catalysts

MDPI

Article

Nepenthes mirabilis Fractionated Pitcher Fluid Use for Mixed Agro-Waste Pretreatment: Advocacy for Non-Chemical Use in Biorefineries

Justine O. Angadam [1], Mahomet Njoya [1], Seteno K. O. Ntwampe [2,*], Boredi S. Chidi [1], Jun-Wei Lim [3], Vincent I. Okudoh [1] and Peter L. Hewitt [4]

[1] Bioresource Engineering Research Group (*BioERG*), Cape Peninsula University of Technology, Cape Town 8000, South Africa; omajustine@gmail.com (J.O.A.); mahomet.njoya@gmail.com (M.N.); chidib@cput.ac.za (B.S.C.); okudohv@cput.ac.za (V.I.O.)
[2] Centre of Excellence for Carbon-Based Fuels, School of Chemical and Minerals Engineering, North-West University, Private Bag X 1290, Potchefstroom 2520, South Africa
[3] HICoE-Centre for Biofuel and Biochemical Research, Institute of Self-Sustainable Building, Department of Fundamental and Applied Sciences, University Teknologi PETRONAS, Seri Iskandar 32610, Malaysia; junwei.lim@utp.edu.my
[4] Pan's Carnivores Plant Nursery, 21 Kirstenhof, Tokai, Cape Town 7945, South Africa; peter@panscarnivores.com
* Correspondence: karabo.ntwampe@nwu.ac.za; Tel.: +27182991762

Abstract: This study determined whether it is feasible to pretreat mixed agro-waste of different particle sizes using the pitcher fluid of *Nepenthes mirabilis* (*N. mirabilis*), which is known to digest leaf litter due to the enzyme cocktail contained in the fluid. This is due to the need for the holocellulolysis (a source of fermentable sugars) of mixed agro-waste to produce fermentable hydrolysates. The pitcher fluid was fractionated (<3 kDa, >3 kDa, <10 kDa, >10 kDa) and slurrified with the mixed agro-waste, i.e., 25% (w/w) for each waste—orange peels, apple peels, maize cobs, grape pomace, and oak plant leaf litter of various particle sizes, i.e., >75 µm x < 106 µm and >106 µm. The process of producing a high concentration of total reducible sugars (TRSs) with the lowest production of total phenolic compounds (TPCs) was determined to be a particle size of >106 µm, pretreatment for 72 h, and an enzyme fraction of <10 kDa, whereby 97 g/L of TRSs were produced with a significantly lower TPCs load (1 g/L). Furthermore, the <10 kDa showed preferable physico-chemical properties, with the highest reduction-oxidation potential including acidity. Several enzymes, i.e., β-1,3-Glucanase, Putative peroxidase 27, Thaumatin-like protein, among others, were identified in the <10 kDa fraction, i.e., enzymes known to perform various functions in plant-based waste. Therefore, there is a need for the renewable energy industry to consider solely using pitcher fluids to pretreat mixed agro-waste for fermentable hydrolysates' production, which can be used as liquid feedstock for the bioenergy and/or biorefinery industries for environmental pollution reduction.

Keywords: agro-waste; *Nepenthes mirabilis*; pitcher fluid; proteomics; biorefinery

Citation: Angadam, J.O.; Njoya, M.; Ntwampe, S.K.O.; Chidi, B.S.; Lim, J.-W.; Okudoh, V.I.; Hewitt, P.L. *Nepenthes mirabilis* Fractionated Pitcher Fluid Use for Mixed Agro-Waste Pretreatment: Advocacy for Non-Chemical Use in Biorefineries. *Catalysts* **2022**, *12*, 726. https://doi.org/10.3390/catal12070726

Academic Editor: Pedro Maireles-Torres

Received: 19 May 2022
Accepted: 28 June 2022
Published: 30 June 2022

1. Introduction

Nowadays, sustainable energy generation is paramount for energy security globally. Hence, the availability and utilisation of bioenergy from waste materials, have resulted in its generation receiving considerable attention from industries and researchers [1]. This has become a focus area for researchers, as there is a need to find feasible substitutes for fossil-based fuels and thus the development of biorefineries with a focus on the co-manufacturing of biofuels, eco-clean energy, and chemicals from renewable biomass sources [2]. Recent studies have proven that some processes used in green energy generation are not always eco-friendly. The practices involving biomass conversion to biofuels in biorefineries also

result in a wide range of pollutants that pollute the environment and cause humans harm via chemical use. These are known as biorefinery-derived pollutants [3,4].

Furthermore, biorefineries are water-intensive, thus placing substantial stress on available water supplies. For example, maize-based biofuel refineries produce 13 litres of wastewater for each litre of ethanol produced. Some experts cautioned that biofuel production puts additional pressures on natural resources. The wastewater produced can cause pollution that extends throughout the ecosystem, resulting in the deterioration of rivers and land with contaminated silt and sediment translocating by natural means to pristine areas [5], mostly because of chemical residues emanating from biomass/agro-waste pretreatment processes.

There are several agro-waste pretreatment methods, among which chemical pretreatment methods, i.e., alkaline/caustic, dilute acid/organic acid, steam explosion (autohydrolysis), carbon dioxide (CO_2) explosion, liquid hot-water (LHW), microwave-chemical, organosolv, and wet oxidation, are among the favourites. Some of these pretreatment methods generate hazardous wastes that are detrimental to human health and the environment (soil, air, water). For example, LHW pretreatment produces solid residue that needs disposal, and processes such as organosolv need solvents to be drained and recycled with solvent residues being in the solid waste [6,7]. These methods can be replaced by plant-based enzyme containing digestive fluids, such as those found in the "monkey cups" of some *Nepenthes sp*.

Nepenthes sp., also known as pitcher plants, is a group of carnivorous plants that grow in tropical regions; about 130 species have been identified, and numerous species are being discovered annually [8]. One of these species is *Nepenthes mirabilis* (*N. mirabilis*), which has "monkey cups" containing pitcher fluid, whereby numerous enzymes are embedded. Indeed, to ascertain large-scale availability, the hydroponic growth method in combination with vertical farming can be used to grow pitcher plants such that they produce the enzyme containing pitcher fluid on a large scale [9,10]. The pitcher fluid has been known to completely digest both insects and leaf litter. Thus, it could be used as the sole means to pretreat agro-waste. The pitcher fluid produced by most pitcher plant species is acidic, with a pH range of 1.5 to 6. The acidity of the pitcher fluid aids in the plants' insects and leaf litter hydrolysing capabilities [11,12]. Enzymes found in the pitcher fluid include β-Xylosidase, Aspartic proteinase nepenthesin I, β-1,3-Glucanase, Class III chitinase, Oxidoreductase, Class IV chitinase, Acid chitinase, Carboxylesterase, Xylanase, Thaumatin-like protein etc. [13–15], with some of these enzymes having characteristics which can enhance delignification and holocellulolysis to produce fermentable hydrolysates from agro-waste. Since pretreatment is an important step involved in the production of bioethanol from lignocellulosic biomass, we envisage that the pitcher fluid can replace some of the current biomass/agro-waste pretreatment methods in which chemicals are used.

The selection of a specific agro-waste depends largely on regional accessibility including availability; thus, this was the primary motivation to use a mixture of apple peels, oak yard (leaf) waste, grape pomace, and maize cobs. These agro-wastes are readily available in South Africa (SA), especially in the Western Cape, where this study was conducted. Several researchers have been reporting on the use of a single agro-waste when performing biomass pretreatment studies. This is not ideal, as agro-waste in most instances consists of two or more different types of waste, especially in an industrial setting. The third leading province in SA producing a large quantity of citrus fruit is the Western Cape, 95% of which being apples [16]. Moreover, there is a plethora of oak trees in the Western Cape, and they produce a large quantity of yard waste. Therefore, the selected agro-wastes were selected with the intention to reduce the reliance on landfilling; hence, transforming such waste into value-added products or crude products, such as hydrolysates containing fermentable sugars, will be advantageous. This is because such hydrolysates can be used in the generation of bioenergy. *N. mirabilis* pitcher fluid was selected to pretreat the mixed agro-waste because other studies have proven that it is not necessary to have a pure form of enzyme cocktails for biomass pretreatment [17]. Furthermore, its "monkey cup", on average, is

larger compared to other *Nepenthes sp.* and can thus store a larger quantity of the pitcher fluid. As elucidated previously, *Nepenthes sp.* pitcher fluid contains an assortment of enzymes which might facilitate the hydrolysis of lignin-containing agro-waste while reducing phenolic reductants. Therefore, the purpose of this study was to assess the feasibility of using *N. mirabilis* pitcher fluid as a suitable agent to pretreat agro-waste for the extraction of total reducible sugars (TRSs) while reducing total phenolic compounds (TPCs), i.e., those known to be toxicants for fermentation systems. Furthermore, it was imperative to also determine optimal conditions, i.e., a suitable particle size of the agro-waste and the fraction in the pitcher fluid responsible for high TRS production while reducing TPCs, including identifying the enzymes in the high performing fraction.

2. Results and Discussion

2.1. Physico-Chemical Characteristics of the Fresh and Fractionated N. mirabilis Pitcher Fluid

The physico-chemical properties of the *N. mirabilis* pitcher fluid was determined with a focus on the following characteristics: redox potential, specific gravity, conductivity, and pH (see Table 1). Even though the *N. mirabilis* pitcher fluid comprises a cocktail of enzymes, the observation was that the enzymes must have acidity-tolerant traits. Furthermore, the pitcher fluid characteristics were determined to be comparable to those of 1% (v/v) dilute sulphuric acid solution, which is usually used in biomass treatment, although the solution has a lower redox potential [18] than the pitcher fluid. When a solution has a high redox potential, it is an indication that reduction-oxidation reactions can occur more rapidly.

Table 1. Physico-chemical properties of *N. mirabilis* pitcher fluid juice before fractionation.

Factors	Values (Units)
pH	2.0–2.09
Specific gravity (S.g)	0.73–0.81
Redox potential (ORP)	501–520 mV
Conductivity (CO)	3.86–4.93 mS/cm

Subsequent to fractionation, the <10 kDa fraction was observed to have somewhat preferable physico-chemical properties, as highlighted in Table 2, with less TPCs formation, including a slightly higher TRSs formation. As such, this fraction was determined to be suitable for the pretreatment of mixed agro-waste; i.e., all fractions were assessed, and a confirmatory statistical analysis was needed.

