Enzyme hydrolysis is advantageous for it requires low energy and mild operational conditions, albeit, it can take extensive periods of time to achieve desired outcomes [11], and would include the high cost of enzymes to be used; however research is being done towards cost-effectiveness by improving enzyme efficiency on cellulolysis [18]. For enzyme hydrolysis, it is important to consider the components of the material to be hydrolyzed, the ratio of biomass to enzyme, type of pretreatment required and the activity of the enzyme used [15].

Fermentation technology is commonly used in the production of industrial enzymes and it may be done in one of two main methods: submerged fermentation (SmF) or solid-state fermentation (SSF). The former is performed by microorganisms in a liquid nutrient broth and the latter on a solid substrate. After fermentation, the enzymes are isolated through various recovery processes, particularly when they are extracellularly produced [19]. The aim of this study was to enrich the pretreatment of WSW for the production of biogas by exploring chemical and biological methods of pretreatment. The highlights include the importance of screening cellulose-degrading bacteria from Zebra (*Equus quagga burchelli*) manure and the use of white-rot-fungi, *P. chrysosporium*, for production of ligninase enzymes through submerged fermentation and of performing reducing sugars assays to evaluate treatment efficacy.

II. Materials and Methods

A. Sample Collection and Preparation

Fresh zebra (*Equus quagga burchelli*) droppings (ZD) were collected from Stellenbosch farm game reserve and winery solid waste (WSW) collected from Agricultural Research Council, Stellenbosch. The ZD was used as source for cellulose and lignin degrading bacteria. The samples were collected in sterile plastic bags and stored in a refrigerator (4˚C) prior to use. The WSW was analyzed for its compositions or constituents, while ZD was soaked in warm water, sieved and incubated at 37˚C (24 h) prior to use [6], [20], [21].

B. Isolation of Cellulose Degrading Bacteria (CDB)

Isolation of Cellulose Degrading Bacteria (CDB) was carried out according to the method of Sethi et al. [22] with modifications. A 1% (m/v) manure solution was prepared by diluting 1g of ZD into 100mL of sterile water. A dilution series (10^{-6}) was carried with 1mL from each tube being plated on Carboxymethylcellulose (CMC) agar (supplemented with 1M NaCl solution: 29.22g NaCl in 500mL of water) in triplicate subsequent to incubation (37^{o} C) for a few days, with growth being monitored daily. The experiment was conducted in duplicate and culture plates that showed most growth (mostly 10^{-3}) were flooded with iodine for 15 minutes and washed (n=3) with 1M NaCl solution. Colonies with a clear zone around it were selected and sub-cultured on CMC agar plates and incubated (37^{o} C) for 24 h.

C. Production of Cellulases

Cellulase production was done by means of SmF. Approximately 0.5 McFarland standard of CDB was grown on an enrichment medium (EM) containing (m/v); 0.03% $MgSO_4$, 0.2% K_2HPO_4, 1% glucose, and 0.25% NH_4SO_4 prepared in 1% (m/v) peptone water and incubated (37^{o} C) overnight. Cellulase was produced by inoculating 1mL of CBD onto 99mL of EM supplemented with 1% (m/v) WSW in place of glucose and incubated at 37^{o} C and 140 rpm for 24 h. Cellulase production was carried out in duplicate; a set was incubated in a shaker incubator and the other in a stationary incubator in order to assess the effect of mechanical agitation on enzyme production. After incubation, the broth was centrifuged at 14, 000 x g for 10 min at 4^{o} C to collect the enzyme solution [23].

D. Cellulase activity assay

Assay for cellulase activity (CA) was done according to Rathore [21] procedure with some modifications. CA was measured against the glucose standard curve at 575 nm wavelength based on enzyme dilution ratio (EDR) required to release 0.5 mg glucose (G) by plotting a graph of glucose liberated against enzyme concentration. CA was calculated using Eq. (1) (unit per liter; U/L):

$$CA = \frac{G}{EDR} \quad (1)$$

E. Production of Ligninases

Phanerochaete chrysosporium BKMF 1767 (ATCC 24725) culture was used for Ligninase production according to the methods described by Ntwampe et al. [20]. Lignin peroxidase activity (1 µmol veratryl alcohol oxidized to veratraldehyde per minute) was measured. Activity (U/L) was calculated every 10s using Eq. (s) (2) and (3):

$$Activity\left(\frac{U}{L}\right) = \left[\frac{dA}{dt} X \frac{Dilution\ factor}{\varepsilon}\right] 60\ X\ 10^{6} \quad (2)$$

$$Dilution\ factor = \frac{Total\ volume\ of\ solution}{Total\ volume\ of\ sample} \quad (3)$$

