), $CaCl_2$ (0.30 g/L), $FeSO_4.7H_2O$ (0.005 g/L), $MnSO_4.4H_20$ (0.0016 g/L), protease peptone (1 g/L), urea (0.30 g/L), $ZnCl_2$ (0.0017 g/L), $CoCl_2.6H_2O$ (0.002 g/L), and cellulose (10 g/L) according to Indesh, et al. [17] with minor modifications. Finally, the pH of the medium was adjusted to 5.30. The Erlenmeyer flasks were then autoclaved at 121 °C for 20 min, cooled, and inoculated with 1 mL of inoculum spore suspension and the moisture content was adjusted to 85% (*v/w*) with a sterile double distilled water. Each Erlenmeyer flask was gently tapped, mixed vigorously using a sterile spatula, and incubated at 28 ± 2 °C for 5 days, which was optimized in preliminary studies. Different substrate combinations, such as WBRB, WBWS, and RBWS at ratio of 1:0.50, 1:1, 1:1.50, and 1:2 were optimized at different moisture content (70 to 90%), in which 1:1 substrate ratio with 85% moisture content showed a high positive zone of clearance for the secretion of enzymes. The control sample was the individual samples without fungus.

2.4. Extraction of Enzyme

The crude enzyme extraction was performed according to Singhania, et al. [18]. Briefly, sodium phosphate—citrate buffer (50 mL) was added to the fermented substrate and mixed on a rotary flask shaker (BST-AS35, Bionics Scientific Technologies Ltd., Delhi, India) at room temperature for 1 h. The suspensions were filtered using muslin cloth and centrifuged (WT-4M, The Western Electric & Scientific Works, Haryana, India) at 3000× *g* for 15 min. The clear crude enzyme extracts were stored at −20 °C until further analysis. All experiments were performed in triplicate under the same conditions.

2.5. Purification of Crude Enzyme

The crude enzyme extracts were purified by a gel filtration chromatography according to Iqbal, et al. [19]. Briefly, the extracts were passed through a Sephadex®G-100 (Merck, Darmstadt, Germany) column (45 × 3 cm) and eluted with phosphate buffer (pH 6.50) at a flow rate of 0.50 mL/min, resulting from the gravity force. The purified enzyme extracts were collected and immediately used for further analysis, including protein estimation and enzymes activity.

2.6. Protein Content

Protein content was determined according to the Bradford method as described by Bradford [20]. Briefly, appropriately diluted enzyme samples (0.50 mL) were thoroughly mixed with Coomassie-Brilliant blue (1 mL) and incubated at room temperature for 30 min. After incubation, the absorbance was measured at 595 nm using a UV/Vis spectrophotometer (Shimadzu 1700, Shimadzu Corporation, Kyoto, Japan). Protein content was determined using bovine serum albumin as a standard and expressed as mg/g dry substrate.

2.7. Analysis of Enzyme Activities

Carboxymethyl cellulase (CMCase) and xylanase activities were determined according to the method described by Pal, et al. [21] with modifications. For CMCase activity, diluted enzyme samples (0.50 mL; diluted in 50 mM acetate buffer at pH 5) were thoroughly mixed with 0.50 mL carboxymethyl cellulose (2% carboxymethyl cellulose dissolved in 50 mM acetate buffer) at 50 °C for 1 h. The DNS (3,5-dinitrosalicylic acid) reagent (3 mL) was then added to the reaction mixture and boiled in a water bath (LE110, GPC Medical Ltd., New Delhi, India) at 100 °C for 15 min. After cooling to room temperature, the color intensity was measured at 540 nm using a UV/Vis spectrophotometer and the amount of reducing sugar was calculated using a glucose standard curve.

For xylanase activity, appropriate diluted enzyme samples (100 µL) were mixed with 9 mL of xylan (1%, *w/v*) and incubated at 50 °C for 10 min. The DNS reagent (3 mL) was then added to the reaction mixture and boiled in a water bath at 100 °C for 15 min. After cooling to room temperature, the intensity of color was measured at 540 nm using a UV/Vis spectrophotometer and the amount of reducing sugar was determined with xylose as a standard. The CMCase and xylanase activities were expressed as U/g dry substrate. One unit (U) of enzyme activity is defined as the amount of enzyme required to liberate 1 µM of reducing sugar from the appropriate substrate per min under the assay conditions.

Specific activity of enzymes is calculated as the number of enzyme units per mL divided by the concentration of protein in mg/mL and expressed as U/mg protein.

2.8. Application of Purified Enzyme Extracts in Clarification of Pumpkin Juice

A fully matured pumpkin (*Cucurbita maxima*) without any mechanical injuries was purchased from the local market. Fresh samples were washed thoroughly with running tap water and then gently blotted with a food-grade bibulous paper to remove any surface water. Samples were peeled manually, deseeded, cut longitudinally into halves, and macerated. Pumpkin juice was then extracted in a house-hold electric grinder until a homogeneous fruit juice was obtained. For enzymatic treatments, the extracted juice (50 g) was treated with a purified enzyme extract at different concentrations (0.50, 0.70, 0.90, 1.20, 1.50, 1.80, 2.10, and 2.40%). The samples (pH 4) were mixed thoroughly and incubated in a water bath at 50 °C for 1 h. It is worth mentioning that these optimum conditions were based on a high juice yield of 54.50%, browning index of 0.75, and clarity of 49.03 %T obtained in a preliminary study with the temperature ranging from 30 to 70 °C, time from 0.50 to 3 h, and pH from 3 to 7. Then, the samples were pasteurized at 80 °C for 15 min, cooled to room temperature, and centrifuged at 2800× *g* for 15 min. The juice samples were packaged in food-grade aseptic bottles and stored in a refrigerator at 4 °C. Sample without enzymatic treatment was considered as a control. The experiments were replicated at least three times.

