Sustainable *Nepenthes mirabilis* Facilitated Recovery of Reducing Sugars from Grape Pomace

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Grape pomace (GP) generated from the winery industry is one of the abundant agro-waste in the Western Cape, South Africa and other regions globally. GP contains a significant quantity of holocelluloses that can be converted into fermentable total reducing sugars (TRS). This study reports on the recovery of TRS from GP treated *Nepenthes mirabilis* digestive fluids for mediated biovalorisation in comparison to a combination of conventional pretreatment methods (hot water pretreatment, dilute acid pretreatment, and cellulase pretreatment) in a single pot system. The recovery of TRS was facilitated while also reducing total residual phenolics (TRPCs) in the samples. Furthermore, powder X-ray diffraction (pXRD) was used to measure the crystallinity index and the functional groups of pre- and post-pre-treated GP were determined using Fourier-transform infrared spectroscopy (FTIR) to ascertain the efficiency of the pre-treatment methods, with quantification of lignin, holocellulose and ash being conducted. Overall, the TRS yield for *N. mirabilis* pre-treated agro-waste was 951±4.7 mg/L, with biomass having a lower CrI of 33%, and 62% residual lignin content. Furthermore, reduced TRPCs were observed in hydrolysate, suggesting limited inhibitory by-product formation during *N. mirabilis* pre-treatment.

Keywords: Agro-waste; Grape pomace; Nepenthes mirabilis; Total reducing sugars

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INTRODUCTION

The generation of waste is an indisputable part of human anthropogenic activity. As such, environmental pollution culminates as a result of waste landfilling, generating pollutant containing leachate (Chandrasekaran and Bahkali 2013; Liguori *et al*. 2013; Mirabella *et al*. 2014). Similarly, the dumping and landfilling of agro-waste in pristine environments is of concern. Overall, the repurposing and utilization of agro-waste is desirable and can limit its disposal, as such waste containing phenolics and other toxicants, which can pollute the environment.

Agro-waste contains micro- and macro-nutrients that can be used as a feedstock in the production of other value-added products by using environmentally benign processes. This can be achieved using numerous approaches that can include process integration, pre-treating the agro-waste appropriately for nutritional component extraction, exposing holocellulosic materials for further biodegradation, and use in downstream processes (fermentation) for high value bio-products production.

Studies have shown that many techniques are used to pre-treat holocelluloses embedded in agro-waste. There is a need to reduce the pre-treatment time to exploit the extraction of fermentable sugars as total reducible sugars (TRS), to reduce the energy intensity input, and to eliminate the use of synthetic chemicals, thus reducing residual toxicants and reducing operating costs (Lee *et al*. 2009). In addition, some important applications of TRS includes transformation of TRS in biorefineries for added value product production such as alcohols (Dlangamandla *et al.* 2019). Despite the fact that currently utilised pre-treatment processes are successful, the utilisation of chemicals, mechanised processes, high energy input systems, in combination with biological pre-treatment techniques, cannot yet be justified at an industrial scale even if they are either used as independent or inter-mixed processes (Lee *et al*. 2009; Cheng *et al*. 2012; Chiaramonti *et al*. 2012; Narayanaswamy *et al*. 2013; Kumar *et al*. 2016; Procentese *et al*. 2017) with numerous challenges being encountered.

Presently, agro-waste pretreatment is carried-out in a number of processes, in which deligno-cellulolysis of the waste to fermentable sugars is facilitated; albeit producing inhibitors associated with the souring of downstream fermentations including enzymatic hydrolysis. This requires the development of alternative and environmental benign holocelluloses valorisation methods for the pretreatment of agro-waste, for the production of value-added products, while limiting the production of toxicants from the lignin component of the waste which is a new promising alternative strategy towards the sustainable and efficient processing of numerous lignocellulose waste types (Chandrasekaran and Bahkali 2013; Nayak *et al*. 2016). Up to this point there has not yet been developed a single stage or a pretreatment reactor system and an ecologically benign method free of chemical use and high temperature for the pretreatment of agro-waste, to efficiently address challenges associated with delignification, cellulolysis and production of inhibitors which in turn affects the downstream process (Jönsson and Martín 2016).

In this research study, plant digestive fluids from *Nepenthes mirabilis* were shown to be effective in targeting holocelluloses extraction, with a significant proportion of residual lignin and ash being left behind. The plant digestive fluids contained digestive aliquots (peroxidases, nucleases, phosphatases, phospholipases, a glucanase, chitinases, and proteolytic enzymes) (Schulze *et al.* 2012; Buch *et al.* 2015), which have the potential to biodegrade complex and polymeric molecules (Chan *et al*. 2016). This proposed pretreatment method requires less energy, as it was operated at ambient temperature, and it eliminates the use of hazardous chemicals such as dilute inorganic acids; albeit the digestive fluid is acidic, with an added advantage of reducing the production of inhibitory compounds such as phenolics (Siragusa *et al*. 2007).

Therefore, the general aim of the study was to develop a suitable process for the holocellulosic extraction of grape pomace (GP) in a single reaction vessel, using *N. mirabilis* digestive fluids as pretreatment aliquots, to exploit the extraction of the TRS. This process was compared to commonly use pre-treatment method, using a single reaction vessel strategy.

The findings of the current study will contribute significantly in ensuring environmental and health safety, particularly whereby plant digestive enzymes are employed on agrowaste to produce fermentable TRS for the production of products such as ethanol and butanol.

