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Cassava as feedstock for ethanol production in South Africa

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South Africa's economy is primarily coal-based, but the high ash content is a contributing factor to the high per capita production of green house gases. Rising crude oil prices, lower crop prices on world markets and the realisation that coal and oil are limiting energy resources has led to the decision to substitute a minimum of 2% of the country's transportation fuel with biomass based fuels. The biofuels industrial strategy of South Africa suggests the use of sugar based crops, but due to the tropical climate preferable for these crops, alternative crops need to be found that can be grown in the more arid and marginal parts of the country. Cassava *(Manihot esculent)* is rich in starch and is not a staple food in South Africa. It can be grown on marginal lands where frost is not prevalent. In this study, the production of ethanol from unpeeled Cassava roots and cassava peels were investigated. It was found that temperature; pH and biomass loading had a significant effect on glucose yield during hydrolysis. Simultaneous saccharification and fermentation (SSF) showed the highest ethanol yield and direct fermentation the lowest. A final ethanol yield of 530 L of ethanol per ton of unpeeled cassava roots or 2400 L/ha were obtained.

Key words: Cassava, bio-ethanol, yield, separate hydrolysis and fermentation, simultaneous saccharification and fermentation.

INTRODUCTION

Growing environmental concerns and fluctuations in crude oil prices has initiated the investigation into the diversification of the energy supply pool in many countries. South Africa hopes to have a 2% blending of bio-fuels in the national liquid fuel supply by 2013 (Department of Minerals and Energy Affairs, 2007). Development of the biofuels industry in South Africa is primarily aimed at creating jobs in the energy sector and empowering impoverished communities to first economic status. Sugarcane and sugarbeet were proposed as suitable crops for bioethanol production in South Africa (Department of Minerals and Energy Affairs. 2007). Sugarcane is largely produced in only two provinces in South Africa and cannot be cultivated on arid, marginal land in, for example, the North West province of the country. Sugarbeet needs irrigation to produce yields per hectare that is economically feasible and is also prone to crop diseases. An alternative crop thus needs to be found to produce bioethanol for economic empowerment in provinces with large marginal land areas.

Cassava (*Manihot esculenta*) is a tuberous root plant that is native to South America and is cultivated around the world as a primary source of starch, as well as, a low-grade animal feed (Putthacharoen et al., 1998). Cassava is considered to be the sixth most important staple food in the world (Sriroth et al., 2000). Cassava is not considered to be a staple food in South Africa and is thus, also not commercially cultivated for food purposes. Cassava can be grown in arid, marginal soil where other crops, such as, sugarcane and sugarbeet fail (Sriroth et al., 2010;

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Zhang et al., 2003). Dai et al. (2006) and De Vries et al. (2010) showed that production of bioethanol from cassava is energy and renewable energy efficient. Various studies (Leng et al., 2008; Nguyen et al., 2007; Yu and Tao, 2009; Zhou et al., 2007) have shown that production of ethanol from cassava is both economical and sustainable. Cassava is thus a good crop to be considered for ethanol production in arid regions in South Africa without compromising food security.

Amutha and Gunasekaran (2001) investigated the use of co-immobilized yeast cells to ferment cassava starch to ethanol. It was shown (Amutha and Ganusekaran, 2001) that co-immobilized yeast cells of *Zymomonas mobilis* and *Saccharomyces diastaticus* could retain their activity during a continuous fermentation cycle of cassava and a final ethanol yield of approximately 0.3 $g.g^{-1}$ could be obtained. Kosugi et al. (2009) showed that ethanol yields as high as 0.46 $g.g^{-1}$ could be obtained by fermenting cassava pulp (starch and peels) to ethanol with a surface-engineered strain of *Saccharomyces cerevisiae*. Nitayavardhana et al. (2010) used ultrasound to try and increase the ethanol yield and overall ethanol conversion efficiency when converting cassava starch to ethanol using *S. cerevisiae*, but an ethanol yield of only 0.43 $g.g^{-1}$ could be obtained although an overall ethanol conversion efficiency of 95.7% with sonification was reported.

In this study, *S. cerevisiae* was used to ferment sugar rich hydrolysates from unpeeled Cassava roots and Cassava peels (inner flesh and peels) to ethanol. *Schwanniomyces occidentalis/castellii* was used to directly ferment unpeeld Cassava roots to ethanol.