2.9. Characteristics of Pumpkin Juice

The yield (%, *w/w*) was calculated according to Equation (1). Browning index (BI) is generally expressed as absorbance units of the visible spectrum from 400 to 590 nm and was determined according to Dorris, et al. [22]. Briefly, the maximum absorbance of the juice (472 nm) to the absorbance at 420 nm was calculated according to Equation (2).

$$\text{Yield } (\%) = \left[\left(\frac{\text{Weight of the clear juice (g)}}{\text{Weight of the sample (g)}} \right) \times 100 \right] \quad (1)$$

$$\text{BI} = \left[\left(\frac{\text{Absorbance}_{472\text{ nm}}}{\text{Absorbance}_{420\text{ nm}}} \right) \right] \quad (2)$$

Clarity of the juice was determined according to Abdullah, et al. [23] with modifications. Briefly, the transmittance (%T) of the clarified juice was measured at 660 nm using distilled water as a reference. The %T was considered as an indicator of juice clarity.

2.10. Principal Component Analysis (PCA)

PCA is a linear model of multivariate statistical technique used to analyze interrelationships among variables that can form parsimonious and manageable groups. We performed PCA (Kaiser-Meyer-Olkin value of 0.70, $p < 0.05$) in order to understand the relationship within the enzyme concentrations and with substrates (WBRB, WBWS, and RBWS) based on pumpkin juice yield, BI, and clarity using Origin® 2019b version 9.65 (OriginLab Corporation, Northampton, MA, USA). The findings were projected by a PCA bi-plot, a combination of the PCA scores and loading plots, contributing to the differences between/among the variables.

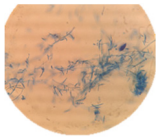
2.11. Statistical Analysis

All experiments were conducted in triplicate and the results were expressed as a mean ± standard deviation of triplicate analyses. The multivariate analysis of variance (MANOVA) and Duncan's multiple range tests were performed to evaluate significant differences ($p < 0.05$) among the substrates using the IBM® SPSS® Statistics version 22.0 (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

Macro-microscopic characteristics of the *Fusarium oxysporum* (MTCC 7229) were analyzed under the compound microscope at 40× using lactophenol cotton blue staining (Table 1). The fungus was irregular in form and creamy white in colour with raised elevation and pink tinge on the reverse. Based on microscopic characteristics, fungi were found to be macroconidia on conidiophores with well-marked foot cells at the attachment end of the spore and tapered towards both ends, confirming *Fusarium oxysporum* MTCC 7229 [24] for further use in SSF along with WBRB, WBWS, and RBWS as dry solid substrates.

Table 1. Macro-microscopic observation of *Fusarium oxysporum* MTCC 7229 [1].

	Macroscopic characteristics				
Fusarium oxysporum MTCC 7229	Color	Form	Elevation	Texture	Color on reverse side
	Creamy white	Irregular	Raised	Cottony and loosely packed	Pink tinge
	Microscopic characteristics				
	• Boat shaped (canoe shaped) and macroconidia with well-marked foot cells at attachment end of spore. • Tapered towards both ends • The macroconidia (phialospores) are produced on conidiophores.				

[1] Microscopic characteristics were observed under the compound microscope at 40× using lactophenol cotton blue staining.

3.1. Protein Content

The protein content in substrates (WBRB, WBWS, RBWS, and control) is presented in Table 2. The statistical analysis showed that the growth of fungus on agro-industrial wastes had a significant effect ($p < 0.05$) on protein content. The protein content significantly ($p < 0.05$) ranged from 0.98 to 3.90 mg/g with the lowest and the highest protein content in control (0.98 mg/g) and WBWS (3.90 mg/g), respectively. The protein content in the control was significantly ($p < 0.05$) lower than those of substrates with agro-industrial waste (WBRB, WBWS, and RBWS). This indicated that the addition of fungus on agro-industrial waste increased the protein content. Addition of chosen substrates caused a statistically significant difference ($p < 0.05$) in protein content, which led to >3.51-times higher protein content compared to control (0.98 mg/g). The protein content of individual control samples (WBRB, WBWS, and RBWS) is shown in Supplementary Table S1. The increased trend in protein content was reported in an earlier study by Brozzoli, et al. [25], in which authors demonstrated 1.17-times higher levels of protein content in stoned olive pomace mixed with field bean and crimson clover compared to control (non-inoculated). Pal et al. [21] reported slightly lower protein content (0.34–0.88 mg/mL) compared to our findings using mustard stalk and straw as a substrate in SSF for production of lignocellulolytic enzymes. Generally, SSF is a suitable long-term sustainable process to increase the protein content, which could be related to the mineralization of the carbon sources, the nitrogen immobilization into mycelial components, and bioconversion of endogenous nitrogen into mycelial proteins, resulting an improved protein digestion [25,26]. Moreover, wheat bran and rice straw are good sources of protein, which further promoted the increase in protein content [21]. There are, however, other possible explanations for increased protein content and could be related to production of lignocellulolytic enzymes from the substrate or fungi. Overall, the increased protein content is considered as a potential value as a low-cost diet ingredient in the area of feed science and processing technology.

Table 2. Production profile of protein and lignocellulolytic enzyme activities produced by solid-state fermentation of different agro-industrial waste as substrates [1].