Table 2. Averaged (n =3) physico-chemical properties of the different fractions of *N. mirabilis* pitcher fluid in comparison to dilute (1% v/v) sulphuric acid (A), and the initial assessment of the best performing fraction in terms of TRSs and TPCs formation (B).

Factors	<3 kDa	>3 kDa	<10 kDa	>10 kDa	[a] 1% (v/v) H_2SO_4
			A		
pH	2.04	2.02	**2.00**	2.06	0.70
S.g.	0.73	0.73	**0.81**	0.80	1.08
ORP	503	501	**510**	511	354.2
CO	3.91	3.86	**4.93**	3.97	[c] n/d
			B		
[b] TRSs	31.31	33.50	**33.87**	30.82	-
[b] TPCs	13.42	11.66	**11.45**	16.95	-

[a] Angadam et al. [18], [b] Conditions (initial evaluation) = 72 h, >106 µm particle size, ambient temperature, without TES, [c] n/d–not determined.

To hydrolyse lignin-containing agro-waste into fermentable sugars, both oxidative and hydrolytic enzymes are needed, as well as an acidity that can culminate in the dissolution of some constituents in the waste. Therefore, when you have a cocktail of enzymes that are acid tolerant within the pitcher fluid, which is highly acidic, numerous advantages can be gained, including: (1) the ease of bond decoupling, (2) the lysis of lignin, (3) the extraction of cellulose and hemicellulose (holocellulolysis), (4) the decomposition of toxicants produced as by-products, and (5) the deactivation of agro-waste decomposers, e.g., fungi and bacteria.

2.2. Identified Enzymes in the N. mirabilis Pitcher Fluid

Several researchers have confirmed the existence of some enzymes in numerous pitcher plants fluid including the pitcher fluid of *N. mirabilis*. Examples include β-1,3-Glucanase, Class III chitinase, Class IV chitinase, and a Thaumatin-like protein. Class III acid endochitinase was also identified from the "monkey cup" of the carnivorous pitcher plant by another researcher [19]. β-1,3-Glucosidase, Xylanase, and Carboxylesterase were also determined to be in the pitcher fluid of *N. mirabilis* [20]. The presence of two or more different chitinases within the pitcher fluid was proven by Senevirathna et al. [21]. Another study also reported the prevalence of Putative peroxidase, Class III chitinase, Glucanase, Oxidoreductase, Class IV chitinase, and Acid chitinase from *Nepenthes sp.* pitcher fluid [14]. In a study carried out by Hatano and Hamada [13], Thaumatin-like protein, Aspartic proteinase nepenthesin I, Chitinase precursor, and β-1,3-Glucanase, were observed [22].

Consequently, in this study, using proteomic analyses performed on the <10 kDa fraction (which was determined to produce a high concentration of TRSs with a low TPCs load) of the pitcher fluid from both opened and closed pitchers, the following enzymes were identified: β-1,3-Glucanase, Purple acid phosphatases, Putative peroxidase 27, Class IV chitinase, Aspartic protease, Thaumatin-like protein, and Class III chitinase. Some of these enzymes have functional attributes that can be advantageous when pre-treating mixed agro-waste (see Table 3).

Table 3. Enzymes identified in the <10 kDa *N. mirabilis* pitcher fluid via a Byonic™ software search and their rankings based on the best score.

Rank	Enzyme	Accession Number	Functional Attributes in Relation to Agro-Waste Pretreatment
1	β-1,3-Glucanase	BAM28611	Degrades glucan into oligosaccharides or reducing sugars [23]
2	Purple acid phosphatase	BAW35430	Degrades phosphate monoesters in plant tissue [24]
3	Class IV chitinase	QBC75407	Prevents agro-waste decomposers such as fungi to proliferate [25]
4	Putative peroxidase 27	AMN14864	Oxidation of toxic reductants and degradation of lignin [26]
5	Aspartic protease nepenthesin I	AFV26024	Decouples proteins associated with some agro-products into amino acids [27]
6	Thaumatin-like protein	ACU31850	Degrades polyphenols into phenolic acids [28]
7	Class III chitinase	BAM28610	Has an ability to break down β-1–4 glycosidic bonds [29]

Putative peroxidase 27 can degrade lignin to water (H_2O) and carbon dioxide (CO_2) and H_2O_2 to H_2O and oxygen (O_2) [30]. Overall, such peroxidases are an exceptional group of ligninolytic enzymes, and due to their high redox potential, they have an ability to oxidise lignin [31]. When combined with β-1,3-Glucanase, they can play a significant role whereby a lignin-glucan-rich substrate can be decoupled into simple saccharides [32]. Overall, β-1,3-Glucanase can hydrolyse some constituents in the agro-waste by targeting 1,3-linked glucose polysaccharides using an inverting mechanism for glucan hydrolysis [33]. A similar trait can also be attributed to Thaumatin-like proteins, which have an ability to break down polymeric β-1,3-Glucans via hydrolysis to oligosaccharides [34], and they can also facilitate the degradation of p-hydroxybenzoic acid, which is a component of lignin [35]. Class III and IV

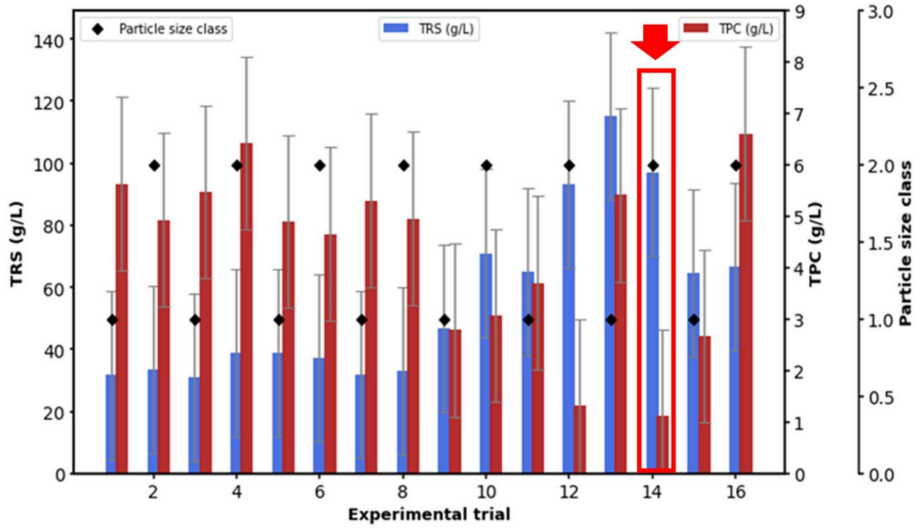
chitinases hydrolyse glycosidic bonds, albeit those associated with chitin with a specificity of decoupling glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety [19]. Therefore, all of the combined attributes of the individual enzymes identified can facilitate an effective way to pretreat agro-waste for the production of crude hydrolysates, which can be further processed without the use of chemicals.

2.3. Analysis of Reducible Sugar Production and Residual Phenolic Compounds in Hydrolysates from Mixed Agro-Waste Pretreatment

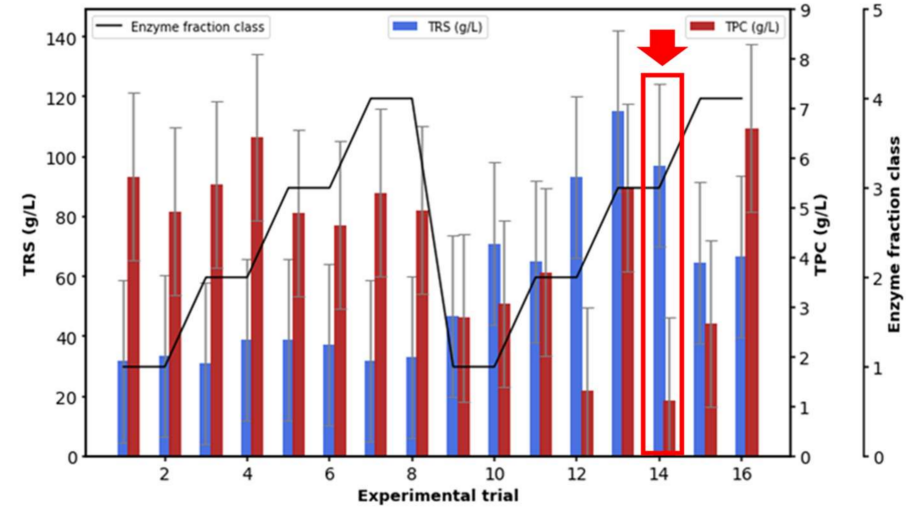
Table 4 provides a statistical summary of the influence of the assessed parameters on the production of TRSs and TPCs, which are residual toxicants in the pretreated agro-waste hydrolysates. Overall, the mean production of TRSs was higher when the agro-waste particle size was >106 µm than that which was >75 µm x < 106 µm (Experiment 13 and 14; see Figure 1), albeit using the <10 kDa enzyme fraction. The <10 kDa enzyme fraction in combination with the >75 µm x < 106 µm particle size produced hydrolysates with a higher concentration of TPCs at 5 g/L (Experiment 13).

Table 4. Statistical summary of the study with respect to the parameters assessed, providing standard deviations to inform the validity of the distribution for the production of TRSs and TPCs for each experiment.