EXPERIMENTAL

Grape Pomace (GP)

Collection and preparation

GP (*Vitis vinifera*) waste, was collected from ARC's Nietvoorbij experimental cellar farm (with permission), Stellenbosch, Cape Town (Western Cape, South Africa). The GP was immediately stored in a plastic bag and placed on ice prior to transportation, and subsequent to storage at -20 ℃. These samples were dried in an oven at 80 ℃ until a constant mass was achieved after 3 days. The samples were milled for 4 min to a powder form (>45 µm to<100 µm) using a ball mill without a pre-washing step. The samples were sieved for 12 min. A mass (2 g) of the milled GP was weighed and transferred into Scott bottles (200 mL) of sterile distilled water in a slurrification process (6 h).

Grape pomace lignin, holocellulose and ash content analyses

The total lignin content, *i.e.* the acid soluble lignin (ASL) and the acid insoluble residue/lignin (AIR), were quantified as residual lignin content of the un- and pre-treated GP. For this, a volume (1 mL) of 72% sulphuric acid was added to beakers containing the homogenised milled GP (300 mg) samples. The analyses were done using the Biorefinery Test Method (L 2:2016). The samples were stirred using a glass rod until the test samples began to dissolve (TAPPI 1991). A volume (28 mL) of sterile distilled water was added to the samples subsequent to placement in a water-bath (30 ℃) for 1 h. Thereafter, autoclavation at 15 psi and 121 ℃ was used for 1h subsequent to cooling to 80 ℃. The samples were each filtered using a pre-weighed compacted glass fibre filters. The ASL was then quantified by measuring the filtrate absorbance (205 nm) using the Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer, UK). The extinction coefficient of lignin to determine ASL in this method was as per TAPPI UM 250 method (TAPPI 1991) using Eq.1. The residual biomass on the filters was oven dried (105 ℃) for 2 h subsequent to reweighing. The AIR was then determined. Lastly the samples were ashed at 800 ℃ for 2 h using a furnace.

$$ASL = \frac{A.V}{\varepsilon.l.M} D_f.C_{GP} \quad (1)$$

Grape Pomace Pretreatment Using Conventional Methods

For comparative analysis of the new pretreatment method being developed, conventional methods, *i.e.* hot water, dilute acid, and commercial cellulase pretreatment (HWP/DAP/CP) methods were sequentially used in a single reaction vessel, *i.e.* using Schott bottles as prepared above, a process analogous to the proposed *N. mirabilis* pre-treatment process being developed herein. During HWP, samples were autoclaved at 121 ℃ for 15 min (Taherzadeh and Karimi 2008; Selvaraj and Vasan 2017), subsequent to sample cooling to ambient temperature. Subsequently, dilute sulphuric acid for DAP was added to the Schott bottles containing the samples to constitute a 1% (v/v) concentration, with heating being instituted for 30 min at 121 ℃ (Kootstra *et al*. 2009; Alvira *et al*. 2010; Idrees *et al*. 2013; Maurya *et al*. 2015). Thereafter, samples were also cooled to ambient temperature prior to the commencement of the CP.

Prior to CP, the samples pH (pH=4.5) was adjusted by using a sodium acetate buffer (pH 5.6) with a volume (600 µL) of commercial cellulases (25 U/mL) being added into the Schott bottles containing the samples. Heating (55 ℃) ensued thereafter for 72 h (Zeng *et al*. 2011). After this, the samples were cooled, subsequent to centrifugation at 4000 xg for

5 min, with total reducible sugars (TRS), total residual phenolic compounds (TRPCs), powder X-ray diffraction (pXRD), Fourier transform infrared Spectroscopy (FTIR) analyses being conducted thereafter, using liquid samples and the recovered residual GP biomass. This process was denoted the classification HWP/DAP/CP.

Grape Pomace Pre-treatment Using *N. mirabilis* Digestive Fluids

Nepenthes mirabilis: *Collection and preparation*

N. mirabilis digestive fluids were collected from Pan's Carnivores Plant Nursery (Tokai, Cape Town, South Africa). The pitcher plants were grown in a greenhouse under controlled environmental conditions. The digestive fluids were collected from both open and closed pitcher plant cups and pooled to form a composite sample. The digestive fluids were carefully collected into sterile 50 mL conical tubes and were immediately stored in ice prior to transportation to the laboratory. Thereafter, the digestive fluids were filtered (0.2 µm) and stored at 4 °C. Up to 15 to 35 mL of *N. mirabilis* digestive fluids can be collected from each monkey cup depending on the sizes of the cups (Hua and Li 2005).

N. mirabilis digestive fluid physico-chemical characteristics

Instruments used to measure the different physio-chemical characteristics of the digestive fluids from *N. mirabilis* cups were, a pH meter to measure both pH and redox potential, with the specific gravity being measured by using a hydrometer, while an electrical conductivity meter was used to measure the conductivity.