Substrate	Protein (mg/g Substrate)	CMCase Activity (U/g Substrate)	Specific Activity (U/mg Protein)	Xylanase Activity (U/g Substrate)	Specific Activity (U/mg Protein)
Control	0.98 ± 0.15^{a}	ND [†]	ND	ND	ND
WBRB	3.07 ± 0.02^{b}	5.89 ± 0.06^{a}	1.91 ± 0.25^{b}	13.77 ± 0.28^{c}	4.48 ± 0.16^{c}
WBWS	3.90 ± 0.02^{d}	6.84 ± 0.07^{c}	1.75 ± 0.03^{a}	12.82 ± 0.10^{b}	3.29 ± 0.14^{b}
RBWS	3.35 ± 0.01^{c}	6.43 ± 0.47^{b}	1.92 ± 0.10^{b}	10.08 ± 0.03^{a}	3 ± 0.07^{a}

[1] The results were expressed as a mean ± standard deviation (n = 3). Mean values bearing different lowercase letters (a–d) within a column indicates significant difference ($p < 0.05$) based on multivariate analysis of variance (MANOVA) and Duncan's multiple range tests. [†] ND = not detected. CMCase = carboxymethyl cellulase, WBRB = wheat bran + rice bran, WBWS = wheat bran + wheat straw, RBWS = rice bran + wheat straw, and Control = individual samples of WBRB, WBWS, and RBWS without fungus.

3.2. Production of Lignocellulolytic Enzymes

We performed qualitative analysis to understand the presence and/or absence of lignocellulolytic enzymes after SSF. Samples (WBRB, WBWS, and RBWS) demonstrated the clear circle around the disc that indicated the secretion and high activity of lignocellulolytic enzymes; however, control sample clearly showed the absence of circle around the disc, which could be related to the absence of lignocellulolytic enzymes (Figure 1). This confirmed that the agro-industrial waste as a substrate in SSF may promote the release of lignocellulolytic enzymes. Thus, WBRB, WBWS, and RBWS were selected for the subsequent quantitative studies based on their positive zone of clearance and enzyme secretion on the CMC as well as xylan-cultivated plates.

The production of lignocellulolytic enzymes, such as CMCase and xylanase after SSF is shown in Table 2. The CMCase activity of all the samples significantly ($p < 0.05$) varied and ranged from 5.89 to 6.84 U/g. The WBRB as a source of carbon produced 5.89 U/g of CMCase activity, which was 1.09 and 1.16-folds lower than RBWS and WBWS, respectively.

Although relatively less difference (0.41 U/g) was observed in CMCase activity of WBWS and RBWS, the mean differences were found to be significant at $p < 0.05$. For CMCase specific activity, WBRB (1.91 U/mg protein) and RBWS (1.92 U/mg protein) reported insignificant values ($p > 0.05$) than the WBWS (1.75 U/mg protein). The xylanase activity was significantly ($p < 0.05$) influenced by the samples containing different composition of agro-industrial waste (Table 2). The xylanase activity was found the lowest in RBWS (10.08 U/g) and the highest was in WBRB (13.77 U/g). For xylanase specific activity, the maximum was recorded in WBRB (4.48 U/mg protein), followed by WBWS (3.29 U/mg protein) and RBWS (3 U/mg protein).

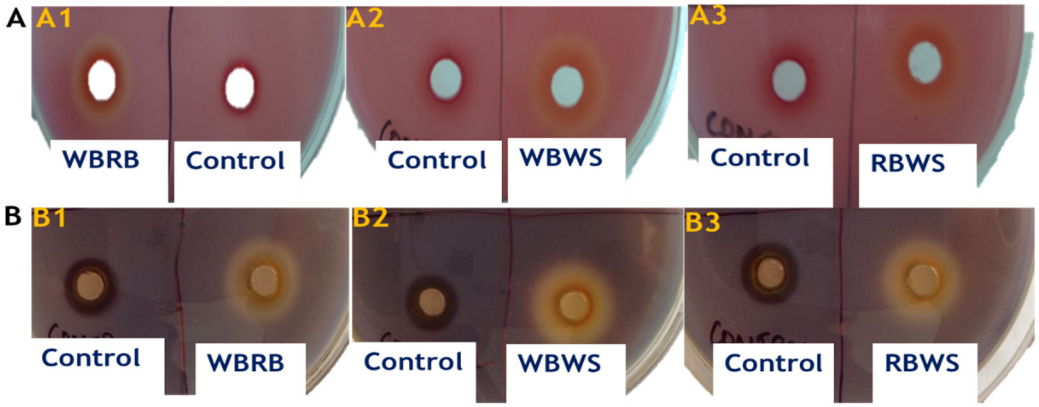


Figure 1. Qualitative determination of lignocellulolytic enzyme activities including carboxymethyl cellulase (CMCase) activity by disc diffusion method (**A**) and xylanase activity by xylan agar dye staining (**B**) in the presence of WBRB (A1, B1), WBWS (A2, B2), and RBWS (A3, B3). A clear-looking circle around the disc indicates the presence of enzymes, while the nonappearance of the circle around the disc shows the absence of enzymes. Agro-industrial waste as a substrate in solid-state fermentation (SSF) by *Fusarium oxysporum* MTCC 7229: wheat bran + rice bran (WBRB), wheat bran + wheat straw (WBWS), rice bran + wheat straw (RBWS), and control = individual combinations of agro-industrial waste without fungi.