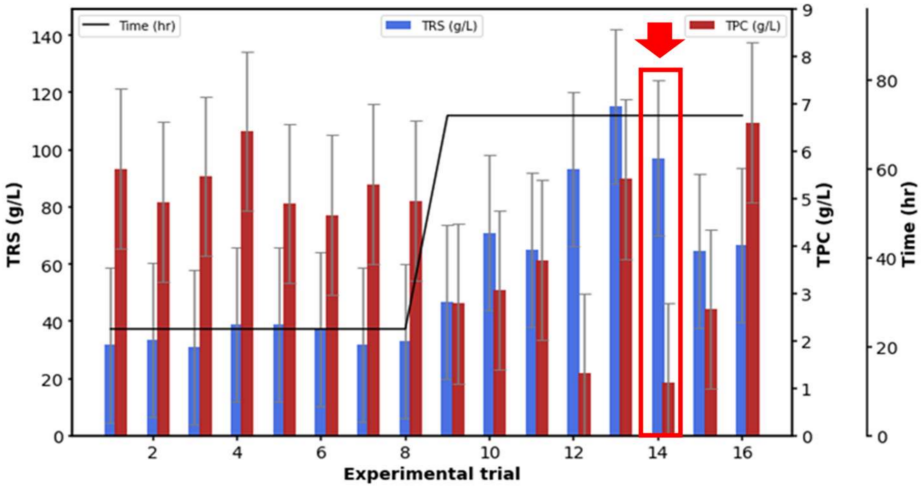
	Count	Mean	Standard Deviation	Min.	25%	50%	75%	Max.
TRS production (>106 µm)	8	53.13	28.73	31.08	31.78	42.73	64.80	115.19
TRS production (>75 µm x < 106 µm)	8	58.88	26.80	33.08	36.41	52.79	76.59	97.13
TPC production (>106 µm)	8	4.48	1.24	2.67	3.48	5.10	5.43	5.63
TPC production (>75 µm x < 106 µm)	8	4.13	2.11	1.11	2.64	4.79	5.32	6.61
TRS production (24 h)	4	33.35	3.68	31.08	31.50	31.74	33.58	38.84
TRS production (72 h)	4	72.91	29.48	46.62	60.17	64.91	77.64	115.19
TPC production (24 h)	4	5.33	0.32	4.89	5.20	5.39	5.51	5.63
TPC production (72 hr)s	4	3.64	1.27	2.67	2.76	3.25	4.13	5.41
TRS production (>10 kDa)	2	39.14	10.59	31.65	35.39	39.14	42.88	46.62
TRS production (<10 kDa)	2	48.10	24.08	31.08	39.59	48.10	56.61	65.13
TRS production (>3 kDa)	2	77.01	53.99	38.84	57.93	77.01	96.10	115.19
TRS production (<3 kDa)	2	48.26	23.24	31.83	40.04	48.26	56.47	64.69
TPC production (>10 kDa)	2	4.21	2.01	2.79	3.50	4.21	4.92	5.63
TPC production (<10 kDa)	2	4.59	1.25	3.71	4.15	4.59	5.03	5.48
TPC production (>3 kDa)	2	5.15	0.37	4.89	5.02	5.15	5.28	5.41
TPC production (<3 kDa)	2	3.99	1.86	2.67	3.33	3.99	4.65	5.31



(**A**)



(**B**)



(**C**)

Figure 1. Variation of TRS and TPC production with (**A**) particle size, (**B**) enzyme fraction, and (**C**) time.

There was an indication that the mean generation of TPCs was higher when the agro-waste particle size was smaller and after 24 h than when using the 72 h pretreatment regime (Figure 1A). An indication that the *N. mirabilis* pitcher fluid might have provided some enzymes that biodegrade TPCs is reported elsewhere [20]. Furthermore, the mean production of the TRSs was higher after 72 h than at 24 h (Figure 1C) using the enzyme fraction class 3 (Figure 1B). Therefore, there was a need to ascertain which of the parameters were better suited for downstream processes such as fermentation—that is, those with the lowest quantity of toxicants, i.e., TPCs. Refer to Table 4 for a statistical validation of the distribution between TRSs and TPCs production.

To ascertain the veracity of the experimental data, a Pearson correlation matrix (Figure 2) between the amendable parameters was done, as it provides the linear correlation between the parameters evaluated.

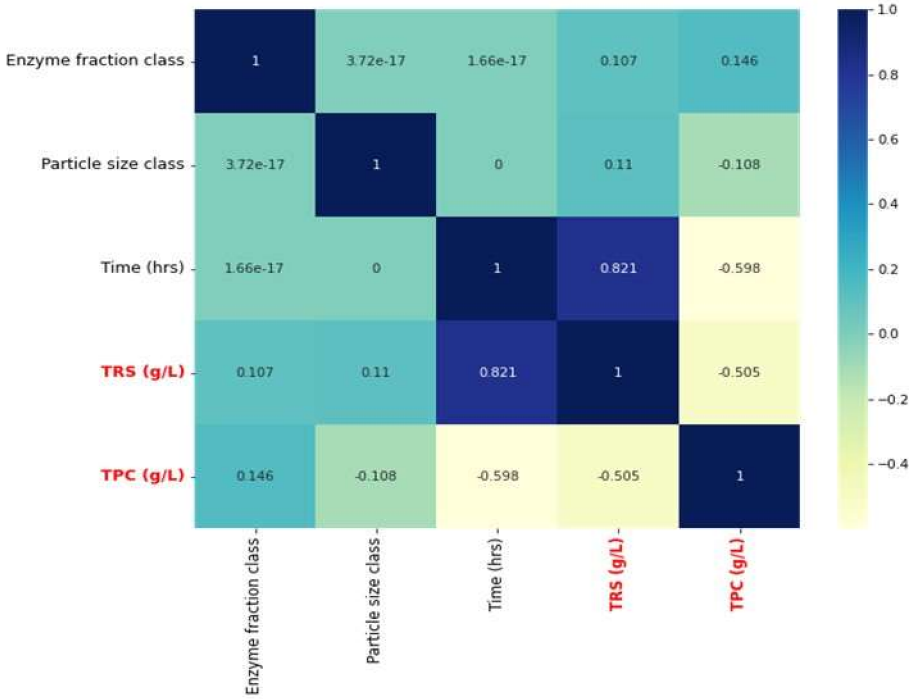


Figure 2. Correlation matrix between the amendable parameters for TRSs and TPCs production.

This correlation matrix was used as a diagnostic tool to attest a correlation between variables (n = 2). It was observed that the contact time was the parameter most correlated with the TRSs production (Pearson Product–Moment correlation of 0.82), while the TPCs production in the hydrolysates was unrelated to the contact time, with a coefficient of −0.6. Normally, it is expected that as the incubation period ensues, the generation of TPCs must cumulatively increase in the hydrolysates. This suggested the hydrolysis of some phenolic components by the pitcher fluid, albeit further verification of this assertion is needed. Carboxylesterases with the potential to hydrolyse phenolic-type compounds, which are present in *N. mirabilis* digestive fluids, were suggested to be candidate phenolic acid esterases with TPCs bioconversion potential [36]. Furthermore, after evaluating the output of each experiment in relation to the parameters that were modified, individually and then collectively, it was observed that a trade-off between the production of a high concentration of TRSs and the lowest quantity of TPCs was ideal, and this was achieved under the following conditions: (1) a particle size of >106 µm, (2) a contact time of 72 h, and (3) an enzyme fraction <10 kDa (Experiment trial 14; refer to Figure 1); although (1) a particle size of >75 µm *x* <106 µm, (2) a contact time of 72 h, and (3) an enzyme fraction <10 kDa produced hydrolysates with 115 g/L of TRSs while the concentration of TPCs was high. These results were further confirmed with the 3-D plots (Figure 3A–D) showing the

variation of TRS production given the variation of the individual parameter in each experimental trial. From the observed literature, there are no studies associating the production of TRSs with a particular enzyme fraction, <10 kDa, with no further association with the waste particle size, as observed in Figure 3A. Experiment 9 indicated the lowest TRSs production when compared to Experiment 13, with the highest TRSs production. However, there is a linkage between TRSs production and the particle size of the milled agro-waste (Figure 3B); in some studies, it was reported that the crystallinity of cellulose can be reduced by milling, with the accessibility of the hemicellulose increasing by hydrolysing enzymes.

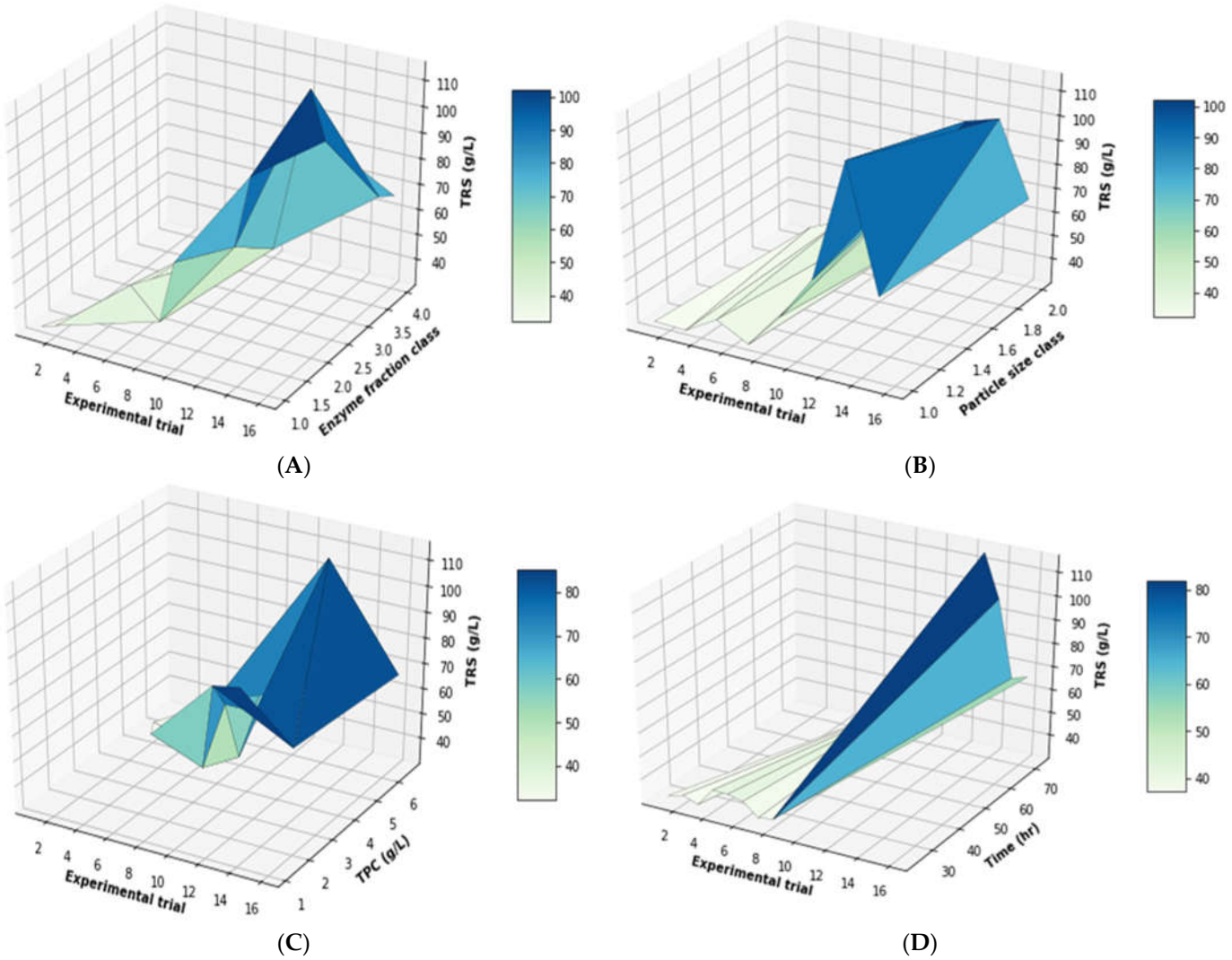


Figure 3. 3-D plots of the variation of TRS production with the enzyme fraction class (**A**), particle size class (**B**), time (**C**), and TPC generation (**D**). Refer to Table 5 for the enzyme fraction and particle size classes.

