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Fractionated digestive juices of *Nepenthes mirabilis* for reducible sugar production and phenolic compound's reduction from mixed agrowaste pretreatment

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ABSTRACT

This paper provides a performance assessment of different fractions of crude plant digestive juices produced by *Nepenthes mirabilis* (*N. mirabilis*) which were used to pretreat mixed agro-waste to determine which fraction produces more holocelluloses while reducing phenolic compounds in the hydrolysate. Fractionation (<3kDa, > 3kDa and <10kDa, > 10kDa) was done with the different fractions being used to pretreat the mixed agro-waste (1:1 ratio, i.e., 25% (w/w) for each; 1 g orange peels, 1 g apples peels, 1 g maize cobs, 1 g grape pomace and 1 g oak plant yard litter) of various particle sizes, i.e., >106µm, >75µm <106µm. The highest results obtained indicated 46,63 (min) to 115,19 (max) g/L for total reducing sugars (TRS) production with a maximum of 6,61 g/L for total residual phenolic compounds (TRPCs) being observed using the <10 kDa/>106µm fraction/particle size of N. mirabilis/agrowaste after 72 hrs, revealing the efficacy of different juice fractions to pretreat the agro-waste. Overall, the digestive enzymes in *N. mirabilis* pods must be fractionated for agro-waste pretreatment, i.e., using a green chemistry approach for the development of biorefineries, for sustainable renewable energy generation and security.

Keywords: Green biomass pretreatment, lignocellulosic waste, *Nepenthes mirabilis*, phenolic compounds, reducible sugars.

1. INTRODUCTION

Enzymes are life's great facilitators. They are protein constituents synthesized by living organisms, facilitating indefinite metabolic and biochemical reactions. Enzymes have been used for centuries to produce food and beverages and even in waste treatment. Nonetheless, in the last century, the biocatalytic activity of enzymes has been exploited to include usage in biorefineries for biofuel production, therefore renewable energy constituents used in fuels. Some enzymes from various bacteria are known to degrade recalcitrant polymeric substances by hydrolytic action. The main end-products in most cases are fermentable hydrolysates, i.e. disaccharides, and monosaccharides, which can be fermented to produce fuel additives for energy generation. Overall, enzyme engineering has also enhanced enzyme efficacy and increased the number of biotransformation products that can be produced.

The enzymes found in the acidic fluid of pitcher plants can therefore be utilized. Recent studies have clarified some previously unknown information, indicating that a diverse and complex enzymatic cocktail does exit with a high concentration of digestive/hydrolytic enzymes from within the pods ("monkey cups") of *Nepenthes* species. The type and activity of the digestive enzymes including whether they can be used in bio-delignification process of lignocellulosic biomass, and agro-waste for a biorefinery, is still unclear [1]; albeit fractionation of such a cocktail of hydrolytic enzymes might be necessary.

Fractionation is a separation procedure used for the division of mixtures into several smaller quantities (fractions) based on differences in specific properties of the individual fractions. Fractionation makes it possible to isolate more than two components in a mixture in a single run [2, 3]. It has been proven that enzymes in the pod juice from the *Nepenthes* species are capable of degrading plant litter in the environment in which they grow in; however, it is not clear which fraction within the digestive pod juice is responsible for this.

There are abundant agro-waste that are continuously being dumped into the environment by various industries. Agro-processing industries are among the top commercial and significant agro-economic conglomerates globally, producing a diverse quantity of lignin-holocellulose containing waste. After waste generation, the inappropriate disposal practices of agricultural-based wastes that contains mostly peels, seeds, and stems which are the main constituents of holocellulose, can lead to environmental pollution with landfilling being potentially harmful to human and animal health. These agro-waste residues are considered the cheapest and most copious organic waste that can be easily transformed to value-added products as they are mostly made-up of holocellulosic material which is considered a fundamental source of fermentable sugars for the manufacturing of value-added products [4], and fuel additives for energy generation. The deficiency of traditional sources of energy to meet the ever-increasing needs of people has led to researchers discovering other potentials of harnessing energy from numerous other renewable resources, including agro-waste.

Some research has been done on the effect of biological pretreatment of milled agrowaste to fermentable sugar and then into fuel or additives in biorefineries. This research focused on the effect of biological pretreatment of a defined particle size milled mixed agrowaste using plant digestive enzymes found in *N. mirabilis* pods for the extraction of fermentable sugars with minimal phenolic compounds formation, or conversion as reported elsewhere [5], which are known to be inhibitory for fermenters in fermentation systems.