Although CMCase and its specific activity followed the dissimilar trend, xylanase and its specific activity were in similar trend for all substrates. These findings agreed with those of Pal et al. [21], who showed production of lignocellulolytic enzymes on mustard stalk and straw as solid substrate, which was further increased by 2 to 10-folds when mustard stalk and straw supplemented with wheat bran and rice straw. Likewise, the results of Shruthi, et al. [27] were found to be slightly lower than our findings, where authors concluded the CMCase activity from 0.20 to 4.10 U/g of agro-industrial waste as a solid substrate in SSF. The dissimilarities in both enzyme activities could be related to different combinations of solid substrates that had different effects on production of lignocellulolytic enzymes [28]. Moreover, the higher release of xylanase compared to CMCase may be due to shorter fermentation time in SSF [29]. Wheat straw has been known for being ideally suitable candidate for high production of xylanase with high stability of >50% of its original activity at 70 °C for 4 h at pH 7–8 [30]. Likewise, moisture content and carbon source may improve the production of xylanase during SSF. A study by Yang et al. [30] reported no CMCase activity on wheat straw in SSF using *Paecilomyces themophila* J18 strains. This could be related to the thickness of substrate cell wall structure. We assume that the longer fermentation time (>10 days) may favor the release of endocellulases by opening up the cell wall structure.

For both enzyme activities, all three substrates provided the suitable environment for production of lignocellulolytic enzymes. Generally, wheat bran and rice bran are good sources of cellulosic composition, proteins, and sufficient nitrogen, which favors the production of lignocellulolytic enzymes by the fungus [21]. The increased production of cellulase enzymes was investigated on soybean hulls supplemented with wheat bran. The authors concluded that the major contribution in increased production of cellulase enzymes was linked with the presence of wheat bran as a solid substrate [31]. Moreover, wheat

straw is the cellulose-rich substrate, which would allow the efficient endocellulases production. The lignocellulolytic enzymes produced by SSF showed high thermal stability and resistance to alkaline or acidic conditions and thus suitable for industrial applications [32]. In brief, the studied agro-industrial waste showed the feasibility as a solid substrate in sustainable production of low-cost lignocellulolytic enzymes by fungus and thus can have an alternative to commercial substrates.

3.3. Application of Purified Enzyme Extracts in Clarification of Pumpkin Juice

The effect of enzyme concentration (0.50–2.40%) on characteristics of pumpkin juice is shown in Table 3. The multivariate analysis of variance of samples showed that the addition of enzyme (0.50–2.40%) had a significant effect ($p < 0.05$) on pumpkin juice yield, BI, and clarity. This could be related to purified enzyme extracts that removed the impurities, improved the purity, and clarification efficiency. All substrate samples showed variations in the characteristics of pumpkin juice as affected by the addition of enzyme concentration. The juice yield, BI, and clarity ranged from 16.30 to 55.60%, 0.70 to 1.63, and 5.31 to 46.83%T, respectively for all samples. In comparison, control samples exhibited a low juice yield (16.30 to 29.60%), high BI (1.03 to 1.63), and low clarity (5.31 to 20.96 %T) compared to other samples treated with purified enzyme extracts (Table 3). For example, control showed an average juice yield of 23.30%, which was raised to ~34.80% after addition of 0.50% enzyme extract. The 2.50-fold increase in enzyme concentration further resulted in an average juice yield of 45.20%, which was 1.30-times higher yield compared to initial concentration of enzyme added (Table 3). The same trend was followed with the increase in enzyme concentration from 1.50 to 2.40% and the highest juice yield of ~ 53% was recorded at the enzyme concentration of 2.40% for all substrates.

The BI for control was found to be highest with an average of 1.32, while enzyme treated samples demonstrated a less BI with an average of 0.72 for all three substrates at varied enzyme concentration. The enzyme concentrations of 0.50 and 0.70% insignificantly ($p > 0.05$) reduced the BI (0.74 to 0.81) of pumpkin juice, but was found to be significant ($p < 0.05$) compared to control (1.03 to 1.63). The rise in enzyme concentration from 0.90 to 2.40% showed an average insignificant ($p > 0.05$) BI of 0.71 for all samples, indicating the no influence of increased enzyme concentration on BI of pumpkin juice.

On the other hand, control samples demonstrated a lower clarity (an average of 12.84 %T) compared to the clarity (an average of 41.29 %T) obtained with juice treated with varied enzyme concentration, which was significant at $p < 0.05$. The clarity of pumpkin juice increased proportionally with an enzyme concentration from 0.50 to 2.40 %T for all samples. The lowest clarity of 37.20 %T was noticed at a lower enzyme concentration of 0.50%, whereas the highest clarity value of 55.60 %T was recorded at the final enzyme concentration of 2.40% for WBRB. Additionally, the same juice clarity tendency was observed for WBWS and RBWS at a final enzyme concentration of 2.40%. Moreover, these results reflect those of Shahrestani, et al. [33] and Shrestha, et al. [34], who also conducted studies to improve the juice yield and clarity as well as prevent browning. Authors reported that the juices treated with enzyme extracts favors juice yield, clarity, and reduces browning. The findings in our study could be related to the enzymatic reactions that hydrolysis the fruit cell wall with consequent release of bounded water molecules, polysaccharides, and substituted celluloses, which resulted in an increase in juice yield [35]. Sandri, et al. [36] used an enzyme extract produced by *Aspergillus niger* LB-02-SF in SSF to increase the juice yield by 23.10% and clarity by 70.60%, which resulted a clarified juice with high yield.

Table 3. Effect of purified enzyme extract on characteristics of pumpkin juice [1].