F. Alkaline Hydrogen Peroxide Pretreatment

WSW for biogas production was pretreated according to Cabrera et al. [24], with minor changes. A volume (50 mL) of H_2O_2 (7.5%) adjusted to pH of 11.5 using 5M NaOH was mixed with 3 g of dry WSW and heated in the water bath at 90^{o} C for 1 h. Samples with water only were used as negative control. The mixture was filtered with Whatman No. 1 filter paper and pH adjusted to neutral. Liquid sample was used for a reducing sugar assay as described by Miller [23], after adjustment to pH5 using 1M HCl. The solid residue was autoclaved, and dried in an incubator for 5-7 days.

G. Enzyme Hydrolysis

Enzyme hydrolysis was carried out on the AHP pretreated WSW samples according to Rathore [21] with some modifications. Acetate buffer (0.05M) was used to make an enzyme dilution of 2:10. Three sets of flasks were autoclaved and prepared in duplicates for the enzyme hydrolysis, along with controls, as shown in Table I.

TABLE I: STEPS IN ENZYME HYDROLYSIS OF WINERY SOLID WASTE

AHP pretreated WSW samples	Biomass (g)	Enzyme solution		Buffer (mL)
		Day 1	Day 4	
1	0.1	2mL ligninase	2mL cellulase	8
2	0.2	2mL ligninase	2mL cellulase	8
3	0.4	2mL ligninase	2mL cellulase	8
Control 1	0.1	1mL ligninase + 1 mL cellulase	No enzyme	8
Control 2	0.1	2mL ligninase	No enzyme	8
Control 3	0.1	2mL cellulase	No enzyme	8
Non-AHP pretreated	0.1	2mL ligninase	2mL cellulase.	8

Ligninase was added first to the sample and incubated at 35° C for 72 h. Lignin is the structure to be broken down before hemicellulose and cellulose hydrolysis. Samples were put in a water-bath at 90° C for 20 min, in order to deactivate active enzymes. Sugar reducing assay was done on samples before cellulase was added and further incubated using rotary incubator (60 rpm) at 50° C. After 4 days, all sample reactions were terminated by repeating the deactivation procedure of active enzymes, and the reducing sugars measured. Controls were made to determine the effect of each enzyme as well as that of the AHP pretreatment on hydrolysis. The concentration of reducing sugar (x) in the samples was determined from absorbance values (y) using the glucose standard curve –See Eq. (4):

$$y = 0.1952x + 0.0053; x = \frac{y}{0.1952} - 0.0053$$

$$y = \frac{Sample\ A + Sample\ B}{2} \quad (4)$$

III. Results

A. Characterization of Winery Solid Waste

The components of winery solid waste (WSW) used in this study were 95.92% and 83.86% of total solids and volatile solids, respectively. The main constituents were sodium (1191.9mg/kg), total carbon (50.40%), iron (28.05mg/kg), ash (15.95%) and protein (11%) as shown in Table II.

TABLE II: COMPOSITION OF WINERY SOLID WASTE

Characteristics	Unit	Dried winery waste
Moisture content	%	1.15
Total solids	%	95.92
Volatile solids	%	83.86
Protein	%	11
Total nitrogen	%	1.76
Total carbon	%	50.40
Ash	%	15.95
Calcium	%	0.06
Phosphorus	%	0.16
Potassium	%	1.77
Iron	mg/kg	28.05
Sodium	mg/kg	1191.9
Cyanide	mg/kg	0.92

B. Cellulose Degrading Bacteria

Cellulose degrading bacteria (CDB) successfully isolated from ZD were identified mainly as actinomycetes and heterotrophs (Fig. 2). In a bid to increase the yield of cellulase enzymes, the effect of mechanical agitation on their production from isolated CDB was assessed. The results showed a significant difference in absorbance at 575 nm between the agitated and non-agitated samples. The non-agitated samples had higher reducing sugars indicating a higher quantity and activity of the CDB than in agitated cultures, which were subsequently used for further enzyme assay (Table III).