Mechanical agro-waste reduction can result in reduced energy consumption, with an increase in the accessibility of the enzymes to the biomass and thus the digestibility of lignocellulosic waste, a result that can impart moderate chemical or physicochemical co-treatments [37]. However, it was observed that, when using ionic liquids as a green chemistry approach to biomass, there is a likelihood that reducing the waste to be much smaller (<0.150 µm) might reduce glucose yields, albeit this can vary with the type of pre-treatment liquid [38]. It was also observed that when particle sizes range from 38 to 105 µm, similar sugar yields can be attained using commercial microcrystalline cellulose [39]. Overall, the hydrolysis is time-dependent (Figure 3D), with the observations being that: (1) there

are lower hydrolysis rates with smaller particle sizes, including those in excess of 300 µm, (2) longer hydrolysis times are preferable at near ambient temperature conditions [40]. While a longer hydrolysis period might be preferable, there is an increasing chance that TPCs might accumulate, as seen in Experimental run 17 (Figure 3C), even when using a >106 µm mixed agro-waste, albeit with a >10 kDa enzyme fraction, unless mitigation mechanisms or enzymes such as laccases are supplemented to reduce the phenolics [41]. An assertion was made by Dlangamandla et al. [20] that the *N. mirabilis* pitcher fluid has enzymes that reduce TPCs.

By using a probability density function (Figure 4), i.e., normal distribution (Gaussian), of the TRSs and TPCs production, a further confirmation was ascertained of the observations achieved, i.e., the variation of various individual experimental conditions for the agro-waste pretreatment using the pitcher fluid of *N. mirabilis*. At 72 h, both the TRSs and TPCs had a fairly symmetrical probability density function PDF (Figure 4C,D), with attributes of a Platykurtic (kurtosis < 3) profile, which further suggested the lack of outliers. Previously, Lai et al. [42] indicated that a 72 h process for an enzyme hydrolysis scheme was appropriate, with the hydrolysis of hard- and softwoods being assessed. In another process, i.e., organosolv-enzyme hydrolysis whereby a lignocellulose of feedstock was used to produce butanol and ethanol, a similar enzymatic hydrolysis incubation period was used [43]. Therefore, the 72 h enzyme incubation period was used as a baseline for probability analysis. The 72 h incubation period was also confirmed in this study as being suited for agro-waste pretreatment using pitcher fluids.

Since TPCs are a challenge downstream in fermentation processes, the minimum and maximum threshold—or a range—of the total phenolics in the hydrolysates needed to be verified, i.e., its probability assessment using the PDF. Although the quantification of the type of phenolics was not conducted in this study, it is imperative to assess the TPCs formation probability, i.e., their presence in the hydrolysates, as this influences fermentation outcomes, especially when using the popular ethanol-producing strain *Saccharomyces cerevisiae* (*S. cerevisiae*). Although different classes of by-products might be present in the hydrolysates of pretreated agro-waste, TPCs were the focus of this study, for which 4-Hydroxy-3-methoxycinnamaldehyde, coniferyl alcohol, and p-coumaric acid are prevalent, among others [44]. It was determined that 4-Hydroxy-3-methoxycinnamaldehyde (coniferyl aldehyde) inhibited *S. cerevisiae* at 0.32 g/L [45], a very low threshold. Furthermore, Klinke et al. [46] mentioned that by using alkaline wet oxidation treating wheat straw, 0.27 g/L of phenols were being produced. An IC_{50} of 0.46 g/L (50% inhibition) for *S. cerevisiae* was also reported elsewhere [47]. As such, two values, i.e., 0.3 (min) and 1 g/L (max), as observed in this study, were used to assess the probability of TPCs formation using these values at 72 h for different particles sizes and including the enzyme fractions used.

For the TPCs formation using >106 µm agro-waste particles, PDF had a slight negative skewness with a semi-bimodal profile (Figure 4B), further suggesting a near normal distribution. However, for TRSs (Figure 4A), a positive skewness was observed, suggesting that the mean was slightly greater than the median, with the >75 µm x < 106 µm agro-waste particles having a higher mode than the >106 µm particle size with a near log normal profile.

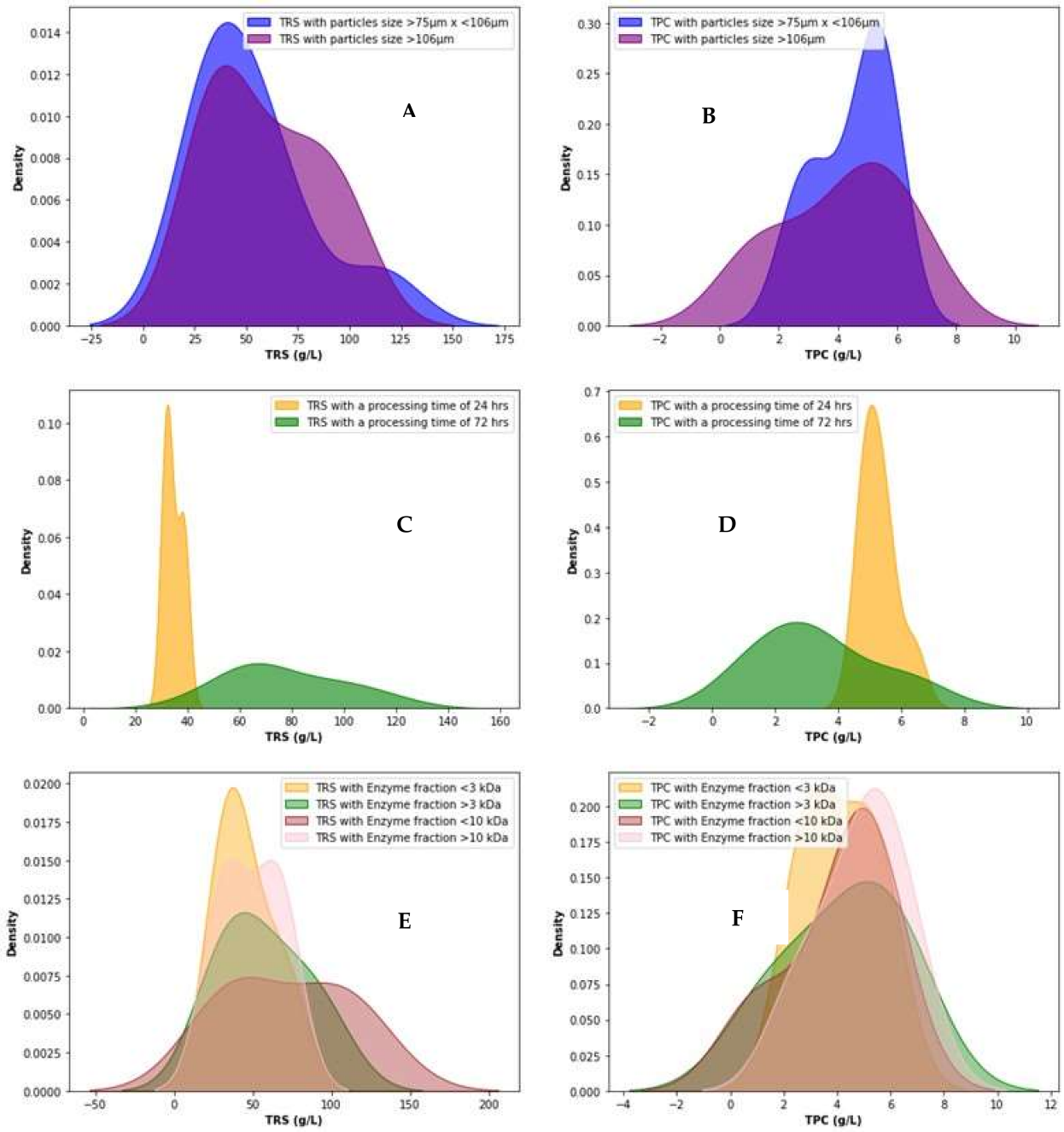


Figure 4. Probability density function of the TRSs and TPCs production with respect to various parametric conditions (**A**–**F**).

For the >106 µm particle size, the probability of their concentration being above 0.3 g/L is almost certain (100%), with standard deviations being 3.37 below the mean (z-score). This is reduced minutely (4%) when considering the >75 µm x < 106 µm agro-waste particles. A similar trend was also observed for TPCs at a concentration of 1 g/L for both particle sizes assessed. Additionally, the TRSs (<3 kDa/>3 kDa) and TPCs (>10 kDa/<3 kDa) in Figure 4E,F are centred around the mean, with higher modes suggesting a likelihood that there is an equivalent frequency such that when the aforementioned fractions are used, a similar outcome will ensue, e.g., a higher TPCs production at a lower TRSs production when compared to the <10 kDa fraction. Since toxicant by-products from agro-waste pretreatment such as phenolics are known to inhibit fermentation processes—e.g., the inhibition of fermenters such as *S. cerevisiae* including enzymic conversion—the use of

inhibitor resistant strains might provide a prudent outcome in such downstream systems, i.e., developing strains via tolerance engineering [48].

2.4. Chemical Compounds and the Use of Pitcher Fluids for Agro-Waste Pretreatment in Biorefineries

Traditional lignocellulosic biomass pretreatment methods such as chemical pretreatment methods are not environmentally friendly and can thus be classified as being unsuitable for biorefineries that are cognizant of the environmental burden imparted by the use of chemicals. Therefore, a green chemistry approach will be warranted in such cases, whereby a strategy can be implemented that uses a less harmful approach while achieving similar results in terms of producing similar hydrolysates to those obtained using chemicals. Generally, the ultimate aim of the pretreatment stage in agro-waste repurposing requires that a high concentration of TRSs be produced at a low cost while preventing the loss of fermentable carbohydrates, using minimal chemical reagents and energy requirements. When using a high temperature, there is a risk of TRSs decomposition, which leads to the formation of Levoglucosan, a six-carbon ring compound generated when carbohydrates are pyrolysed [49].