Enzyme Extract (%)	Substrate								
	WBRB			WBWS			RBWS		
	Juice Yield (%)	Browning Index	Clarity (%T)	Juice Yield (%)	Browning Index	Clarity (%T)	Juice Yield (%)	Browning Index	Clarity (%T)
Control	29.60 ± 1.08 [a]	1.03 ± 0.08 [c]	20.96 ± 1.62 [a]	23.70 ± 1.15 [a]	1.30 ± 0.28 [c]	12.26 ± 0.62 [a]	16.30 ± 0.34 [a]	1.63 ± 0.26 [c]	5.31 ± 1.22 [a]
0.50	37.20 ± 0.14 [b]	0.77 ± 0.10 [b]	38.73 ± 1.24 [b]	35.20 ± 2.10 [b]	0.78 ± 0.08 [b]	37.83 ± 1.10 [b]	32 ± 1.47 [b]	0.81 ± 0.06 [b]	35.75 ± 2.90 [b]
0.70	40.10 ± 0.04 [c]	0.74 ± 0.15 [b]	41.12 ± 1.73 [c]	38.90 ± 3.17 [c]	0.76 ± 1.08 [b]	39.41 ± 0.04 [c]	36.40 ± 0.86 [b]	0.78 ± 0.11 [b]	38.29 ± 1.08 [c]
0.90	45.20 ± 0.17 [d]	0.73 ± 0.19 [a]	42.91 ± 2.11 [d]	41.30 ± 1.50 [d]	0.74 ± 1.65 [a]	41.80 ± 1.28 [d]	40.30 ± 0.72 [d]	0.74 ± 0.23 [a]	41.22 ± 1.93 [d]
1.20	47.30 ± 0.09 [e]	0.72 ± 0.03 [a]	44.24 ± 2.09 [e]	44.40 ± 1.75 [e]	0.73 ± 0.09 [a]	42.76 ± 2.28 [e]	43.90 ± 0.58 [e]	0.73 ± 0.13 [a]	42.31 ± 1.52 [e]
1.50	50.30 ± 0.13 [f]	0.71 ± 0.21 [a]	45.12 ± 1.18 [f]	47.30 ± 1.82 [f]	0.71 ± 0.04 [a]	44.29 ± 2.26 [f]	45.70 ± 0.81 [f]	0.73 ± 0.28 [a]	43.13 ± 1.09 [f]
1.80	53.30 ± 0.18 [g]	0.71 ± 0.13 [a]	45.91 ± 1.10 [g]	50.40 ± 2.21 [g]	0.70 ± 0.05 [a]	45.48 ± 3.58 [g]	48.70 ± 1.93 [g]	0.71 ± 0.14 [a]	44.34 ± 1.66 [g]
2.10	54.70 ± 0.77 [h]	0.70 ± 0.18 [a]	46.28 ± 2.08 [h]	51.80 ± 2.08 [h]	0.70 ± 0.08 [a]	45.61 ± 3.92 [h]	49.40 ± 1.06 [h]	0.71 ± 0.19 [a]	44.81 ± 1.82 [h]
2.40	55.60 ± 1.54 [i]	0.70 ± 0.03 [a]	46.83 ± 2.10 [i]	53.10 ± 2.68 [i]	0.70 ± 0.10 [a]	45.78 ± 3.30 [i]	50.30 ± 1.15 [i]	0.70 ± 0.11 [a]	45.53 ± 1.16 [i]

[1] The results were expressed as a mean ± standard deviation (n = 3). Mean values bearing different lowercase letters (a–i) in a column indicates significant difference ($p < 0.05$) based on multivariate analysis of variance (MANOVA) and Duncan's multiple range tests. Percentage T for juice clarity indicates transmittance at 600 nm (%T). Control = pumpkin juice without enzyme extract, WBRB = wheat bran + rice bran, WBWS = wheat bran + wheat straw, and RBWS = rice bran + wheat straw.

High enzyme concentration may rapidly increase the rate of clarification by breakdown of pectin molecules, which enable the formation of pectin-protein flocs, leaving a clear homogeneous solution after the complete removal of all suspended solids [37]. Overall, enzyme concentration is the most important factor and positively influences the characteristics of pumpkin juice in terms of increased juice yield, reduced BI, and an increase in juice clarity. Therefore, application of enzyme extracts produced from agro-industrial waste as solid substrate by fungus might be useful to maintain the high quality of beverages with homogeneity and increased yield. However, industrially, the optimization of enzyme addition should be necessary in order to make the process viable with low enzymatic concentration and high juice yield. Currently, there is an interest in the reuse of enzymes for cost reduction at an industrial scale. The lignocellulolytic enzymes produced through SSF may be suitable for reuse due to their binding forces via non-covalent bonds. All these should be investigated to in-crease the reuse activity of immobilized and free enzymes.