N. mirabilis digestive fluid microbial population

Prior to digestive fluid filtration, the fluid were analysed for microbial population proliferation using a VITEK 2 systems VO7:01 (BioMérieux, France), utilising gram-negative and positive cards (GN cards), as per the manufacturer instructions. DNA sequencing was further utilised to identify strains in the digestive fluid, which were not identified by the vitek system. Cultures were activated by adding a volume (1µL) of the *N. mirabilis* digestive fluids into 15 mL glass test tubes that contained Luria broth (5 mL) subsequent to incubation at 37 °C for 24 h. The cultures broth were then inoculated onto Luria Bertani agar (LBA) plates at 30 °C for 24 h, with pure cultures sub-cultured for species purification and identification. DNA was isolated from cultures using ZR Bacterial DNA kitTM (Zymo Research). PCR was performed by using DreamTaqTM DNA polymerase (Thermo ScientificTM) and primers (16S-27F 5' – AGAGTTTGAT-CMTGGCTCAG– 3' and 16S-1492R 5' – CGGTTACCTTGTTACGACTT – 3') were used to amplify the 16S rDNA target region. ZymocleanTM Gel DNA Recovery Kit (Zymo Research) was used to extract the PCR products (amplicons) from agarose gel, which was sequenced on the ABI PRISMTM 3500xl Genetic Analyser in both forward and reverse direction. The sequenced products were purified with ZR-96 DNA Sequencing Clean-up KitTM (Zymo Research) and analysed with CLC Main Workbench 7. A BLAST search was also done on the NCBI website.

Biocatalytic activities of N. mirabilis digestive fluid

Biocatalytic activity of enzymes of interest was undertaken based on their ability to degrade the agro-waste constituents including by-products formed, with the enzymes of interest being β-glucosidases, xylanases, and carboxylesterases determined earlier for their importance, and identified (Rottloff *et al.* 2016) as; 1) having an essential cellulose biodecomposing component for the biocatalytic conversion of cellobiose, which is a

reducing sugar; 2) having the capacity to biodegrade holocelluloses (García-Huante *et al*. 2017), and 3) having the potential for hydrolytic activity against carboxylester bonds between holocellulose bound sugars and lignin monomers (Manavalan *et al*. 2017). All assay mixtures were mixed in 3 mL Eppendorf tubes, prior to assay mixture transfer in to glass cuvettes for absorbance reading, in a kinetic mode.

Since the *N. mirabilis* pre-treatment was conducted at ambient temperature (25 °C), the enzyme activity quantification was also conducted at ambient temperature. The compound *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) was used as a substrate to measure the activity of β-glucosidase in the *N. mirabilis* digestive fluids (Kim *et al*. 2012; Bailey *et al*. 1992), with the rate of formation of xylose from endo-xylan being determined as an indication of xylanase (Sudffldt *et al.* 1990). Additionally, *p*-nitrophenyl acetate (*p*NPA) was used as a substrate to determine carboxylesterase activity. Overall, the *N. mirabilis* digestive fluids were used to perform the above assays in buffers (i.e. Sodium acetate (pH 6), Citrate (pH 5) and mM Tris-HCl (pH 7.8) at ambient temperature, using a Jenway 6405 UV/Vis spectrophotometer (Cole-Parmer, UK), with a temperature controlled cuvette holder, set in a kinetics mode (Ljungquist and Augustinsson 1971; Wheelock *et al*. 2001; Gilham and Lehner 2005; Brenda 2018). For β-glucosidase, the reaction mixture contained 600 µL 50 mM sodium acetate (pH 6) containing 0.35 mM *p*NPG, enzyme (200 µL) with the reaction being performed at 420 nm, using an extinction coefficient of 18100 $M^{-1}.cm^{-1}$ to monitor the product produced, *i.e*. *ρ*-nitrophenol (*p*NP) (Kim *et al*. 2012; Bailey *et al*. 1992). Similarly, the activity of xylanase was determined using a reaction mixture constituted by 100 mM Mcllvaine's citrate phosphate buffer (pH 5) in which 54.2 mM endo-xylan was dissolved, at 25 °C and 586 nm using an extinction coefficient of 135 $M^{-1}.cm^{-1}$ (Sudffldt *et al*. 1990) to quantify the product formed, *i.e*. xylose. To quantify the activity of carboxylesterases, the reaction mixture contained 200 mM Tris-HCl buffer (pH 7.8) with 0.5 mM *p*NPA as a substrate. The reaction was monitored at 410 nm, using extinction coefficient of 17000 $M^{-1}.cm^{-1}$ for the product formed, i.e. ^{p}NP, (Ljungquist and Augustinsson 1971; Wheelock *et al.* 2001; Gilham and Lehner 2005; Brenda 2018). The enzyme activity was quantified using Eq. 2.

$$Enzyme\ activity\ (U/L) = \frac{dA/dt}{\varepsilon}.Df \rightharpoonup .600 \quad (2)$$

Grape pomace pre-treatment

The agro-waste was allowed to initially undergo a slurrification process at 25 °C for 72 h. The first sample was taken at 6 h to quantify readily dissolvable sugars in the form of TRS from the unwashed slurried GP prior to the direct supplementation of the *N. mirabilis* digestive fluids (1%) into each of the Erlenmeyer flasks used, a process analogous to when a 1% DAP is implemented. The flasks were incubated in a shaking incubator (120 rpm) at 25 °C, with sampling aseptically conducted under the laminar flow hood after 6, 72, 96, and 120 h. The final mass after pre-treatment was 86 mg. The TRPCs, were measured alongside the TRS; afterward, the residual GP biomass was recovered by centrifugation (4000 g, 5 min, for 5 days) and oven dried (80 °C) to reduce the moisture content prior to the evaluation of structural deformation using Fourier transform infrared Spectroscopy (FTIR) and powder X-ray diffraction (XRD) systems. The HWP/DAP/CP samples were used as a reference to compare the efficacy of the *N. mirabilis* (NmGP) treated samples. All biomass samples were mixed to form a composite sample for XRD and FTIR analyses.

