Phanerochaete Chrysosporium Supported Biovalorisation of Grape Pomace for Hyper Reducible Sugar Extraction

J.O. Angadam, S.K.O. Ntwampe* and N. Dlangamandla

Abstract **—In the Western Cape, South Africa and other regions globally, grape pomace (GP) is one of the abundant agro-waste from the winery industry. This study reports on the hyper-extraction of fermentable sugars from GP treated with white rot fungi (WRF) *Phanerochaete chrysosporium* BKMF 1767 to facilitate improved biovalorisation for total reducing sugars (TRS) extraction. TRS were quantified using the 3,5-dinitrosalicylic acid (DNS) reagent method. The free readily dissolvable sugars from the GP recorded for the bio-treated (BT) samples was 206.39 ± 0.06 mg/L and for the untreated (UT) samples was 271.05 ± 0.02 mg/L. Overall, the TRS yield for the Bio-treated (BT) and untreated (UT) samples was recorded as 205.68 ± 0.09 and 380.93 ± 0.14 mg/L, respectively, using hot water pretreatment (HWP) with 2266.00 ± 0.73 (BT) and 2850.68 ± 0.31 mg/L (UT), respectively, for dilute acid pretreatment (DAP); with 2068.49 ± 6.02 (BT) and 2969.61 ± 8.054 mg/L (UT) respectively, using the cellulase pretreatment (CP) method. Using the HWP as a reference, the relative increases imparted by the bio-treatment was higher (51%) for DAP and low (33%) for CP.**

Keywords—Agro-waste, Grape pomace, *Phanerochaete chrysosporium*, Total reducing sugars.

I. Introduction

There are bountiful, inexpensive and renewable lignocellulosic biomass being landfilled continuously by the winery industry. The winery and juicing industries are among the profitable and substantial agro-economic operations worldwide, processing a variety of grape berries. After the extraction of the juice from grapes, approximately 20% end-up as grape pomace (GP) containing skins, seeds, and stems-major components of holocellulose, which are landfilled, culminating in growing environmental pollution concerns [1]. Instead of land filling, this GP can be used as animal feed or as a source of fermentable sugars [2, 3]. If the GP is not treated or handled accordingly, it can result in deleterious environmental pollution challenges including release of extracts which will seep into ground-water bodies; this being among the challenges identified [4]. Thus, the repurposing of GP, will serve as a means to remove waste containing phenolics and other potential toxicants from the environment and also as an alternative nutrient source for the production of value-added products [5].

GP is composed mostly of holocellulosic material which is categorized as a key source of fermentable sugars for the production of value-added products [6]; albeit, it is challenging to degrade because of the presence of lignin [7]. The effect of milling and biological pre-treatment (bio-treatment) on fermentable sugar extraction from such GP has not been studied effectively. Some pretreatment methods, can be used for fermentable sugar extraction, with hot water, dilute sulphuric acid and cellulase pretreatment methods being preferred [8-11]. Recent studies have shown that, pre-treatment techniques used to pre-treat lignocellulosic biomass encompassing agro-waste, are making emphases on the reduced pre-treatment time, exploiting the removal of fermentable sugars while reducing energy intensity usage, including the utilization of environmentally benign processes by eliminating the use of chemicals and at reduced operating cost [12]. These processes can be utilized either as independent or as combined processes [13-19].

This research presents the effect of bio-pretreatment using *Phanerochaete chrysosporium* BKMF1767 on different commonly used pretreatments methods for hyper-extraction of TRS from GP, since they are deemed to be easily fermentable.

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II. Materials and Methods

A. Collection and Preparation: Grape Pomace as a Holocellulosic Biomass Feedstock

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 ºC. The samples were thawed prior to drying in an oven at 80°C for 3days. The samples were milled to a powder using a ball mill to >45µm <100µm without a pre-washing step. A mass (10g) of the milled sample was weighed and mixed with *Phanerochaete chrysosporium* BKMF1767 inoculum (10% v/v) grown in agar plates as described and highlighted in Ntwampe [20] and placed in an incubator for 7days. Mass (2g) of the bio-treated and an untreated samples, were weighed and transferred into Schott bottles. A volume (200mL) of sterile distilled water was added to the samples for slurrification and readily dissolvable sugars were measured after 6h. This was followed by HWP.