3.4. Principal Components Analysis (PCA)

The PCA of all datasets yielded two major and more significant principal components (PCs), which explained 84.83% of the total variance. Total of 64.06% by PC1 and 20.77% by PC2 showed a remarkable change and considered as the most significant PCs of the total variance, indicating the close interrelation within the enzyme concentrations and with substrates (WBRB, WBWS, and RBWS). Figure 2 illustrates the bi-plot of variables and scores on PCs 1 and 2, which demonstrated a clear separation of substrates (WBRB, WBWS, and RBWS) within the three quadrants (quadrants 1, 2, and 4). PC1 had positive loadings for juice yield (>0.30) and clarity (~0.34), while BI projected positively towards PC2 with loading values of 0.26 to 0.35 for substrates WBRB, WBWS, and RBWS (Supplementary Table S2). Moreover, samples WBRB, WBWS, and RBWS showed a less angle for their juice yield, BI, and clarity, indicating the high correlation among them. Therefore, samples WBRB, WBWS, and RBWS formed well-separated individual groups for juice yield (group 1), BI (group 2), and clarity (group 3) (Figure 2). Likewise, PCA predictive scores exclusively showed differences in enzyme concentration and were clearly separated on PCs 1 and 2, showing that the increase in enzyme concentration affected the juice yield, BI, and clarity. These results further support the idea of using PCA in understanding the relationships among the parameters in SSF; however, these results were not comparative with existing literature on production of lignocellulolytic enzymes through SSF, where many studies were highly centered to understand relationships among process parameters in SSF [5,26]. Nonetheless, our approach thoroughly investigated the enzyme concentration and substrate interrelationships used in quality characteristics of pumpkin juice. Thus, based on the above observations, we concluded that the enzyme concentration had a positive influence on the quality characteristics of pumpkin juice.

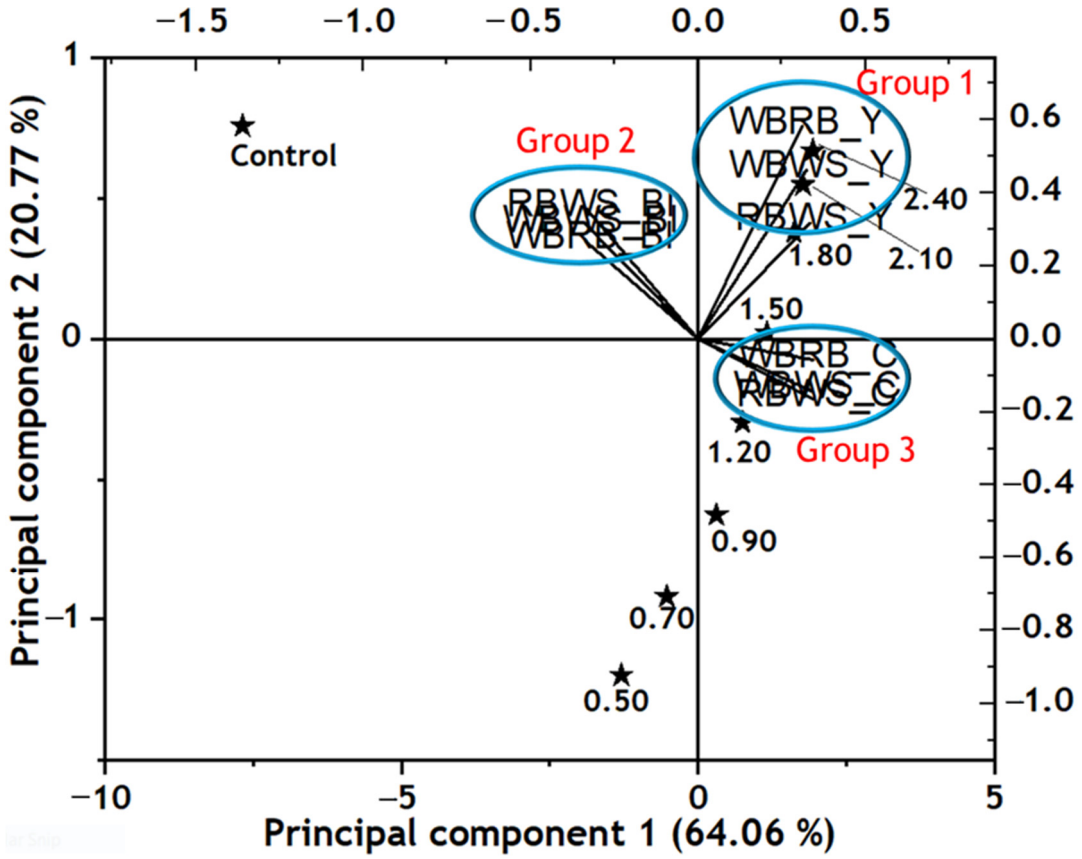


Figure 2. Bi-plot variables and scores of the first two principal components. WBRB = wheat bran + rice bran, WBWS = wheat bran + wheat straw, RBWS = rice bran + wheat straw, Y = yield, BI = browning index, and C = clarity. Control = pumpkin juice without enzyme extract, while ★ represents concentration of enzyme extracts (0.50 to 2.40) added to pumpkin juice. WBWS_C and RBWS_C in group 2 as well as WBRB_BI and WBRS_BI in group 3 were superimposed due to high similarity in their characteristics as shown by principal component analysis.

4. Conclusions

The study showed the potential of using agro-industrial waste for conversion into lignocellulolytic enzymes by using fungi *Fusarium oxysporum* in SSF and their subsequent application in the clarification of pumpkin juice. The use of agro-industrial waste as a solid substrate significantly increased the protein content and favored the release of CMCase and xylanase. Additionally, the purified enzyme extracts improved the characteristics of pumpkin juice, including increased juice yield, reduced BI, and an increase in juice clarity, which was also confirmed by the PCA bi-plot (a total variance of 84.83%). Among the agro-industrial waste substrates, WBRB and WBWS yielded high enzyme activity and thus recommended for industrial application in the production of low-cost lignocellulolytic enzymes. The use of agro-industrial waste in SSF is an alternative substrate for cost-efficient and sustainable enzyme production, such as lignocellulolytic enzymes. Hence, this approach could be used as a sustainable and circular source to valorize agro-industrial waste towards the greener future and the preservation of planetary ecosystems.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11122379/s1, Table S1: protein content of control samples without fungus, Table S2: extracted Eigenvectors of the first two principal components.