Quantification

Quantification of total reducible sugars (TRS)

TRS during the pretreatment of GP was measured using a dinitrosalicylic (DNS) based method, with 2 g/200 mL concentration for GP samples. The assay mixture was made up of DNS (10 g), phenol (2 g), sodium sulphite (0.5 g), and sodium hydroxide (10 g), in 1000 mL of sterile distilled water. Equivalent volumes (1.5 mL) of the diluted samples (1:9) and the DNS mixture were transferred into sterile test tubes and heated in a water bath for 10 min at 90 °C, subsequent to cooling to ambient temperature. Thereafter, a 40% (v/v) sodium potassium tartrate (0.5 mL) solution was added into the test tubes. Subsequently, the TRS concentration was analysed using a Jenway 7305 UV/Vis spectrophotometer (575nm Cole-Parmer, UK) (Dlangamandla *et al.* 2019) using a calibration curve developed by different glucose concentration standards (0 to 1000 mg/L), to quantify TRS at different sampling intervals. The control used was distilled water and DNS mixture without the diluted pretreatment samples.

Quantification of total residual phenolic compounds (TRPCs)

Agro-waste contains inhibitors known as phenolic compounds that are predominantly accountable for inhibiting the functionality of β-glucosidase (Kim *et al*. 2011), a key enzymatic agent which facilitates the breaking down of oligosaccharides to simpler sugars (Singhania *et al*. 2013). The Folin-Ciocalteu assay, composed of 15 g of lithium sulphate in 5 mL of water, and a drop of bromine, was used to quantify TRPCs in the samples (1:9) (Singleton *et al*. 1999; Ainsworth and Gillespie 2007; Blainski *et al*. 2013). A volume (100 µL) of the diluted samples was transferred into test tubes whereby a volume (250 µL) of the Folin-Ciocalteu reagent and a volume (1.5 mL) of sterile distilled water were added to each sample test tube allowing for 3 min reaction time to lapse, subsequent to addition of 20% (w/v) sodium carbonate (1mL). The assay mixture was kept in the dark for 1h prior to absorbance (650 nm) analyses using a Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer, UK). Thereafter, the TRPCs were quantified, using a calibration curve generated with 2 to 10 mg/L of 1,2-dihydroxybenzene, a hydroxylation by-product of phenol.

Powder X-ray diffraction (pXRD) and Fourier Transform Infrared Spectroscopy (FTIR) analysis

The pooled residual GP biomass obtained pre- and post- pre-treatment were oven dried (80 °C for 24 h) and the crystallinity index (*CrI*%) was quantified. This was done using a pXRD (Bruker Pty Ltd, SA) at 40 kV and 40 Ma with a D2 phaser with a Lynxeye, which provided a suitable peak-to-background ratio. The scanning range (2θ) was 10 to 50° at a ramping scale 0.017°, using a zero background holder plate (50 µm depth), with the crystallinity index (*CrI* %) being determined using Eq. 2. A mass of (5 mg) of biomass as indicated in Eq. 3,

$$CrI(\%) = \frac{\Delta I}{I002}.100 \quad (3)$$

where ΔI is $I_{002} - I_{am}$ with I_{002} being an intensity for portion of the agro-waste at a 2θ of 21°, while I_{am} is the peak of the agro-waste at a 2θ of 18°.

Furthermore, to ascertain the efficiency of the pre-treatment method developed, an α-FTIR spectrometer (Bruker Pty Ltd, SA) and smart iTR with a diamond crystal window was used to measure the organic, polymeric, and in some cases, inorganic materials

including functional groups in the untreated and pre-treated GP. First the measurements were taken against a background spectrum of the diamond window without the agro-waste sample. A scan range of 400 to 4000 cm^{-1} at a spectral resolution of 4 cm^{-1} was used, at 100 scans per min (Zeng *et al*. 2011).

Experimental Data Handling, Computations and Statistical Evaluation

The pretreatment of GP was done with the total experimental time being 120 h with recurring sampling being at 6, 72, 96, and 120 h to perform different analyses. The supplementation of *N. mirabilis* digestive fluids was at 52 h after determining that readily dissolvable TRS concentration solubilised in the sterile distilled water stabilised at 52 to 72 h, and the experiment was terminated at 120 h. The mean value, and standard error of the mean (SEM) was determined (Eq. 4) from the data produced from the various analyses. Experimental data were all computed in order to take into account sample dilutions, which were used to measure the actual concentrations for parameters monitored. All analyses where performed in triplicates.

$$SEM = \frac{\text{Standard Deviation}}{\sqrt{\text{Number of samples tested}}} \quad (4)$$

RESULTS AND DISCUSSION

Selection of Grape Pomace (GP) and its Constituents

From an availability perspective, GP is a regionally available feedstock, as in South Africa more than 80% of table grapes are produced in the Western Cape region of South Africa (Daff *et al*. 2012). Research has shown that GP (*Vitis vinifera* waste) encompasses adequate quantities of readily dissolvable and fermentable sugars. These readily dissolvable sugars (rdTRS) are free soluble sugars that dissolve into solution during the slurrification process without any pretreatment method being implemented on the GP. The results seen after 6 h shows that the UGP released some quantity of free sugars greater than the previously reported *Phanerochaete chrysosporium* pre-treated GP (Angadam *et al*. 2018). A stabilised concentration in the free sugars was seen after 48 h, culminating in the supplementation of *N. mirabilis* digestive fluids, after 52 h. After 72 h, the fermentable sugars concentration further increased.

The residual lignin, holocellulose, and ash content of different pre-treated GP samples are also shown in Table 1 with UGP being determined to have one of the highest content of holocelluloses (63.8%) in comparison to other agro-waste, *e.g.* rice straw (62.7%) (Montusiewicz *et al*. 2017). Generally, softwood lignin content ranges from 30 to 60% with ash content being up to 0.50 %, which is similar to values observed for the UGP.