2. MATERIALS AND METHODS

2.1Collection and preparation of the mixed agro-waste

Agro-waste comprised of pomace from *Vitis vinifera* (grape) fruit, cobs from *Zea mays*, *Malus domestica* (apple) peels, *Quercus robur* (oak) yard waste, and *Citrus sinensis* (orange) peels was sourced from marketplaces around the neighborhood and the garden of the Cape Peninsula University of Technology (CPUT), District 6 campus (Western Cape, Cape Town, SA). After drying all the waste at 80°C for 24 h, *C. sinensis* peels were re-dried for 48 h. The individual agro-waste were milled and screened to a >75 µm < 106 µm size without a preliminary rinsing step. These agro-waste were mixed in equal proportions of 1:1 ratio, i.e., i.e., 25% (w/w) for each; 1 g orange peels, 1 g apples peels, 1 g maize cobs, 1 g grape pomace and 1 g oak plant yard litter.

2.2 Collection and characterization of the N. mirabilis juice

N. mirabilis plants can grow in a greenhouse under regulated conditions. These plants selected were grown in Pan's Carnivores Plant Nursery (21 Kirstenhof, Tokai, Cape Town, SA). The plants digestive juice from individual pods were collected into sterile 50 mL conical tubes, and immediately stored in ice, prior to transportation to the laboratory. In the laboratory, the plants digestive juice was centrifuged at 4000 x g for 15 min and filter sterilized with a 0.22 µm Millipore membrane filter with subsequent pooling to make a single batch and subsequently stored at -20 °C prior to use, i.e., without dilution or the use of a buffer. Approximately 10 to 40 mL of the digestive juice was collected per pod, i.e., "monkey cup", of the *N. mirabilis* plants, a volume which was dependent on the size of the individual cups. The specific gravity, pH, redox potential, and conductivity which are the physico-chemical properties of the *N. mirabilis* digestive pod juices, were measured by making use of a multi-parameter instrument (Eutech Instruments Pty Ltd, Thermo Fisher Scientific, Singapore).

There are some factors which can promote the functionality of enzymes even when in a cocktail, with trace element's being one of the solutions which can facilitate enzyme co-factor availability thus enhance the digestive juices performance.

2.3 Trace element stock solution preparation

Trace elements are essential in small amounts for biological functioning of enzymes as co-factors. Some metallic ions such as iron and copper contribute to oxidation-reduction reactions in biomass breakdown [6].

The trace element solution used was prepared by dissolving 1.5 g of Nitrilotriacetate in 800 mL sterile distilled water. Thereafter, the pH was adjusted to 6.5 by using 1M KOH (8 g/500 mL). The following compounds, i.e., $MgSO_4$ (3 g), $MnSO_4$ (0.5 g), NaCl (1 g), $FeSO_4.7H_2O$ (0.1 g), $CoCl_2$ (0.1 g), $ZnSO_4.7H_2O$ (0.1 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), were added to the solution and the solution was made up to 1000 mL. The solution was filter sterilized using a 0.22 µm filter and autoclaved. It was then stored at 4 °C prior to use. The color of the solution was light yellow.

2.4 Enzyme facilitated hydrolysis of the mixed agro-waste

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 fractions was added to each Schott bottle. Thereafter, the trace element solution (0.1 mL) was added as a supplement. The mixed agro-waste and the digestive juices were mixed by swirling in a shaking incubator at a temperature maintained at 25-30 °C, to mimic ambient temperature. Sampling was done in 24 h intervals and the samples were centrifuged at 4000 x g for 10 min. The supernatant collected was analyzed for total reducible sugars (TRS) using a Dinitrosalicylic acid (DNS) assay protocol. Phenolic content, which is known as inhibitory compounds to enzymes such as β-glucosidase in the degradation of polysaccharides to simpler sugars, was quantify using the Folin-Ciocalteu assay protocol.

The depiction of the experimental process is highlighted in Fig. 1.

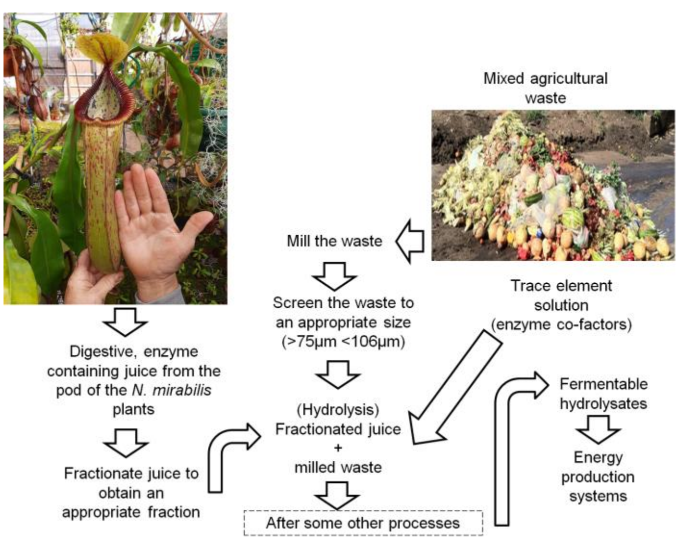

Figure 1. Experimental process undertaken for the study.