Author Contributions: Conceptualization, P.R. and S.G.; methodology, P.R.; software, K.S. and B.S.I.; validation, S.G., K.S. and P.R.; formal analysis, P.R. and K.S.; investigation, P.R.; resources, P.R. and K.S.; data curation, P.R.; writing—original draft preparation, P.R. and K.S.; writing—review and editing, S.G., K.S. and B.S.I.; visualization, K.S. and B.S.I.; supervision, S.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no internal/external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the primary corresponding author upon reasonable request.

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

References

1. AboSiada, O.A.; Negm, M.; Basiouny, M.; Fouad, M.; Elagroudy, S. Nutrient enrichment of agro–industrial waste using solid state fermentation. *Microbiol. Res. J. Int.* **2017**, *22*, 1–11. [CrossRef]
2. Wang, B.; Dong, F.; Chen, M.; Zhu, J.; Tan, J.; Fu, X.; Wang, Y.; Chen, S. Advances in recycling and utilization of agricultural wastes in china: Based on environmental risk, crucial pathways, influencing factors, policy mechanism. *Proc. Environ. Sci.* **2016**, *31*, 12–17. [CrossRef]
3. FEECO. Waste Transformation. Available online: https://feeco.com/waste-transformation/ (accessed on 29 April 2021).
4. Freitas, L.C.; Barbosa, J.R.; da Costa, A.L.C.; Bezerra, F.W.F.; Pinto, R.H.H.; Carvalho Junior, R.N.D. From waste to sustainable industry: How can agro-industrial wastes help in the development of new products? *Res. Conserv. Recycl.* **2021**, *169*, 105466. [CrossRef]
5. Oliveira, F.; Souza, C.E.; Peclat, V.R.O.L.; Salgado, J.M.; Ribeiro, B.D.; Coelho, M.A.Z.; Venâncio, A.; Belo, I. Optimization of lipase production by *Aspergillus ibericus* from oil cakes and its application in esterification reactions. *Food Bioprod. Proc.* **2017**, *102*, 268–277. [CrossRef]
6. Chilakamarry, C.R.; Mimi Sakinah, A.M.; Zularisam, A.W.; Sirohi, R.; Khilji, I.A.; Ahmad, N.; Pandey, A. Advances in solid-state fermentation for bioconversion of agricultural wastes to value-added products: Opportunities and challenges. *Biores. Technol.* **2022**, *343*, 126065. [CrossRef]
7. Saratale, G.D.; Kshirsagar, S.D.; Sampange, V.T.; Saratale, R.G.; Oh, S.-E.; Govindwar, S.P.; Oh, M.-K. Cellulolytic enzymes production by utilizing agricultural wastes under solid state fermentation and its application for biohydrogen production. *App. Biochem. Biotechnol.* **2014**, *174*, 2801–2817. [CrossRef] [PubMed]
8. Ferreira, J.A.; Mahboubi, A.; Lennartsson, P.R.; Taherzadeh, M.J. Waste biorefineries using filamentous ascomycetes fungi: Present status and future prospects. *Biores. Technol.* **2016**, *215*, 334–345. [CrossRef] [PubMed]
9. Ramamoorthy, N.K.; Sambavi, T.R.; Renganathan, S. A study on cellulase production from a mixture of lignocellulosic wastes. *Process Biochem.* **2019**, *83*, 148–158. [CrossRef]
10. Marín, M.; Sánchez, A.; Artola, A. Production and recovery of cellulases through solid-state fermentation of selected lignocellulosic wastes. *J. Clean. Product.* **2019**, *209*, 937–946. [CrossRef]
11. Mtui, G.Y. Recent advances in pretreatment of lignocellulosic wastes and production of value added products. *Afr. J. Biotechnol.* **2009**, *8*, 1398–1415.
12. Putri, D.N.; Khootama, A.; Perdani, M.S.; Utami, T.S.; Hermansyah, H. Optimization of *Aspergillus niger* lipase production by solid state fermentation of agro-industrial waste. *Energy Rep.* **2020**, *6*, 331–335. [CrossRef]
13. De la Rosa, O.; Múñiz-Marquez, D.B.; Contreras-Esquivel, J.C.; Wong-Paz, J.E.; Rodríguez-Herrera, R.; Aguilar, C.N. Improving the fructooligosaccharides production by solid-state fermentation. *Biocat. Agric. Biotechnol.* **2020**, *27*, 101704. [CrossRef]
14. Marzo, C.; Díaz, A.B.; Caro, I.; Blandino, A. Valorization of agro-industrial wastes to produce hydrolytic enzymes by fungal solid-state fermentation. *Waste Manag. Res. J. Int. Solid Wastes Public Clean. Assoc.* **2019**, *37*, 149–156. [CrossRef] [PubMed]
15. Toushik, S.H.; Lee, K.-T.; Lee, J.-S.; Kim, K.-S. Functional applications of lignocellulolytic enzymes in the fruit and vegetable processing industries. *J. Food Sci.* **2017**, *82*, 585–593. [CrossRef] [PubMed]
16. Llimós, J.; Martínez-Avila, O.; Marti, E.; Corchado-Lopo, C.; Llenas, L.; Gea, T.; Ponsá, S. Brewer's spent grain biotransformation to produce lignocellulolytic enzymes and polyhydroxyalkanoates in a two-stage valorization scheme. *Biom. Convers. Biorefin.* **2020**, 1–12. [CrossRef]
17. Indesh, A.; Sushma, G.; Sawinder, K.; Swetlana, N.; Monika, T. Utilization of orange peel and wheat straw for cellulase enzyme production from *Aspergillus terreus* MTCC7600. *Eco. Environ. Cons* **2016**, *22*, 307–313.
18. Singhania, R.R.; Sukumaran, R.K.; Pillai, A.; Prema, P.; Szakacs, G.; Pandey, A. Solid-state fermentation of lignocellulosic substrates for cellulase production by *Trichoderma reesei* NRRL 11460. *Indian J. Biotechnol.* **2006**, *5*, 332–336.