TABLE III: ABSORBANCE VALUES (575 nm) OF CDB CULTURES DURING ENZYME PRODUCTION

Treatment	Samples	A_{575}	*SD*
Agitated CDB culture	A1	0.436	0.01
	A2	0.455	
	A3	0.421	
Non-agitated CDB Culture	B1	0.627*	0.00
	B2	-	
	B3	0.623	

* CDB supernatant with the highest absorbance was used for enzyme (cellulase) assay; -, discarded.

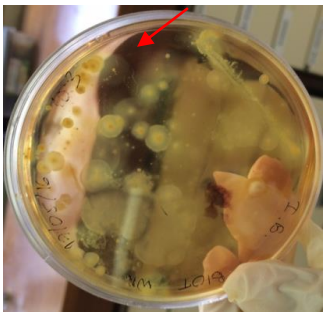

Fig. 2. CDB screening using Gram's iodine. Arrow indicates clear zone

around the colonies after applying dye

C. Production and Activity of Cellulase and Ligninase

The introduction of *Phanerochaete chrysosporium* activated a steady increase in enzyme (ligninase) production. The activity of cellulase produced during SmF of CDB is also shown in Table IV.

TABLE IV: CELLULASE AND LIGNINASE ACTIVITIES OF THE ISOLATED CDB AND *P. chrysosporium*

Cellulase activity (A_{575})			**Ligninase activity** (A_{310})
Enzyme dil.	**Absorbance**	**Glucose (mg/ml)**	**Activity (U/L)**
0.1	0.043	0.21	21.48
0.2	0.055	0.27	13.58
0.4	0.066	0.32	10.70
0.6	0.078	0.38	3.58
0.8	0.092	0.45	2.86
1.0	0.104	0.51	2.08

D. Effects of Pretreatment

WSW pretreated with alkaline hydrogen peroxide (AHP), yielded high amount of reducing sugar (1.900 mg/mL reducing sugars on average) which was indicated by an increased absorbance values of 2.121±0.22 (Mean: 0.1900) as shown in Table V.

TABLE V: ABSORBANCE VALUES OF RELEASED REDUCING SUGARS

Pretreated samples	**Absorbance (575 nm)**
Blank	0.00
1	1.851±0.05
2	1.730±0.171
3	2.121±0.22
Control (Water + AHP)	0.371
Mean	*0.1900*
Standard deviation (σ)	*0.1699*

E. Enzyme Cocktail Hydrolysis

The highest concentration (0.66 mg/mL) of reducing sugar was observed on day 4 when the cocktail of cellulase and ligninase enzymes (Table I) were used for hydrolysis, as opposed to the individual enzymes with lower concentrations (Fig. 4). There was a higher concentration of reducing sugar on day 7 for all samples except with the non-pretreated WSW biomass. The concentration of reducing sugar determined by means of the glucose standard curve linear equation is shown in Table VI.

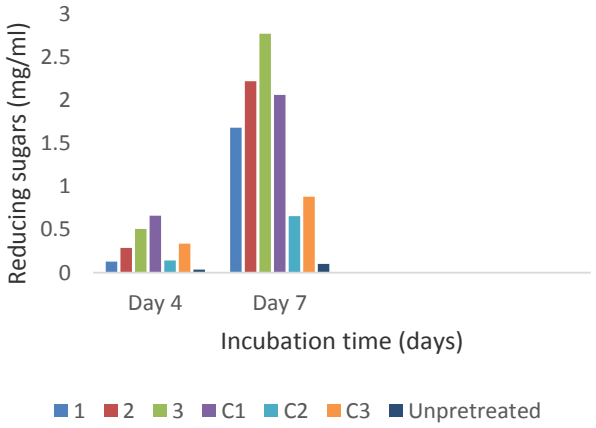

Fig.4. Reducing sugars yield over time during enzyme hydrolysis. Calculation of reducing sugars from absorbance values based on Glucose standard curve equation y = 0.1952x + 0.0053 e.g. x=0.130/0.1952-0.0053 = 0.660 mg/ml