B. Hot Water Pretreatment (HWP)

The samples were then autoclaved at 121ºC for 15mins. The samples were cooled and samples (6mL) from a homogenous mixture were centrifuged at 4000xg for 5mins. A volume sample (1mL) was then added to distilled water (9mL) for dilution. The essay mixture was composed of equivalent aliquots of the diluted sample and DNS (1,5mL) in sterile test tubes, with a 7305 UV/Vis spectrometer (Cole-Parmer, UK) being used to measure the absorbance at 575nm; hence, TRS quantification.

C. Dilute Acid Pretreatment (DAP)

DAP commenced immediately after HWP. A volume (1mL) of 1% (v/v) dilute sulphuric acid was added to the Schott bottles containing the samples. It was heated for 30mins at 121ºC. The samples were cooled subsequent to centrifuging at 4000xg for 5mins. A volume of samples (1mL) was then added to distilled water (9mL) for dilution. Thereafter, TRS was quantified.

D. Cellulase Pretreatment (CP)

After the DAP process, CP commenced. The pH of the samples was adjusted to 4.5 using sodium acetate buffer (pH=5.6). A volume 600uL/g waste of commercial cellulase (24.67U/mL) was pipetted into the Schott bottles containing the samples. It was heated to 55ºC for 72h. Thereafter, TRS was quantified and some of the filtrate was stored at -20 ºC for future use.

E. Quantification of Total Reducible Sugars (TRS)

The TRS that were extracted during the pretreatment of the GP was measured using a DNS based method, with the assay mixture being composed of DNS (10g), phenol (2g), sodium sulphite (0.5g) and sodium hydroxide (10g), made-up to 1000mL using sterile distilled water [21]. Aliquots of the diluted sample and DNS (1,5mL) was transferred into sterile test tubes and heated in a water bath for 10mins at 90ºC, subsequent to cooling to ambient temperature followed by the addition of a 40%(v/v) sodium potassium tartrate (0.5mL) solution into test tubes for TRS concentration analyses using a spectrophotometer (575nm). Different glucose concentration standards (0-1000mg/L) were used to produce an appropriate calibration curve. The control was distilled water and DNS solution, without the diluted samples. Figure 1A to 1C, highlight the results of TRS concentration obtained using different pre-treatment methods.

III. Data Handling

A. Effectiveness of P. Chrysosporium Bio-treatment on TRS Extraction using Mild Acid and Cellulases

In order to assess the effectiveness of biologically pre-treating the GP, comparative absolute relative increases between mild acid and cellulase pre-treated GP was assessed using the HWP processes as a reference, as it had a minor or insignificant contribution to the TRS extracted from the GP. This was computed using Eq.1:

$$\%\ \text{Relative Increase} = \left(\frac{\Delta TRS_{CP}}{\Delta TRS_{DAP}} - 1\right) \text{X } 100 \quad (1)$$

Whereby ΔTRS for both cellulases (ΔTRS_{CP}) and mild acid (ΔTRS_{DAP}) pretreatment can be estimated using Eq. 2:

$$\Delta TRS_{CP\,/DAP} = \left(\Delta TRS^{*\backslash x}_{CP\,/DAP} - \Delta TRS^{*\backslash x}_{HWP}\right)/\Delta TRS^{*\backslash x}_{HWP} \quad (2)$$

With $*/x$ being ΔTRS for *P. chrysosporium* treated (*) or untreated GP (x). Standard deviation from a triplicate set of experiments was used in this study to account for variations in datasets.

IV. Results And Discussion

A. Selection of Agro-waste

Studies have identified holocellulosic materials as one of the major source of fermentable sugars for the production of bio-ethanol and other value-added products [6]. GP is a regionally available and inexpensive feedstock in the Western Cape; albeit, its availability is seasonal. Furthermore, although GP can be used to produce fermentable sugars, it does contain some inhibitory compounds such as ρ-coumaric, ferulic, acetic, glucuronic acids including furfural and phenolics, which are released during pre-treatment processes [22]. Alternatively, cellulases can reduce some of these fermentation inhibiting

by-products, while catalyzing fermentable sugar extraction from different agro-waste [23].