The acid solution hydrolysis of agro-waste targets hemicellulose, whereby the hemicelluloses are more readily hydrolysed than cellulose and lignin. As such, maize cobs containing up to 30% xylan can be easily hydrolysed to produce xylose. Others (Hassanet al. [50]) have also demonstrated that 2.9 g/L of TRSs can be obtained using an *Aspergillus niger*-facilitated decomposition of maize cobs, with up to 35 g/L TRSs production when 5.5% (v/v) dilute sulphuric acid is used [51], albeit this can lead to lower enzymatic hydrolysis yields downstream of the pretreatment process, suggesting the generation of toxicants [52]. Likewise, 10.26 g/L were generated from 5% (w/v) sulphuric acid (3% w/v)-grape pomace hydrolysates. However, when the acid solution concentration was increased to beyond 5% (w/v), the TRS content in the grape pomace hydrolysate was reduced to 8 g/L [53], suggesting TRSs decomposition when a high acid concentration solution is used for grape pomace pretreatment. Furthermore, since most yard waste in the form of leaves contains a higher concentration of hemicellulose with minimal lignin, glucose and fructose will be the dominant TRSs when such waste is hydrolysed using dilute acid solutions, with traces of xylose and sucrose being observed [54]. This will not be the case for orange and apple peels, which have a slightly higher cellulose content than hemicellulose [7]. For orange peels, the main reducing sugars are glucose > fructose > sucrose, albeit with a TPCs content between 0.6–7.3% (dry biomass wt.), which can contribute significantly to the souring of a fermentation process [55]. Additionally, apple peels generally have higher TPCs, irrespective of the source cultivar [56].

Evidently, acid hydrolysis uses high temperatures exceeding 80 °C, albeit with a shorter pretreatment period in excess of 30 min (see Appendix A Table A3). Some of the research studies also use a single feedstock, i.e., sugarcane bagasse, rice hulls, corn stover, etc., which, in terms of the management and repurposing of the agro-waste, is much simpler in its composition when compared to mixed waste. Some processes also use secondary pretreatment processes such as sonication and enzyme hydrolysis [57,58]. This can be mitigated by using the *N. mirabilis* pitcher fluid, which is highly acidic and contains some enzymes with hydrolysis capabilities. Furthermore, reducible sugars from the dilute sulphuric acid pretreatment of different wastes include xylose from hemicellulose [59]. By increasing the acid concentration, the quantity of galactose in the hydrolysates can increase [60]. Thus, an observation was made when 5.5 g/L galactose was formed from a 3.3% (v/v) dilute sulphuric acid pretreatment of mixed hardwoods [61]. Although the individual reducible sugars had not been identified, in our previous study, it was determined that a similar mixed agro-waste hydrolysate as that obtained in this study—*S. cerevisiae* fermentation for the production of ethanol—was much improved when the *N. mirabilis* pitcher fluid was used instead of dilute sulphuric acid solutions [62]. Overall, the *N. mirabilis* pitcher fluid with its cocktail of enzymes has demonstrated an ability to decompose the components of the mixed agro-waste. For biorefineries and other similar

sustainable energy process systems, it is imperative that they assess the use of pitcher fluids to ascertain whether applicability on a large scale is feasible. Furthermore, a trade-off between using ambient conditions and high temperatures must be further investigated to determine which of the processes might be financially beneficial while considering the environmental impact of chemical use for agro-waste pretreatment.

3. Materials and Methods

3.1. Collection and Processing of the Mixed Agro-Waste

Agro-waste such as *Malus domestica* (apple) peels, *Quercus robur* (oak) yard waste, *Citrus sinensis* (orange) peels, *Vitis vinifera* (grape) pomace, and cobs from *Zea mays* (maize) were obtained from a local fruit/vegetable store, while yard waste was gathered from the garden of the Cape Peninsula University of Technology (CPUT), District 6 campus (Western Cape, Cape Town, South Africa). These agro-wastes were dried separately in an oven at 80 °C for 24 h, with the exception of *C. sinensis* peels, which were dried for 72 h. The different agro-wastes were pulverised and screened to >75 µm x < 106 µm and >106 µm particle sizes, eliminating a pre-cleansing step. A composite was made by accurately weighing 10 g of each of the milled wastes by pooling in equal proportions of 25% (w/w) and by subsequent homogenous mixing. The basis for using mixed agro-waste is that mixed lignocellulosic biomass (MLB) was demonstrated to contribute to cost savings with bench scale experiments, indicating a better ethanol yield when MLB was used in comparison to single feedstocks [63].

3.2. Collection, Physico-Chemical Characterisation, and Sample Fractionation of the N. mirabilis Pitcher Fluid

N. mirabilis pitcher plants were cultivated hydroponically in a greenhouse under ambient conditions (25–30 °C). These plants were grown at Pan's Carnivores Plant Nursery (21 Kirstenhof, Tokai, Cape Town, South Africa). Pitcher fluid samples (10 to 40 mL) were collected from both the opened and closed "monkey cups" depending on the size of the individual cups. This fluid was transferred from the "monkey cups" into sterile 50 mL conical tubes and instantly stored in ice prior to transportation to the laboratory. The plant's pitcher fluid was then centrifuged at 4000× g for 15 min and filter-sterilised with a 0.22 µm Millipore membrane filter (Merck, Burlington, MA, USA), followed by pooling to make a single batch. From the single batch, the pitcher fluid was filtered through a 10 kDa filter via centrifugation at 4000× g for 10 min. The flow-through from the 10 kDa filter was then concentrated on a 3 kDa filter (Pall OD003C34) by centrifugation at 4000× g for 10 min. The different fractions, i.e., <3 kDa, >3 kDa, <10 kDa, >10 kDa, were stored at −20 °C before further processing, i.e., without dilution or the use of a buffer. The basis of the selection for the 10 kDa fraction was based on a method used by [64], whereby a 10 kDa Amicon ultra molecular weight cut-off centrifugal filter (Millipore) was used. Furthermore, since the nominal molecular weight limit (NMWLs) below the 10 kDa is the 3 kDa, this fraction was also assessed. Overall, the characterisation of the *N. mirabilis* pitcher fluid was carried out to determine the physico-chemical properties, i.e., the pH, redox potential, and conductivity of the pooled samples and the individual fractions, by using a multi-parameter meter (Eutech Instruments Pty Ltd., Thermo Fisher Scientific, Singapore). The specific gravity of the pooled pitcher fluid was determined by weighing 1 mL of the fluid. Protein quantification was accomplished by using the QuantiPro BCA assay kit (Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa) according to the manufacturer's instructions to ascertain the presence of enzymes (proteins) within the fluid.

3.3. Pitcher Fluid Fraction in-Solution Digestion and Proteome Analysis via LC-MS/MS

LC-MS/MS analysis was piloted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Dionex Ultimate 3000 nano-UPLC system. The data were developed using Xcalibur v4.1.31.9, Chromelean v6.8 (SR13), Orbitrap MS v2.9 (build 2926), and Thermo Foundations 3.1 (SP4). The mobile phase was made by dissolving Peptides in 0.1% Formic acid (FA, Sigma 56302) and 2% Acetonitrile (ACN, Burdick & Jackson BJLC015CS, Muskegon, MI, USA) and loaded on a C18 trap column (PepMap100, 9027905000, 300 µm × 5 mm × 5 µm). The volume of the samples injected was approximately 400 ng of peptide for each sample. The samples were trapped onto the column and washed for 3 min before the valve was switched on and the peptides eluted onto the analytical column. Chromatographic separation was performed with a Waters nanoEase (Zenfit) M/Z Peptide CSH C18 column (186008810, 75 µm × 25 cm × 1.7 µm). The solvent system used was solvent A: LC water (Burdick and Jackson BJLC365, Muskegon, MI, USA), 0.1% FA and solvent B: ACN, 0.1% FA. All of the data were obtained using Proxeon stainless steel emitters (Thermo Fisher TFES523, Waltham, MA, USA). The multi-step gradient for peptide separation was generated at 300 nL/min with a time change of 5 min, a gradient change of 2–5% Solvent B, a time change of 40 min, a gradient change of 5–18% Solvent B, a time change of 10 min, a gradient change of 18–30% Solvent B, a time change of 2 min, and a gradient change of 30–80%. The gradient was then held at 80% Solvent B for 10 min before returning it to 2% Solvent B for 15 min. The mass spectrometer was operated in a positive ion mode with a capillary temperature of 320 °C. The applied electrospray voltage was 1.95 kV. Details of the data acquisition conditions are shown in Appendix A Table A1. Furthermore, database interrogation was performed with Byonic™ Software v3.8.13 (Protein Metrics, Cupertino, CA, USA) using the *Nepenthes sp.* database of reviewed and unreviewed proteins containing 1790 proteins sourced from the NCBI (www.ncbi.nlm.nih.gov, accessed on 19 October 2021). Details of search parameters are displayed in Appendix A Table A2.

3.4. Trace Element (co-Factor) Solution Preparation for Pitcher Fluid Supplementation

The enzyme's performance can be enhanced by several factors, with a trace elements solution (TES) being one of the solutions that can provide enzyme co-factors, thus improving the digestive fluid performance. Trace elements are believed to work as co-factors and are essential in minute quantities to enhance the biological functioning of enzymes [65]. The oxidation-reduction reactions of biomass degradation can be enhanced by some metallic ions; examples include iron and copper [66]. The trace element solution used in this study was prepared by dissolving 1.5 g of Nitrilotriacetate in 800 mL of sterile distilled water. Subsequently, the pH was adjusted to 6.5 by using 1M KOH (8 g/500 mL). These compounds, i.e., $ZnSO_4.7H_2O$ (0.1 g), $FeSO_4.7H_2O$ (0.1 g), $MgSO_4$ (3 g), $MnSO_4$ (0.5 g), NaCl (1 g), $CuSO_4$ (0.1 g), $AlK(SO_2)_2.12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MnO_4.2H_2O$ (0.01 g), $MgSO_4.7H_2O$ (6.14 g), $MnSO_4.H_2O$ (0.56 g), $CoCl_2.6H_2O$ (0.187 g), and $CoCl_2$ (0.1 g), were weighed as specified and added to the Nitrilotriacetate solution, and the solution was made up to 1000 mL. The solution was filter-sterilised using a 0.22 µm filter and autoclaved. It was then stored at 4 °C prior to use.