3. RESULTS AND DISCUSSION

3.1 Physico-chemical properties of N. mirabilis pod juices and the juices fractions

The specific gravity, pH, redox potential, and conductivity, i.e., the physico-chemical properties of the fresh *N. mirabilis* digestive juice collected, are listed in Table 1. Although, *N. mirabilis* digestive juice contains a cocktail of enzymes, its properties, are similar to those of 1% (v/v) dilute sulphuric acid solution; albeit, with a higher redox potential [7]. This is a trait associated with the ability of a mixture to facilitate reduction-oxidation reactions. Overall, *N. mirabilis* plants are categorized by their "monkey cups" which can charm, trap, and break down insects' exoskeletons, via a digestive, acidic enzyme facilitated process. This is due to the acidic nature of the *N. mirabilis* pod juices, which also facilitate leaf litter digestion for the plant's nutrition [8]. Thus, the notion that such enzyme containing acidic juices can be used to pretreat mixed agro-waste for TRS production from a variety of lignocellulose containing biomass, is valid.

Table 1. Physico-chemical properties of *N. mirabilis* digestive juice without 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

Overall, the <10 kDa fraction was observed to have the best physico-chemical properties as highlighted in Table 2, which led to it performing better in terms of agro-waste pretreatment for TRS production and total residual phenolic compounds (TRPCs) reduction.

Table 2. Physico-chemical properties of the different fractions of *N. mirabilis* extract.

Factors	<3kDa	>3kDa	<10kDa	>10kDa
pH	2.04	2.02	**2.00**	2.06
S.g.	0.73	0.73	**0.81**	0.80

ORP	503	501	**510**	511
CO	3.91	3.86	**4.93**	3.97

3.2 Performance of different enzyme ("monkey cup") fractions

Overall, the suitable conditions were such that, a particle size of >106 µm, using a contact time of 72 hrs, including using an enzyme fraction of < 10 kDa produced the highest TRS with minimized TRPCs, a betterment of other agrowaste pretreatment methods, i.e., without the use of inorganic acids; albeit the mixed agro-waste waste was milled. Others have observed a higher yield of TRS production using dilute inorganic acids, albeit with a higher TRPCs load in the hydrolysates [9]. This was a confirmation of the hypothesis that during pretreatment of agro-waste, there can be a reduction of inhibitory compounds by pretreating the agro-waste with plant-based digestive juices from pitcher plants.

It has been reported that harsh delignification methods, based on chemical pretreatment methods, were inhibitory to subsequent cellulase/enzyme facilitated hydrolysis and fermentation to produce value added products destined for renewable energy generation [10]. Chemical pretreatment methods can be categorized as inappropriate for a less energy intensive biorefinery, and the use of a green chemistry approach will lessen the burden on the environment and thus environmental impact for bio-refineries. Overall, a well-developed green pretreatment system will facilitate ligninolysis and consequently, uncover holocelluloses for the subsequent successful hydrolysis of the agro-waste with negligible energy intake, to accomplish the most favorable outcomes [11, 12]. For biorefineries, assessment of environmental impact based on final products achieved from known mass and energy input, is important. For example, the use of steam in a biorefinery was also recently reported to have a negative impact to human health [13], and is largely recommended for biomass delignification on a large scale.

4. CONCLUSIONS

Exploration for novel scientifically viable plant enzymes is never-ending, and carnivorous plants have shown to be a valuable resource for ongoing research in various fields. In this study, a fractionated portion of *N. mirabilis* extract were used to pre-treat mixed agrowaste of a particular particle size for the breaking down of holocelluloses to produce fermentable sugars and reduction of phenolic compounds thereof. The technique of enzymes fractionation helps to separate enzymes in solution according to their molecular weight. The findings revealed a significant production of fermentable sugars and a substantial reduction in the inhibitory compound produced, i.e., phenolics. Consequently, using different fractions of *N. mirabilis* digestive juices to pre-treat mixed agrowaste of a particular particle size, has the potential to produce fermentable sugars and subsequently other value-added products, a strategy which can be profitable in biorefineries.

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