B. Total Readily Dissolvable Sugars

The freely dissolvable sugar obtained during GP slurrification for the untreated samples was 271.05 ± 0.02 mg/L and 206.39 ± 0.06 mg/L for the *P. chrysosporium* treated samples, respectively. This revealed that *P. chrysosporium* used some of the freely available sugars present in the slurrified samples thus the reduced quantity of the TRS at the initiation of the experiments with differentiation being determined to be miniscule in comparison to the TRS present in the untreated samples.

C. Hot Water Pretreatment (HWP)

This is a commonly used pretreatment method, with its function being to delignify/or loosen the holocellulose in order, to improve its penetrability during hydrolysis. Thermal methods broadens the penetrable and vulnerable surface area of densely, lignified biomass, for improved accessibility by hydrolytic enzymes [24, 25]. For HWP, the TRS concentration for the untreated sample was 380.93 ± 0.14 mg/L, with that observed for bio-treated samples being 205.68 ± 0.09 mg/L. Although this pretreatment method can be classified as advantageous due to non-use of chemicals including independence of the agro-waste particle size, it is however, energy intensive with a higher water requirement than some pretreatment methods and it produces some toxicants such as furfural and phenolics which can sour downstream processes [26, 27].

D. Dilute Acid Pretreatment (DAP)

One of the effective pretreatment methods used to delignify and solubilised agro-waste components to fermentable sugars is the dilute sulphuric acid pretreatment method. Its function is to solubilize hemicellulose to monosaccharides. At a higher temperature, sulphuric acid will also degrade xylose and some waste components into inhibitory compounds such as soluble lignin [8, 10, 22, 27, 28]. The yield for TRS was 2850.68 ± 0.31 mg/L for the untreated sample, with 2266.00 ± 0.73 mg/L being the concentration of TRS for the bio-treated samples. The acid pretreatment in combination with bio-treated samples released a higher concentration of fermentable sugars as compared to the HWP, probably due to the loosened hemicellulose and cellulose (structure) which culminated in the ease of the holocellulose decoupling. Although acid pretreatment is effective and produces a high yield of fermentable sugars, it also has some limitations which include corrosiveness and the degradation of xylose and glucose units at high temperatures [24, 27].

E. Cellulase Pretreatment (CP)

Cellulases are a cocktail of enzymes used to hydrolyze, hemicellulose and cellulose, and are constituted by endoglucanases, cellobiohydrolases (exoglucanases), and β-glucosidases [6]. By using cellulases, the delignified biomass structure, can be easily converted to disaccharides and further into fermentable monosaccharides. As there is a need for environmental benign, efficient, and inexpensive processes, cellulase for agro-waste pretreatment is deemed suitable. Numerous pretreatment practices are required to alter the physical and chemical composition of the lignocellulosic biomass, with enhanced hydrolysis rates being observed for biomass pre-treated with dilute acid prior to cellulase pre-treatment [29]; although, this can culminate into residual toxicants when compared to sole cellulase pre-treatment which produces less harmful by-products. By pretreating the agrowaste with hot water and dilute acid can facilitate efficient cellulase treatment to slacken hemicellulose and celluloses for efficient debonding by the cellulases. After hot water and acid pretreatment, cellulase pretreatment effectively produced 2969.61 ± 8.05 mg/L (untreated samples) and 2068.49 ± 6.02 mg/L TRS for the bio-treated samples. The cumulative TRS obtainable is represented by Eq. 3 (untreated) and Eq. 4 (bio-treated).