3.5. Conditions for Pitcher Fluid-Facilitated Mixed Agro-Waste Pretreatment

A mass (0.5 g) of the mixed agro-waste was weighed into each 100 mL Schott bottle, and a volume (10 mL) of the individual *N. mirabilis* pitcher fluid fractions was added to each Schott bottle to constitute a 5% (w/v) slurry. Thereafter, the trace element solution (0.1 mL) was added as a supplement. The mixed agro-waste and the pitcher fluid were mixed by swirling in a shaking (120 rpm) incubator (LABWIT ZWY-240, Shanghai Zhicheng Analytical, Shanghai, China) to ensure uniformity at a temperature maintained at 25–30 °C to mimic ambient temperature. Sampling (3 mL) was done at 24 h and 72 h, and the samples were centrifuged at 4000× *g* for 10 min. The supernatant collected was used for further analyses, i.e., TRSs and TPCs quantification. All experiments were done in triplicate, and the average values were used for data analyses.

3.6. Quantification of Total Reducible Sugars and Residual Phenolic Compounds in Agro-Waste Pretreatment Hydrolysates

The TRSs were quantified from individual samples collected as the supernatant. This test was performed using a Dinitrosalicylic acid (DNS) assay protocol [67], and the concentration of TRSs was determined by using a calibration curve (R^2 = 0.95). Consequently, the Folin–Ciocalteu method was used to measure TPCs [68], with a calibration curve (R^2 = 0.78) being used to determine the actual concentration in individual samples. The assay protocols were done using a Jenway 7305 UV/Vis spectrophotometer (Cole–Parmer, Staffordshire, UK). All measurements were done in triplicate, and the averages were used in data analysis.

3.7. Data Analytics and Rationale

The data collected from this experiment were analysed with Python using different libraries, i.e., Matplotlib, Pandas, Seaborn, and SciPy. The rationale was to determine which arrangement of parameters yielded the most TRSs while generating the least TPCs. To this end, the enzyme fraction and the particle size were each divided into classes, as observed in Table 5. This classification was geared towards creating a cluster of observations presenting the same characteristics and thus facilitating a categorical analysis meant to inform which arrangement was more effective.

Table 5. Classification of the enzyme fraction and particle size for data analytics.

Enzyme Fraction (kDa)	Enzyme Fraction Class	Particle Size Class	Particle Size (μm)
<3	1	1	>75 x < 106
>3	2	2	>106
<10	3	-	-
>10	4	-	-

To evaluate the correlation between these classes and the contact time on the production of both TPCs and TRSs, the Pearson correlation coefficient was used. The latter shows the correlation between the parameters assessed and the target variables (TRSs, TPCs) without providing causation. When assessing several key parameters, a correlation matrix can be used to show the Pearson correlation coefficient between each of these parameters. Although the Pearson correlation does not inform the causation of observed experimental outputs, it can inform the influences that each of these parameters have on the target variable by providing the degree of covariance between them.

Further data analysis and graphical evaluation was conducted to evaluate the influence that each of these key parameters had on the production of TRSs, including a statistical summary and 3-D plots of the effects of the variation of each class on the production of TRSs. Furthermore, a comparison of the probability density function (PDF) of the production of TRSs and TPCs for each class was also carried out. The statistical summary provided the mean, standard deviation, minimum, p25, p50, p75, and maximum values of the production of TRSs and TPCs with respect to each class of the key parameters. The 3-D plots give a visual representation of this analysis, and the PDF illustrations compare the distribution of the TRSs and TPCs production with respect to each class of the key parameters. The combination of these analyses contributes to understanding the variation of the production of TRSs, informs the most relevant key parameters, and guides the decision of what arrangement to select to minimise the generation of TPCs.

4. Conclusions

In this study, the pretreatment of mixed agro-waste was performed using *N. mirabilis* pitcher fluid, whereby the agro-waste was initially milled, screened into sizes >75 μm x < 106 μm and >106 μm, and subsequently pretreated with fractionated (<3 kDa, >3 kDa, <10 kDa, >10 kDa) pitcher fluid. The best performing fraction was <10 kDa, with the hydrolysates pretreated with this fraction having a higher TRS load with significantly fewer

TPCs. This fraction was further analysed using LC-MS/MS to identify the enzymes contained therein, as it also had significantly better physico-chemical characteristics than the other fractions studied. Putative peroxidase 27, β-1,3-glucanase, Class III and IV chitinases, Thaumatin-like protein, Aspartic protease nepenthesin I, and purple acid phosphatase were identified as being in the <10 kDa fraction. From the investigation, it was concluded that *N. mirabilis* digestive fluid is indeed made-up of a cocktail of digestive/hydrolytic enzymes that are capable of pre-treating milled agro-waste into a significant quantity of fermentable sugars. It is therefore prudent to suggest that the pitcher fluids were able to decouple some carbohydrates into TRS under ambient conditions, limiting the accumulation of TPCs within the hydrolysates. The maximum formed was 97 g/L (TRSs) and 1 g/L (TPCs). This study is relevant to the bioenergy industry as it provides an alternative biomass pretreatment method. This green chemistry method of agro-waste pretreatment can be considered to be eco-friendly and cost-effective. It is recommended that (1) further feasibility studies be undertaken to assess its implementation on a large scale, (2) optimisation be undertaken with the aim of increasing TRSs while reducing TPCs, and (3) the individual reducible sugars and the type of toxicants in the hydrolysates from *N. mirabilis* pretreated agro-waste be identified and quantified.

5. Recommendations

It is recommended that the following be considered in future studies:

- To determine the different enzymes contained within the individual pitcher fluid fractions assessed in order to ascertain the reasons that the <10 kDA fraction performed better than other fractions.
- Since the TRSs reported herein constitute a measure of all reducing sugars within the hydrolysates from pretreated agro-waste, the concentration of the individual reducing sugars must be quantified.

Author Contributions: Conceptualisation, S.K.O.N.; methodology, J.O.A., P.L.H., J.-W.L., S.K.O.N., B.S.C., and V.I.O.; software, M.N.; validation, M.N., S.K.O.N., V.I.O., and B.S.C.; formal analysis, M.N., S.K.O.N., V.I.O., and B.S.C.; investigation, S.K.O.N., V.I.O., and B.S.C.; resources, V.I.O. and P.L.H.; data curation, P.L.H., J.-W.L., and M.N.; writing—original draft preparation, J.O.A.; writing—review and editing, S.K.O.N., B.S.C., V.I.O., and J.-W.L.; visualisation, M.N. and J.O.A.; supervision, S.K.O.N., V.I.O., and B.S.C.; project administration, V.I.O., S.K.O.N., and B.S.C.; funding acquisition, V.I.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Foundation of South Africa as well as the CPUT University Research Fund (URF RK16/RK45), and the support of the CPUT Vice-Chancellor's Prestigious Award programme is also acknowledged. The Bioresource Engineering Research Group (BioERG) at CPUT also contributed additional funding for the study.

Data Availability Statement: Not applicable.

Acknowledgments: Ken Barris is hereby acknowledged for his service as an English language editor for the manuscript.

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

Declaration: The short version of the paper was presented at the ICAE2021, Nov 29–Dec 5, 2021. This paper is a substantial extension of the short version of the conference paper. The title of the shorted version of the paper was "Fractionated digestive juices of Nepenthes mirabilis for reducible sugar release and phenolic compound's reduction from mixed agro-waste pretreatment". We hereby confirm that the title of the extended paper now is "*Nepenthes mirabilis* fractionated pitcher fluid use for mixed agro-waste pretreatment: Advocacy for non-chemical use in biorefineries", with a significant extension of more than 50% of the text in the conference paper, including content.

Appendix A

Table A1. Mass spectrometry data acquisition parameters.

Full Scan	
Resolution	70,000 (@m/z 200)
AGC target value	3×10^6
Scan range	350–2000 m/z
Maximal injection time (ms)	100
Data-dependent MS/MS	
Inclusion	off
Resolution	17,000 (@m/z 200)
AGC target value	1×10^5
Maximal injection time (ms)	50
Loop count	10
Isolation Window width (Da)	3
NCE (%)	27
Data-dependent settings	
Underfill rate (%)	1
Charge exclusion	Unassigned 1, 7, 8, >8
Peptide match	Preferred
Exclusion isotopes	on
Dynamics exclusion (s)	60

Table A2. Byonic™ search parameters.

Recorded Protein Search Configuration		
Num	**Rule**	**Value**
0	Protein database	Nepenthes_NCBI_1790 proteins_191021.fasta
1	Spectrum-level FDR	Auto cut
2	Cleavage residues	RK
3	Digest cutter	C-terminal cutter
4	Peptides termini	Fully specific
5	Maximum number of missed cleavages	2
6	Precursor's tolerance	10.0 ppm
7	Fragment tolerance	Frag:qtof_hcd 20.0 ppm
8	Fragment tolerance version	2
9	Charges applied to charge unassigned spectra:	1,2,3
10	Precursor mass max	10,000.0
11	N-glycan search	None
12	O-glycan search	None

Table A2. *Cont.*

Recorded Protein Search Configuration		
Num	**Rule**	**Value**
13	Off by x isotopes	−2, −1, 0, +1, +2
14	Contaminants added	True
15	Decoy added	True
16	**Additional parameters:**	
17	Disulphide enable	False
18	Trisulphide enable	False
19	DSS crosslink enable	False
20	Custom crosslink enable	False
21	Wildcat enable	0
22	Combyne cut-off score	Auto
23	Protein FDR cut-off	1%
24	Focused DB created	False
25	Export mzldentML	True
26	Score version	2
27	Precursor assignment flags	2
28	Po_NumberMONOsReturn	1
29	Lock mass list	None
30	% Modification searches:	
31	Common_Modification_max	1
32	Rare_modifications_max	1

Table A3. Dilute sulphuric acid pretreatment of different lignocellulose wastes under different conditions.