Table 1. Residual Lignin, Holocellulose and Ash Content in Different GP samples

	Residual Lignin (%)	Residual Holocellulose (%)	Ash (%)
UGP (Untreated grape pomace)	36	63.8	0.2
NmGP (*Nepenthes mirabilis* bio-treated grape pomace)	62	38	0.02
HWP/DAP/CP	75	24.6	0.4

For the single reaction vessel HWP/DAP/CP pre-treatment system, significant changes in the organization of the holocelluloses in the residual GP biomass occurred, with a relatively higher residual lignin being observed in comparison to NmGP samples. Similarly, relatively higher residual lignin was also reported by Angadam *et al*. (2018) in *Phanerochaete chrysosporium* treated samples. These levels may be due to pre-treatments with *Phanerochaete chrysosporium*, the conventional methods (HWP/DAP/CP), and *N. mirabilis.*

In the reference materials, a high content of ash is observed in lignin dominant samples than in sample with high hemicellulose and cellulose. This suggest that greater residual lignin than hemicellulose/cellulose can be achieved (Stefanidis *et al*. 2014). Elsewhere, the Klason lignin 45 to 300 (g kg^{-1}) and residual lignin 27 to 59 (%) were obtained (Okeke and Obi 1994; Dlangamandla *et al*. 2018).

Direct Comparative Analysis of TRS Produced Using Conventional and *N. mirabilis* Pre-treatment Process in a Single Reaction Vessel

The agro-waste used was milled, which is another form of pretreatment which alters the structure of the GP, thus the holocelluloses crystallinity index (Taherzadeh and Karimi 2008; Maurya *et al*. 2015). However, the pretreatment of agro-waste into fermentable sugars using the HWP/DAP/CP has been widely researched (Alvira *et al*. 2010; Idrees *et al*. 2013); albeit in a single reaction vessel. The comparison of combined common pretreatment, *i.e.* HWP/DAP/CP used sequentially to pre-treat GP, was directly compared to the pre-treatment method for NmGP.

HWP assists in the delignifying of biomass and the partial break-down of the holocelluloses, making it easier for improved holocellulolysis efficiency, broadening the penetrability and vulnerability of the biomass surface area to cellulases hydrolysis (Taherzadeh and Karimi 2008; Selvaraj and Vasan 2017). Furthermore, one of the successful methods used to delignify agro-waste components to fermentable sugars is using dilute sulphuric acid (DAP) with 1% (v/v) (Alvira *et al*. 2010; Idrees *et al*. 2013). Similarly to the HWP, the function of the DAP in biomass pretreatment is also to delignify and partially solubilize holocelluloses using a high temperature. At a higher temperature, dilute acid can degrade lignin into furfural and other components such as phenolics (Kootstra *et al*. 2009; Alvira *et al*. 2010; Idrees *et al*. 2013; Maurya *et al*. 2015). Previously, it was determined that the DAP releases more fermentable sugars as compared to the HWP when used as a stand-alone process, a phenomena evidenced in this study probably because of the corrosiveness of the acid used, which would lessen the integrated bondage mechanism between holocelluloses and lignin, which in turn makes it easier for the hydrolysis of the polymers of the biomass, resulting in more fermentable sugars extraction. This type of pretreatment has some limitations, which include production of phenolic compounds and causes corrosion to equipment. By subsequently applying cellulases post DAP, further holocellulolysis is facilitated by a cocktail of enzymes constituted primarily by endoglucanases, cellobiohydrolases (exoglucanases), and β-glucosidases. The HWP/DAP/CP has disadvantages as individual processes and when they are used sequentially in single reaction vessels. Consequently, there is a need for more environmental benign and efficient processes such as the NmGP method evaluated. Numerous pretreatment practices are designed to alter the physical and chemical composition of the lignocellulosic biomass, albeit producing toxicants, which reduce hydrolysis rates and efficiency of other downstream fermentation processes (Zeng *et al*. 2011). The results reported herein indicated that the combination of HWP and DAP indeed

made it easier for the CP to effectively hydrolyse the holocellulose. Hence there was a better TRS production result, albeit with higher residual TRPCs -see Table 1. In a single reaction system for conventional (HWP/DAP/CP) method, 3269 ±8.054 mg/L of TRS was recorded when the untreated grape pomace (UGP) was used as a feedstock. On the other hand, total reducible sugars recorded for the *N. mirabilis* digestive fluids treated GP (NmGP) were found to be 951 ±4.7 mg/L, albeit with: 1) reduced TRPCs and 2) without energy usage, i.e. at ambient temperature - see Fig. 1. From the results obtained, it can be deduced that HWP/DAP/CP system performed better than the *N. mirabilis* pretreatment, but the NmGP can be an alternative green method for pre-treating agrowaste for TRS extraction, specifically for targeted holocellulose biovalorisation.