TABLE VI: VALUES OF REDUCING SUGARS AFTER DAY 4 AND 7 PRETREATMENTS WITH ENZYMES

AHP pretreated WW samples	**Ligninase activity (day 4)**	**Cellulase activity (day 7)**
	Reducing sugar (mg/ml)	Reducing sugar (mg/ml)
1	0.128	1.68
2	0.287	2.22
3	0.506	2.77
Control 1	0.660	2.06
Control 2	0.143	0.656
Control 3	0.337	0.880
Non- pretreated	0.036	0.102

IV. DISCUSSION

The impact of agitation on cellulose production was measured based on enzyme activity and it was found that the activity was higher in stationary cultures than in agitated cultures. This may be due to the release of some certain compounds produced intracellularly, that inhibit enzyme productivity or shearing effects [25]. Although activity varies with different species, the activity of cellulase produced by CDB was considerably higher in comparison to the average activity in commercial enzyme mixtures produced using microorganisms such as *T. longibrachiatum* and *A. niger*, as stated by Verardi et al. [15]. Alternatives for better outcomes would include raising the temperature to 40° C and pH 10, as well as adding coconut cake, which was previously determined to act as a stimulus for cellulase production [22].

The ligninase activity was determined to be 21.48 U/L.

Consequential decrease in the activity of these enzymes would reduce the efficacy to degrade lignin. This could be due to influential factors such as the optimum C: N ratio alteration and insufficient exposure to dissolved oxygen [26]. Bioreactor operational conditions play an important role in enzyme activity [20].

AHP had a higher impact on the pretreated samples, attributed to a higher delignification efficacy of the WSW as opposed to the untreated samples. This meant that AHP method was very effective in treating WSW. Several authors have reported similar findings [27], [28]. However, the use of AHP will be disadvantageous for AD plants operation using WSW due to the cost involved in purchasing pretreatment chemicals, as it is advisable to use environmentally benign methods. The best approach will be to combine the use of limited amounts with enzyme hydrolysis in a bid to save cost; albeit AHP has a better outcome as pretreatment method of lignocellulose biomass than other pre-treatment methods such as ammonia fiber expansion (AFEX) since hydrogen peroxide dissociates into oxygen and water, which pose minimal threat to the environment [16], [29].

The two enzymes produced in this study are ligninases and cellulases. Enzyme activity assay carried-out indicated sufficient production for the WSW pretreated, as *Phanerochaete chrysosporium* (white-rot fungus) is widely known for the production of ligninases such as lignin peroxidases for the degradation of lignin [30]. The use of detergents such as Tween 80 or Tween 20 can minimize ligninases deactivation due to mechanical inactivation caused by agitation without any adverse effects on the enzyme production rate or its ability to catalyze reactions [31], [32].

Cellulases include a mixture of various enzymes, but there are three main types: β-1-4-endoglucanases which target areas of low crystallinity; β-1-4-exoglucanases or cellobiohydrolases (CBH) which remove cellobiose units and β-glucosidases (BG) that degrade the cellobiose into glucose [17].

Cellulase catalyzed hydrolysis showed successful conversion, with "Control 1" having the highest concentration of reducing sugars, meaning the enzyme cocktail containing both ligninases and cellulases worked better than when each enzyme was separately. This phenomenon is called synergism; however, the mechanism of action has still not well understood [33].

V. CONCLUSION

The pretreatment of WSW for the production of biogas was effective when carried out with AHP to solubilize lignin and decrystalinize cellulose in the lignocellulosic material, followed by saccharification in submerged fermentation, using an enzyme mixture of ligninases and cellulases. This is important in the biogas industry as the production process becomes much easier for the methanogenic microorganisms.

Of interest to the authors is measuring the percentage of lignin, hemicellulose and cellulose in the lignocellulosic material; running the same experiment using different identified CDBs found in the screening; purifying the enzymes produced, measuring reducing sugars over time as well as enzyme activity and finally using the produced sugars to generate biogas in an anaerobic digester. If this procedure and other forms of pretreatment are optimized to produce higher yields at a lower cost, it will facilitate the further development of a bio-refinery exclusively producing clean biogas for energy generation and bio-based products to boost the South African national bio-economy initiative.

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