19. Iqbal, H.M.N.; Ishtiaq, A.; Muhammad Anjum, Z.; Muhammad, I. Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by *Trichoderma viride* under SSF and its detergent compatibility. *Adv. Biosci. Biotechnol.* **2011**, *2*, 149–156. [CrossRef]
20. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
21. Pal, S.; Banik, S.P.; Khowala, S. Mustard stalk and straw: A new source for production of lignocellulolytic enzymes by the fungus *Termitomyces clypeatus* and as a substrate for saccharification. *Ind. Crop. Prod.* **2013**, *41*, 283–288. [CrossRef]
22. Dorris, M.R.; Voss, D.M.; Bollom, M.A.; Krawiec-Thayer, M.P.; Bolling, B.W. Browning index of anthocyanin-rich fruit juice depends on ph and anthocyanin loss more than the gain of soluble polymeric pigments. *J. Food Sci.* **2018**, *83*, 911–921. [CrossRef]
23. Abdullah, A.G.L.; Sulaiman, N.M.; Aroua, M.K.; Megat Mohd Noor, M.J. Response surface optimization of conditions for clarification of carambola fruit juice using a commercial enzyme. *J. Food Eng.* **2007**, *81*, 65–71. [CrossRef]
24. St-Germain, G.; Summerbell, R. *Identifying Filamentous Fungi: A Clinical Laboratory Handbook*; Star Publishing Company: Belmont, CA, USA, 1996.
25. Brozzoli, V.; Bartocci, S.; Terramoccia, S.; Contò, G.; Federici, F.; D'Annibale, A.; Petruccioli, M. Stoned olive pomace fermentation with *Pleurotus* species and its evaluation as a possible animal feed. *Enzym. Microb. Technol.* **2010**, *46*, 223–228. [CrossRef]
26. Sousa, D.; Venâncio, A.; Belo, I.; Salgado, J.M. Mediterranean agro-industrial wastes as valuable substrates for lignocellulolytic enzymes and protein production by solid-state fermentation. *J. Sci. Food Agric.* **2018**, *98*, 5248–5256. [CrossRef] [PubMed]
27. Shruthi, K.; Yadav, P.S.; Prasad, B.V.S.; Chandra, M.S. Cellulase production by *Aspergillus unguis* in solid state fermentation. *J. For. Res.* **2019**, *30*, 205–212. [CrossRef]
28. Hu, T.; Zhou, Y.; Dai, L.; Wang, Y.; Liu, D.; Zhang, J.; Liu, H. Enhanced cellulase production by solid state fermentation with polyurethane foam as inert supports. *Proc. Eng.* **2011**, *18*, 335–340. [CrossRef]
29. Leite, P.; Salgado, J.M.; Venâncio, A.; Domínguez, J.M.; Belo, I. Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. *Biores. Technol.* **2016**, *214*, 737–746. [CrossRef] [PubMed]
30. Yang, S.Q.; Yan, Q.J.; Jiang, Z.Q.; Li, L.T.; Tian, H.M.; Wang, Y.Z. High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. *Biores. Technol.* **2006**, *97*, 1794–1800. [CrossRef]
31. Brijwani, K.; Oberoi, H.S.; Vadlani, P.V. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process Biochem.* **2010**, *45*, 120–128. [CrossRef]
32. Leite, P.; Sousa, D.; Fernandes, H.; Ferreira, M.; Costa, A.R.; Filipe, D.; Gonçalves, M.; Peres, H.; Belo, I.; Salgado, J.M. Recent advances in production of lignocellulolytic enzymes by solid-state fermentation of agro-industrial wastes. *Curr. Opin. Green Sustain. Chem.* **2021**, *27*, 100407. [CrossRef]
33. Shahrestani, H.; Taheri-Kafrani, A.; Soozanipour, A.; Tavakoli, O. Enzymatic clarification of fruit juices using xylanase immobilized on 1,3,5-triazine-functionalized silica-encapsulated magnetic nanoparticles. *Biochem. Eng. J.* **2016**, *109*, 51–58. [CrossRef]
34. Shrestha, L.; Kulig, B.; Moscetti, R.; Massantini, R.; Pawelzik, E.; Hensel, O.; Sturm, B. Optimisation of physical and chemical treatments to control browning development and enzymatic activity on fresh-cut apple slices. *Foods* **2020**, *9*, 76. [CrossRef]
35. Nagar, S.; Mittal, A.; Gupta, V.K. Enzymatic clarification of fruit juices (apple, pineapple, and tomato) using purified *Bacillus pumilus* SV-85S xylanase. *Biotechnol. Bioproc. Eng.* **2012**, *17*, 1165–1175. [CrossRef]
36. Sandri, I.G.; Piemolini-Barreto, L.T.; Fontana, R.C.; Silveira, M.M.D. Application of enzymatic preparations to produce araçá pulp and juice. *Food Sci. Technol.* **2014**, *34*, 657–662. [CrossRef]
37. Sharma, H.P.; Patel, H.; Sugandha. Enzymatic added extraction and clarification of fruit juices–A review. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 1215–1227. [CrossRef]