$$\Delta TRS_T = 6201.22 \pm 8.50 \text{ mg/L} \quad (3)$$

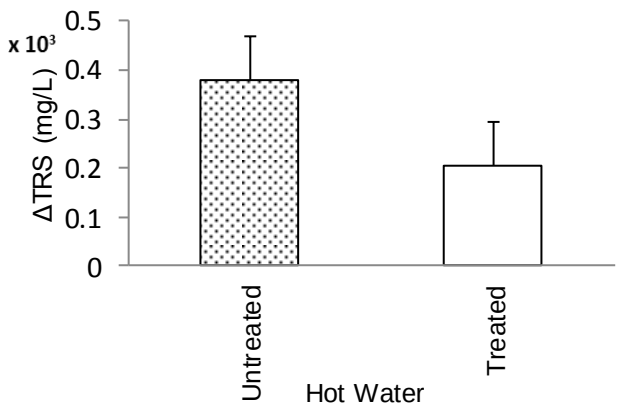
$$\Delta TRS_T = 4540.17 \pm 6.84 \text{ mg/L} \quad (4)$$

F. Relative Increases of TRS by Bio-pretreatment

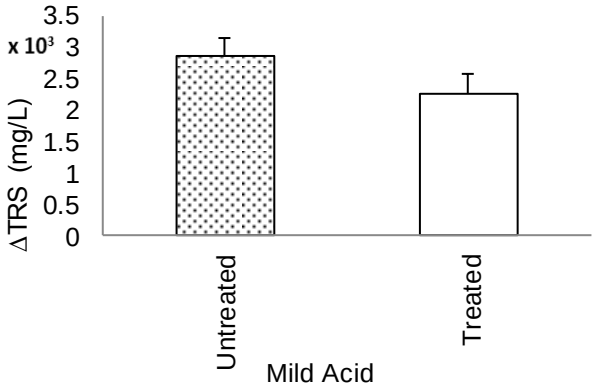
TABLE I: HIGHER RELATIVE INCREASES OF TRS BY BIO-TREATMENT

Pre-treatment methods	Untreated (%)	Treated (%)	Related Increases (%)
Mild acid	650	980	51
Cellulase	682	886	33

Table I, highlights relative increases of TRS. Relative increases are the difference in the change between the different pre-treatment processes for the untreated samples and bio-treated samples, using HWP as a reference.



A



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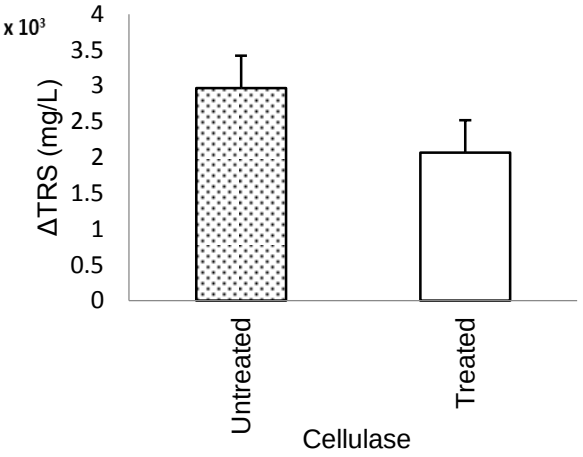

C

Fig 1: TRS results recorded for hot water (A), mild acid (B) and cellulase (C) pretreatments for the untreated and *P. chrysosporium* treated GP.

Overall, the relative increases for the bio-treated samples were higher than those of the untreated samples. This can be attributed to the fact that, the WRF helped in delignifying the samples; hence, making it easy for the pre-treatment procedures to perform optimally.

V. Conclusion

From the results obtained in this study, the cellulase pretreatment was determine to be an effective technique for the extraction of fermentable sugars from GP. This was seen in the TRS produced as 2969.61 ± 8.05 mg/L recorded for the untreated samples and 2068.49 ± 6.02 mg/L recorded for the *Phanerochaete chrysosporium* treated samples. Overall, cumulative TRS was 6201.22± 8.50 mg/L for untreated samples and 4540.17 ± 6.84 mg/L for treated sample. From the results of this research, it can be deduced that *Phanerochaete chrysosporium* used some of the readily available sugars in the samples for growth purposes. Although it contributed to higher relative increases for TRS extraction.

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