Pretreatment Method (Dilute Sulphuric Acid)	Waste Type	Secondary Treatment Method	Temp. (°C)	Time (min)	TRSs (Type/conc.)	TPCs (Type/conc.)	Reference
Acid hydrolysis (2% v/v)	Sugarcane bagasse	-	122	60	Xylose (19.1 g/L) Arabinose (2.2 g/L)	-	[69]
	Rice hulls	-	122		Glucose (33, 5 g/L)	-	
Acid hydrolysis (5.5 % v/v)	Corn stover	-	100	300	Xylose (18.73 g/L) Glucose (6.64 g/L)	Furfural (0.63 g/L)	[70]
Acid hydrolysis (0.73% v/v)	Corn stover	Cellulases	150	30	Glucose and xylose (0.50 g/g)	n/d	[71]
Acid hydrolysis (10% v/v)	Rice straw	Sonication	80	50	TRS (0.32 g/g)	-	[57]
Acid hydrolysis (3.3% v/v)	Mixed hardwoods	-	130	50.2	Galactose (5.5 g/L)	-	[61]

Table A3. *Cont.*

Pretreatment Method (Dilute Sulphuric Acid)	Waste Type	Secondary Treatment Method	Temp. (°C)	Time (min)	TRSs (Type/conc.)	TPCs (Type/conc.)	Reference
Acid hydrolysis (1% v/v)	Sorghum	-	121	120	Xylobiose (18.02 mg/g) Xylose (225 mg/g)	Furfural (4.6 mg/g)	[71]
Acid hydrolysis (4% v/v)	Teft straw	-	120	55	TRS (26.65 mg/g)	-	[72]
Acid/enzyme hydrolysis (100% pitcher fluid) > 10 kDA fraction	**Mixed agro-waste**	-	**25–30 (Ambient)**	**4320**	**TRSs (97 g/L)**	**TPCs (1 g/L)**	**This study**

References

1. Leong, H.Y.; Chang, C.-K.; Khoo, K.S.; Chew, K.W.; Chia, S.R.; Lim, J.W.; Chang, J.-S.; Show, P.L. Waste biorefinery towards a sustainable circular bioeconomy: A solution to global issues. *Biotechnol. Biofuels* **2021**, *14*, 87. [CrossRef] [PubMed]
2. Ghatak, H.R. Biorefineries from the perspective of sustainability: Feedstocks, products, and processes. *Renew. Sustain. Energy Rev.* **2011**, *15*, 4042–4052. [CrossRef]
3. Lee, E.K.; Romeiko, X.X.; Zhang, W.; Feingold, B.J.; Khwaja, H.A.; Zhang, X.; Lin, S. Residential proximity to biorefinery sources of air pollution and respiratory diseases in New York state. *Environ. Sci. Technol.* **2021**, *55*, 10035–10045. [CrossRef]
4. Heath, G. Analyzing Air Pollutant Emissions from the Biofuel Supply Chain. Available online: https://www.nrel.gov/analysis/biofuels-emissions.html (accessed on 25 February 2022).
5. Levidow, L. Eco-efficient biorefineries: Techno-fix for resource constraints? *Économie Rurale. Agric. Aliment. Territ.* **2015**, *349–350*, 1–24. [CrossRef]
6. Capolupo, L.; Faraco, V. Green methods of lignocellulose pretreatment for biorefinery development. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 9451–9467. [CrossRef] [PubMed]
7. Dlangamandla, N. Design of Integrated Processes for a Second Generation Biorefinery Using Mixed Agricultural Waste. Ph.D. Thesis, Cape Peninsula University of Technology, Cape Town, South Africa, 2018.
8. Ellison, A.M. Pitcher Plants of the Old World, Volumes One and Two. *Rhodora* **2010**, *112*, 95–97. [CrossRef]
9. Anonymous. Growing Nepenthes Hydroponically: Preliminary Results. Available online: https://pitcherplants.proboards.com/thread/13543/growing-nepenthes-hydroponically-preliminary-results (accessed on 25 February 2022).
10. Jürkenbeck, K.; Heumann, A.; Spiller, A. Sustainability matters: Consumer acceptance of different vertical farming systems. *Sustainability* **2019**, *11*, 4052. [CrossRef]
11. Takeuchi, Y.; Chaffron, S.; Salcher, M.M.; Shimizu-Inatsugi, R.; Kobayashi, M.J.; Diway, B.; von Mering, C.; Pernthaler, J.; Shimizu, K.K. Bacterial diversity and composition in the fluid of pitcher plants of the genus Nepenthes. *Syst. Appl. Microbiol.* **2015**, *38*, 330–339. [CrossRef]
12. Chan, X.-Y.; Hong, K.-W.; Yin, W.-F.; Chan, K.-G. Microbiome and biocatalytic bacteria in monkey cup (Nepenthes pitcher) digestive fluid. *Sci. Rep.* **2016**, *6*, srep20016. [CrossRef]
13. Hatano, N.; Hamada, T. Proteome analysis of pitcher fluid of the carnivorous plant *Nepenthes alata*. *J. Proteome Res.* **2008**, *7*, 809–816. [CrossRef]
14. Hatano, N.; Hamada, T. Proteomic analysis of secreted protein induced by a component of prey in pitcher fluid of the carnivorous plant *Nepenthes alata*. *J. Proteom.* **2012**, *75*, 4844–4852. [CrossRef] [PubMed]
15. Takeuchi, Y.; Salcher, M.M.; Ushio, M.; Shimizu-Inatsugi, R.; Kobayashi, M.J.; Diway, B.; Von Mering, C.; Pernthaler, J.; Shimizu, K.K. In situ enzyme activity in the dissolved and particulate fraction of the fluid from four pitcher plant species of the genus Nepenthes. *PLoS ONE* **2011**, *6*, e25144. [CrossRef] [PubMed]
16. Hunlun, C.; De Beer, D.; Sigge, G.O.; Van Wyk, J. Characterisation of the flavonoid composition and total antioxidant capacity of juice from different citrus varieties from the Western Cape region. *J. Food Compos. Anal.* **2017**, *62*, 115–125. [CrossRef]
17. Adsul, M.; Sandhu, S.K.; Singhania, R.R.; Gupta, R.; Puri, S.K.; Mathur, A. Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels. *Enzym. Microb. Technol.* **2020**, *133*, 109442. [CrossRef]
18. Angadam, J.O.; Ntwampe, S.K.O.; Chidi, B.S.; Lim, J.W.; Okudoh, V.I. Lignocellulosic waste pretreatment solely via biocatalysis as a partial simultaneous lignino-holocellulolysis process. *Catalysts* **2021**, *11*, 668. [CrossRef]
19. Rottloff, S.; Miguel, S.; Biteau, F.; Nisse, E.; Hammann, P.; Kuhn, L.; Chicher, J.; Bazile, V.; Gaume, L.; Mignard, B.; et al. Proteome analysis of digestive fluids in Nepenthes pitchers. *Ann. Bot.* **2016**, *117*, 479–495. [CrossRef]
20. Dlangamandla, N.; Ntwampe, S.K.O.; Angadam, J.O.; Itoba-Tombo, E.F.; Chidi, B.S.; Mekuto, L. Integrated hydrolysis of mixed agro-waste for a second generation biorefinery using *Nepenthes mirabilis* pod digestive fluids. *Processes* **2019**, *7*, 64. [CrossRef]
21. Senevirathna, R.; Seneviratne, V.; Rajapakse, S. Chitinases from pitcher fluid of *Nepenthes distillatoria*. *Ceylon J. Sci.* **2019**, *48*, 243–249. [CrossRef]