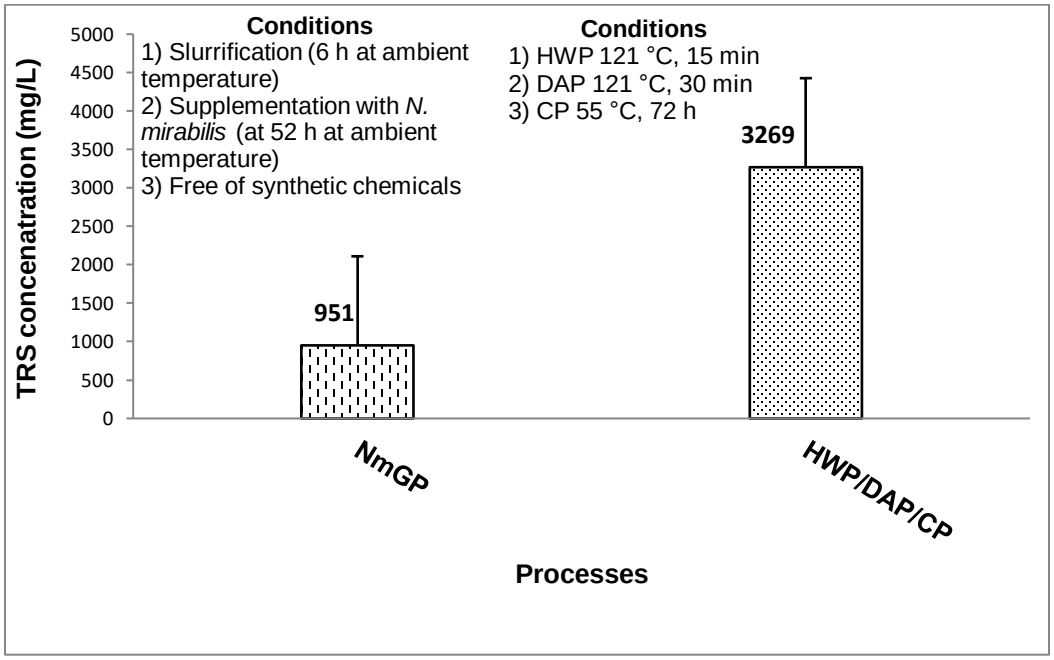

Fig. 1. Direct comparison of pre-treatment processes in a single reaction vessel between the conventional pretreatment methods (HWP/DAP/CP) and the proposed pretreatment method (NmGP), for the production of TRS

Physico-chemical characteristics indicated that the *N. mirabilis* digestive fluids had a pH of 2 (±0.12) (Chou *et al.* 2014), with a specific gravity around 0.745 (±0.04). Furthermore, the redox potential was 519 (±4.04 mV), indicating the oxidative potential of the digestive fluids, at a conductivity averaging 4.695 (±0.69) mS/m, which was relatively high when, compare to that of water, *i.e.* 1.75 (±0.72) mS/cm. Similarly, a 1% (v/v) dilute sulphuric acid, has a pH of 1.65, a redox potential of 681.6 mV, and has a conductivity of 18.23 mS/cm. Generally, these physico-chemical characteristics of the dilute acid used in the DAP, are analogous to those observed for the digestive fluids of *N. mirabilis.*

N. mirabilis Digestive Fluid Characteristics and Microbial Population

In order to evaluate the ability of *N. mirabilis* digestive fluids to degrade the GP, several parameters were evaluated to determine the reduction and the oxidative reaction potential of the *N. mirabilis* digestive fluids. High redox potential means the solution has a high electron accepting tendency. Some of the oxidative reactions might be facilitated by

extracellular bio-products of microorganisms with the digestive fluids of the *N. mirabilis*. This necessitating the identification of the microbial population with the digestive fluids used for NmGP.

Microbial identification and isolation including DNA analysis was done on the *N. mirabilis* digestive fluids to identified different species proliferating in the fluids that included *Bacillus spp*. and *Klebsiella oxytoca* identified *via* 16S rDNA sequencing. The NmGP pre-treatments had a relatively lower residual lignin (62%) and lower ash content (0.02%) compared to the conventional (HWP/DAP/CP), which had the highest ash content (0.4%) and residual lignin (75%) (Table 1). Previously, Klason lignin ranging from 45 to 300 (g kg^{-1}) and residual lignin ranging from 27 to 39% were also observed after hydrolysis (Okeke and Obi 1994). The *Bacillus* spp. were identified as *Bacillus cereus, Bacillus thuringiensis*, and *Bacillus anthracis* with accession numbers KY249126.1, DQ513324.1, and KU948294.1, respectively. All the species were identified as being prevalent in *N. mirabilis* digestive fluids (Chan *et al*. 2016). A confirmation of homology was previously conducted for these isolates and an overall similarity of more than 71.1 percent has been reported previously (Zhong *et al.* 2007). Furthermore, the enzyme activity assays for the *N. mirabilis* digestive fluids contained carboxylesterase, (1260 ±6.63 U/L), β-glucosidase (2645 ±17.647 U/L), and xylanase (360 ±10.418 U/L). These enzymes all have the biocatalytic ability to decompose agro-waste (Zabed *et al*. 2016).

Determination of Total Residual Phenolic Compounds (TRPCs)

Phenolics are one of the known inhibitory compounds in delignification of biomass for TRS extraction, with the inhibition of β-glucosidase being among the most detrimental in biorefineries; hence, the TRPCs quantification. TRPCs were measured at 6, 72, and 120 h for 1) UGP (control), 2) *N. mirabilis*, and 3) HWP/DAP/CP (used as reference) pre-treatment systems in a single reaction vessel system. Observations showed that the TRPCs were lower (2.57 mg/L) compared to the previously reported *Phanerochaete chrysosporium* NmBT (GP) sample (24.3 mg/L) (Angadam *et al*. 2018). On the other hand, the TRPC concentrations for HWP/DAP/CP (2.24 mg/L) and UGP samples (2.16 mg/L) were higher than the NmGP treated samples (1.54 mg/L) after operating the reaction vessels for 120 h. There was an indication that the *N. mirabilis* digestive fluids had the capacity to reduce inhibitants such as phenolics (Table 2), which were lower at the end of the experimentation method (up to 1.54 mg/L). The use of *N. mirabilis* digestive fluids for biological pretreatment of agro-waste was presumed to have the ability to reduce TRPCs formation at ambient temperature (Table 2).

Table 2. TRPCs during Conventional (HWP/DAP/CP), *N. mirabilis* (NmGP) Pretreatment Methods

		TRPCs (mg/L)±SEM
Process/Sample type	Time (h)	
Slurrification	6	2.42 ±0.04
N. mirabilis	72	2.57±0.10
End	120	1.54±0.05
Control Experiments After 120 h		TRPCs (mg/L)±SEM
UGP		2.16±0.02
HWP/DAP/CP		2.24 ±0.33

Powder X-ray and FTIR Evaluation of Grape Pomace

The effects of the pretreatment were further quantified by performing pXRD analysis prior and post pretreatment, to quantify the crystallinity index of the agro-waste samples. The deformation of the crystalline structures can be seen in the reduced crystallinity of biomass structures, into an amorphous phase. The untreated sample (UGP) showed a crystallinity index (*CrI*) of 35% while other pre-treated samples had the following *CrI* (%) order: HWP/DAP/CP - 42% > NmGP – 36.6%. In biological systems, the impact of a high crystallinity might eventually affect biological agent efficacy, especially whereby biodecomposition *via* an enzymatic hydrolysis mechanism is required. The increase in *CrI* values as the pretreatment progresses indicated the removal of more holocelluloses leaving the lignin and ash fraction intact in the pre-treated solid residues (Timung *et al*. 2016).

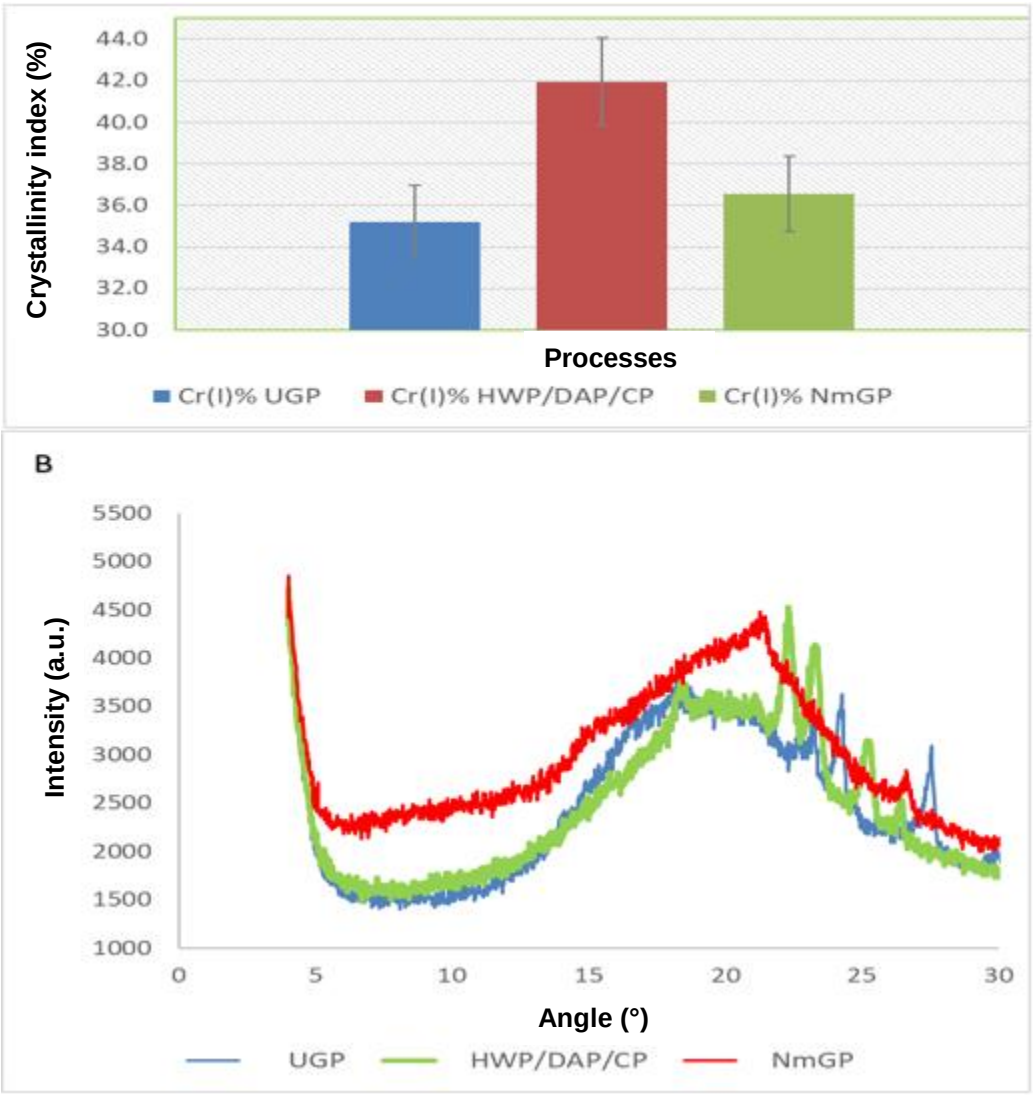

Fig. 2. Graphical representation of the crystallinity index of the different pretreatment methods (a) and the pXRD patterns (b)