22. Athauda, S.B.; Matsumoto, K.; Rajapakshe, S.; Kuribayashi, M.; Kojima, M.; Kubomura-Yoshida, N.; Iwamatsu, A.; Shibata, C.; Inoue, H.; Takahashi, K. Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. *Biochem. J.* **2004**, *381*, 295–306. [CrossRef]
23. Jose, D.; Jayesh, P.; Gopinath, P.; Mohandas, A.; Singh, I. Potential application of β-1, 3 glucanase from an environmental isolate of *Pseudomonas aeruginosa* MCCB 123 in fungal DNA extraction. *Indian J. Exp. Biol.* **2014**, *52*, 89–96.
24. Kong, Y.; Li, X.; Wang, B.; Li, W.; Du, H.; Zhang, C. The soybean purple acid phosphatase GmPAP14 predominantly enhances external phytate utilization in plants. *Front. Plant Sci.* **2018**, *9*, 292. [CrossRef] [PubMed]
25. Slavokhotova, A.A.; Naumann, T.A.; Price, N.P.J.; Rogozhin, E.A.; Andreev, Y.A.; Vassilevski, A.A.; Odintsova, T.I. Novel mode of action of plant defense peptides—hevein-like antimicrobial peptides from wheat inhibit fungal metalloproteases. *FEBS J.* **2014**, *281*, 4754–4764. [CrossRef]
26. Anonymous. UniProtKB—A0A140GMM6 (A0A140GMM6_NEPMI). Available online: https://www.uniprot.org/uniprot/A0A140GMM6 (accessed on 25 February 2022).
27. Anonymous. Aspartic-Type Endopeptidase Activity. Available online: https://www.ebi.ac.uk/QuickGO/term/GO:0004190 (accessed on 25 February 2022).
28. Ma, Y.; Luo, M.; Xu, Y.; Liu, Y.; Liu, X.; Bi, X.; Yuan, Y.; Su, F.; Yin, X. Purification and characterization of a thaumatin-like protein-1 with polyphenol oxidase activity found in *Prunus mume*. *RSC Adv.* **2020**, *48*, 28746–28754. [CrossRef] [PubMed]
29. Hamid, R.; Khan, M.A.; Ahmad, M.; Ahmad, M.M.; Abdin, M.Z.; Musarrat, J.; Javed, S. Chitinases: An update. *J. Pharm. Bioallied Sci.* **2013**, *5*, 21–29. [CrossRef] [PubMed]
30. Isroi, I.; Millati, R.; Niklasson, C.; Cayanto, C.; Taherzadeh, M.J.; Lundquist, K. Biological treatment of Lignocelluloses with white-rot funghi and its applications. *BioResources* **2011**, *6*, 5224–5259. [CrossRef]
31. Ravichandran, A.; Sridhar, M. Insights into the mechanism of lignocellulose degradation by versatile peroxidases. *Curr. Sci.* **2017**, *113*, 35–42. [CrossRef]
32. Michalko, J.; Socha, P.; Mészáros, P.; Blehová, A.; Libantová, J.; Moravčíková, J.; Matušíková, I. Glucan-rich diet is digested and taken up by the carnivorous sundew (*Drosera rotundifolia* L.): Implication for a novel role of plant β-1, 3-glucanases. *Planta* **2013**, *238*, 715–725. [CrossRef]
33. McGrath, C.E.; Wilson, D.B. Characterization of a *Thermobifida fusca* β-1, 3-glucanase (Lam81A) with a potential role in plant biomass degradation. *Biochemistry* **2006**, *45*, 14094–14100. [CrossRef]
34. Grenier, J.; Potvin, C.; Trudel, J.; Asselin, A. Some thaumatin-like proteins hydrolyse polymeric β-1, 3-glucans. *Plant J.* **1999**, *19*, 473–480. [CrossRef]
35. Fierascu, R.C.; Fierascu, I.; Avramescu, S.M.; Sieniawska, E. Recovery of natural antioxidants from agro-industrial side streams through advanced extraction techniques. *Molecules* **2019**, *24*, 4212. [CrossRef]
36. Manavalan, T.; Liu, R.; Zhou, Z.; Zou, G. Optimization of acetyl xylan esterase gene expression in *Trichoderma reesei* and its application to improve the saccharification efficiency on different biomasses. *Process Biochem.* **2017**, *58*, 160–166. [CrossRef]
37. Barakat, A.; Chuetor, S.; Monlau, F.; Solhy, A.; Rouau, X. Eco-friendly dry chemo-mechanical pretreatments of lignocellulosic biomass: Impact on energy and yield of the enzymatic hydrolysis. *Appl. Energy* **2014**, *113*, 97–105. [CrossRef]
38. Bahcegul, E.; Apaydin, S.; Haykir, N.I.; Tatli, E.; Bakir, U. Different ionic liquids favor different lignocellulosic biomass particle sizes during pretreatment to function efficiently. *Green Chem.* **2012**, *14*, 1896–1903. [CrossRef]
39. Peters, L.; Walker, L.; Wilson, D.; Irwin, D. The impact of initial particle size on the fragmentation of cellulose by the cellulase of *Thermomonospora fusca*. *Bioresour. Technol.* **1991**, *35*, 313–319. [CrossRef]
40. Li, S.; Zhang, X.; Andresen, J.M. Production of fermentable sugars from enzymatic hydrolysis of pretreated municipal solid waste after autoclave process. *Fuel* **2012**, *92*, 84–88. [CrossRef]
41. De La Torre, M.; Martín-Sampedro, R.; Fillat, Ú.; Eugenio, M.E.; Blánquez, A.; Hernández, M.; Arias, M.E.; Ibarra, D. Comparison of the efficiency of bacterial and fungal laccases in delignification and detoxification of steam-pretreated lignocellulosic biomass for bioethanol production. *J. Ind. Microbiol. Biotechnol.* **2017**, *44*, 1561–1573. [CrossRef]
42. Lai, C.; Tu, M.; Shi, Z.; Zheng, K.; Olmos, L.G.; Yu, S. Contrasting effects of hardwood and softwood organosolv lignins on enzymatic hydrolysis of lignocellulose. *Bioresour. Technol.* **2014**, *163*, 320–327. [CrossRef]
43. Nanda, S.; Dalai, A.K.; Kozinski, J.A. Butanol and ethanol production from lignocellulosic feedstock: Biomass pretreatment and bioconversion. *Energy Sci. Eng.* **2014**, *2*, 138–148. [CrossRef]
44. Sharma, J.; Kumar, V.; Prasad, R.; Gaur, N.A. Engineering of *Saccharomyces cerevisiae* as a consolidated bioprocessing host to produce cellulosic ethanol: Recent advancements and current challenges. *Biotechnol. Adv.* **2022**, *56*, 107925. [CrossRef]
45. Adeboye, P.T.; Bettiga, M.; Olsson, L. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. *AMB Express* **2014**, *4*, 46. [CrossRef]
46. Klinke, H.B.; Olsson, L.; Thomsen, A.B.; Ahring, B.K. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: Wet oxidation and fermentation by yeast. *Biotechnol. Bioeng.* **2003**, *81*, 738–747. [CrossRef] [PubMed]
47. Hrenovic, J.; Stilinovic, B.; Dvoracek, L. Use of prokaryotic and eukaryotic biotests to sssess toxicity of wastewater from pharmaceutical sources. *Acta Chim. Slov.* **2005**, *52*, 119–125.
48. Wang, S.; Sun, X.; Yuan, Q. Strategies for enhancing microbial tolerance to inhibitors for biofuel production: A review. *Bioresour. Technol.* **2018**, *258*, 302–309. [CrossRef] [PubMed]

49. Harris, J.; Baker, A.; Conner, A.; Jeffries, T.; Minor, J. Two-Stage, Dilute Sulfuric Acid Hydrolysis of Wood: An Investigation of Fundamentals. Technical Report for Forest Products: Madison, WI, USA, 1985; pp. 1–77.
50. Hassan, D.u.B.; Maikano, S.A.; Hoomsuk, R.H.; Issac, A.J.; Muhammad, B.I. Studies on reducing sugar yields produced from corn cob and corn stalk hydrolysis using *Aspergillus niger*. In Proceedings of the 5th International Symposium on "Fusion of Science & Technology", New Delhi, India, 18–22 January 2016; pp. 650–653.
51. Ayeni, A.O.; Daramola, M.O.; Agboola, O.; Ayoola, A.A.; Babalola, R.; Oni, B.A.; Omodara, J.O.; Dick, D.T. A comparative evaluation of fermentable sugars production from oxidative, alkaline, alkaline peroxide oxidation, dilute acid, and molten hydrate salt pretreatments of corn cob biomass. *AIMS Energy* **2021**, *9*, 15–28. [CrossRef]
52. Dziekońska-Kubczak, U.A.; Berłowska, J.; Dziugan, P.T.; Patelski, P.; Balcerek, M.; Pielech-Przybylska, K.J.; Czyżowska, A.I.; Domański, J.T. Comparison of steam explosion, dilute acid, and alkali pretreatments on enzymatic saccharification and fermentation of hardwood sawdust. *BioResources* **2018**, *13*, 6970–6984.
53. Kurt, A.S.; Cekmecelioglu, D. Bacterial cellulase production using grape pomace hydrolysate by shake-flask submerged fermentation. *Biomass Convers. Biorefinery* 2021, *in press*. [CrossRef]
54. Nykvist, N. Leaching and decomposition of water-soluble organic substances from different types of leaf and needle litter. *Studia For. Suec.* **1963**, *3*, 1–31.
55. Tsouko, E.; Maina, S.; Ladakis, D.; Kookos, I.K.; Koutinas, A. Integrated biorefinery development for the extraction of value-added components and bacterial cellulose production from orange peel waste streams. *Renew. Energy* **2020**, *160*, 944–954. [CrossRef]
56. Ergün, Z. Determination of biochemical contents of fresh, oven-dried, and sun-dried peels and pulps of five apple cultivars (Amasya, Braeburn, Golden Delicious, Granny Smith, and Starking). *J. Food Qual.* **2021**, *2021*, 9916694. [CrossRef]
57. Rehman, M.; Kim, I.; Kim, K.H.; Han, J.-I. Optimization of sono-assisted dilute sulfuric acid process for simultaneous pretreatment and saccharification of rice straw. *Int. J. Environ. Sci. Technol.* **2014**, *11*, 543–550. [CrossRef]
58. Chen, S.X.; Yong, Q.; Xu, Y.; Yu, S.Y. Dilute sulfuric acid pretreatment and enzymatic hydrolysis of corn stover into fermentable sugars. *Adv. Mater. Res.* **2012**, *535–537*, 2462–2468. [CrossRef]
59. Jacobsen, S.E.; Wyman, C.E. Xylose monomer and oligomer yields for uncatalyzed hydrolysis of sugarcane bagasse hemicellulose at varying solids concentration. *Ind. Eng. Chem. Res.* **2002**, *41*, 1454–1461. [CrossRef]
60. Sun, Y.; Cheng, J. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresour. Technol.* **2002**, *83*, 1–11. [CrossRef]
61. Park, S.J.; Um, B.H. Optimization study on acid hydrolysis of hardwood-derived hemicellulosic extract for alcohol fermentation using response surface methodology. *Holzforschung* **2015**, *69*, 135–141. [CrossRef]
62. Dlangamandla, N.; Ntwampe, S.K.O.; Angadam, J.O.; Chidi, B.S.; Mewa-Ngongang, M. Kinetic parameters of *Saccharomyces cerevisiae* alcohols production using *Nepenthes mirabilis* pod digestive fluids-mixed agro-waste hydrolysates. *Fermentation* **2019**, *5*, 10. [CrossRef]
63. Oke, M.A.; Annuar, M.S.M.; Simarani, K. Mixed feedstock approach to lignocellulosic ethanol production—prospects and limitations. *BioEnergy Res.* **2016**, *9*, 1189–1203. [CrossRef]
64. Lee, L.; Zhang, Y.; Ozar, B.; Sensen, C.W.; Schriemer, D.C. Carnivorous nutrition in pitcher plants (Nepenthes spp.) via an unusual complement of endogenous enzymes. *J. Proteome Res.* **2016**, *15*, 3108–3117. [CrossRef]
65. Keskin, T.; Arslan, K.; Abubackar, H.N.; Vural, C.; Eroglu, D.; Karaalp, D.; Yanik, J.; Ozdemir, G.; Azbar, N. Determining the effect of trace elements on biohydrogen production from fruit and vegetable wastes. *Int. J. Hydrog. Energy* **2018**, *43*, 10666–10677. [CrossRef]
66. Šelih, V.S.; Strlič, M.; Kolar, J.; Pihlar, B. The role of transition metals in oxidative degradation of cellulose. *Polym. Degrad. Stab.* **2007**, *92*, 1476–1481. [CrossRef]
67. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
68. Agbor, G.A.; Vinson, J.A.; Donnelly, P.E. Folin-Ciocalteau reagent for polyphenolic assay. *Int. J. Food Sci. Nutr. Diet.* **2014**, *3*, 147–156. [CrossRef]
69. Martín, C.; Klinke, H.B.; Thomsen, A.B. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. *Enzym. Microb. Technol.* **2007**, *40*, 426–432. [CrossRef]
70. Lu, X.; Zhang, Y.; Liang, Y.; Yang, J.; Zhang, S.; Suzuki, E. Kinetic studies of hemicellulose hydrolysis of corn stover at atmospheric pressure. *Korean J. Chem. Eng.* **2008**, *25*, 302–307. [CrossRef]
71. Deshavath, N.N.; Mohan, M.; Veeranki, V.D.; Goud, V.V.; Pinnamaneni, S.R.; Benarjee, T. Dilute acid pretreatment of sorghum biomass to maximize the hemicellulose hydrolysis with minimized levels of fermentative inhibitors for bioethanol production. *3 Biotech* **2017**, *7*, 139. [CrossRef] [PubMed]
72. Tesfaw, A.A.; Tizazu, B.Z. Reducing sugar production from *Teff* straw biomass using dilute sulfuric acid hydrolysis: Characterization and optimization using response surface methodology. *Int. J. Biomater.* **2021**, *2021*, 2857764. [CrossRef]