Similarly, FTIR (Fig. 3) was used to quantify the structural changes pre- and post-pretreatment. A broad peak was observed in the region of 3312 cm^{-1} for all the pretreatment

processes. These peaks are associated to the O-H group region of the FTIR spectrum. Also peaks such as 2927 cm^{-1} (C-H stretching), 1745 cm^{-1}, (CH_2/-SH/cellulose, C-H stretch, S-H stretch, C-H stretch associated with cellulose), 1605 cm^{-1} (N-H, CH_2, carbonyl stretching with aromatic rings, O-H stretch, C-H stretch all associated with aromatic lignin), 1440 cm^{-1} (C-H, xylan C-O-C contribution), and 1030 cm^{-1} (C-O stretching vibration) were identified (Pavia *et al*. 2014). The prominent peak at 950 to 1182 cm^{-1} is assigned to C-O, C-C, and C-OH bends in xylan, albeit overlapping with respect to C-O stretching at C-3 and C-O stretching at C-6 associated with cellulose. The FTIR spectra of cellulose and hemicellulose as constituents of holocellulosic material is very similar (Xu *et al*. 2013). In previous studies, the bands formed at 895 to 897 cm^{-1} have been allocated as β-glucosidic bonds (Kim *et al*. 2007; Liu and Kim 2017; Kunusa *et al*. 2018), which were observed at wave number 896 cm^{-1} in this study; albeit they were not more pronounced in the HWP/DAP/CP and NmGP samples. NmGP samples had very high transmittance, which was indicative of a clustering of unexposed bonds, with pronounced inverted peaks at 1500, 1700, 3000 cm^{-1}, related to emissions and lattice-bond strains.

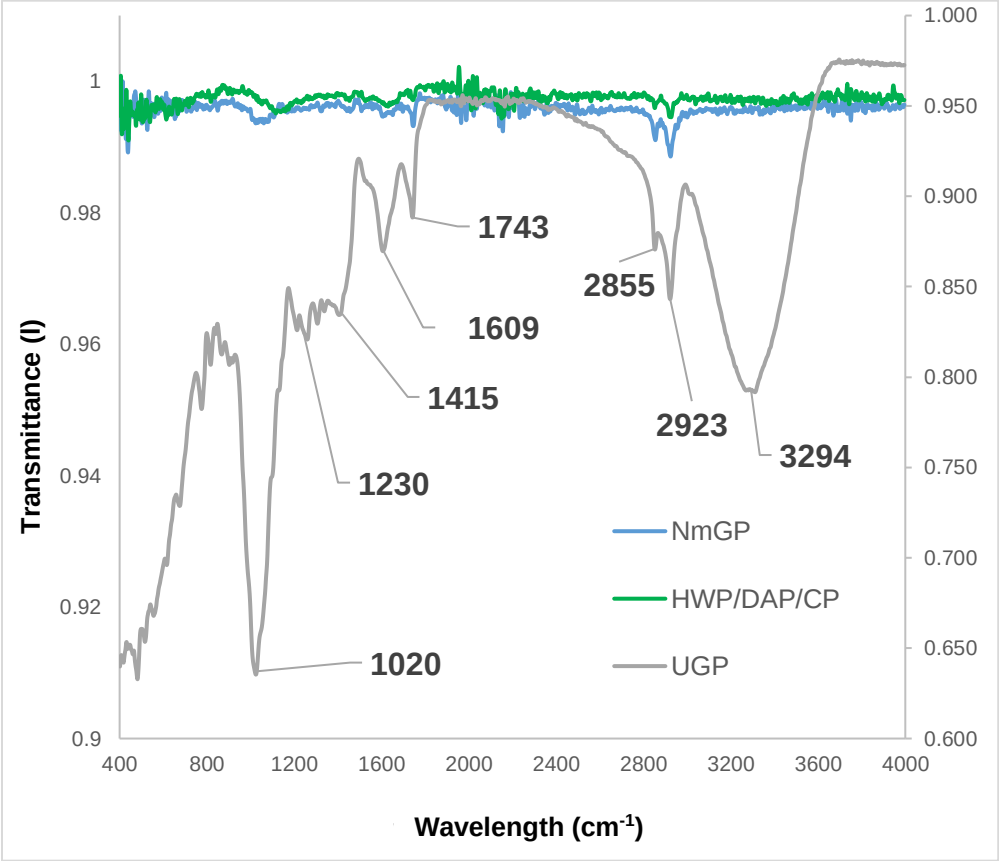

Fig. 3. The FTIR representation for UGP, NmGP and HWP/DAP/CP

CONCLUSIONS

1. The results of this study have shown that *N. mirabilis* digestive fluids contains enzymes that have the ability to biodegrade holocellulose in agro-waste without heating.

2. TRS produced by the conventional methods (HWP/DAP/CP) in a single reaction vessel was higher than that produced by *N. mirabilis* digestive fluids at ambient temperature.
3. Powder XRD diffraction and FTIR were also performed prior and post pretreatment methods to further quantify the efficiency of *N. mirabilis* digestive fluids.
4. FTIR also confirmed the distortion of the GP during the various pretreatment methods.
5. *N. mirabilis* pre-treated agro-waste yielded 951±4.666 mg/L of TRS.
6. Reduced TRPCs were observed in extractants, suggesting limited inhibitory by-product formation during *N. mirabilis* pre-treatment.
7. Optimisation of the process conditions should be done in future studies including fermentations to ascertain whether the fermentation media constituted with *N. mirabilis* pre-treated agro-waste hydrolysates.

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