MATERIALS AND METHODS

Cassava

Unpeeled Cassava roots were obtained from the Agricultural Research Council (ARC) of South Africa. A complete compositional analysis of cassava used in this study was done according to AACC methods by the South African Grain Laboratory (SAGL) and is presented in Table 1. The moisture content of the raw cassava roots were determined to be between 55 and 62 wt% as measured by a Mettler-Toledo HR 83 moisture analyzer according to standard methods. Unpeeled cassava roots and cassava peels were both dried in the sun for 3 days and then milled to flour and sieved with a +1.5 mm sieve.

Enzymes and micro-organisms

The enzyme mixtures Termamyl® SC (α-amylase enzyme mixture), Spiritzyme Fuel® (gluco-amylase enzyme mixture) and Celluclast® 1.5L (cellulase enzyme mixture) were obtained from Novozymes SA and used without further modification. *S. cerevisiae* was obtained from Anchor Yeast South Africa and was revived from the dormant state using the fermentation broth as a growth medium for 10 min before use in batch fermentation experiments. *S. occidentalis/ castellii* ATCC 26706 was preserved and stored on glycerol stocks at 4°C; it was subcultured on malt extract agar plates for 72 h at at 32°C from which an inoculum was prepared. The yeast was grown at 45°C, 150 rpm in malt extract broth (YM broth) containing 0.5 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.5 $g.L^{-1}$ $(NH_4)_2HPO_4$, 1.5 $g.L^{-1}$ yeast extract, 5 $g.L^{-1}$ glucose, 1.5 $g.L^{-1}$ malt extract and 2.5 $g.L^{-1}$ peptone at a pH of 5.5 (Srinorakutara et al., 2004). The concentration of *S. occidentalis/castellii* used was 10% (v/v) of fermentation sample (Saelim et al., 2008).

Experimental procedure

Enzymatic hydrolysis of cassava samples were done according to the methods described by Ayernor et al. (2002) and Mojovic (2006). The experimental procedure followed is illustrated in Figure 1.

Separate hydrolysis and fermentation (SHF)

Milled unpeeled cassava roots were liquefied in an incubator using 7.5 $\mu L.g^{-1}$ Termamyl® SC as enzyme mixture at 85 to 95°C and a pH of 5 to 6 for 1 h. Saccharification of the liquefied unpeeled cassava roots were done using of Spiritzyme Fuel ® (7.5 $\mu L.g^{-1}$) and Celluclast® 1.5L (4 $\mu L.g^{-1}$) as saccharification enzyme mixtures at 55 to 65°C and a pH of 4 to 5.5 for 48 h. The influence of biomass loading on the final sugar yield was investigated by comparing sugar yields after liquefaction and saccharification with an initial biomass loading of 10 and 20 wt%.

All fermentation experiments were done at 32°C for 48 h and 120 rpm. Hydrolysates treated at optimal conditions for liquefaction and saccharification was used with an initial biomass loading of 20 wt%.

Simultaneous saccharification and fermentation (SSF)

In the SSF process, the saccharification step and fermentation steps as described above were carried out simultaneously for 48 h. This shortened the overall conversion of cassava to ethanol with 48 h. During the saccharification and fermentation step, Spiritzyme Fuel® (7.5 $\mu L.g^{-1}$), Celluclast® 1.5L (4 $\mu L.g^{-1}$) and *S. cerevisiae* (8.$g.L^{-1}$) was added simultaneously to the prepared hydrolyzate.

Direct fermentation (DF)

Direct fermentation of unpeeled cassava roots were done with a 20 wt% biomass loading. The slurry was inoculated with 1% peptone and was autoclaved at a temperature of 121°C for 15 min. After heat pretreatment, the slurry was inoculated with 25 ml of the 24 h old inoculum at a pH of 4.5 and was processed in a shaker at 150 rpm and a temperature of 37°C. Samples were taken for 7 days and were centrifuged, filtered and analyzed by HPLC for sugar and ethanol content. The method used for the direct fermentation with *S. occidentalis* was adapted from Rojan et al. (2007).

Analysis

The presence of residual starch in hydrolyzed samples was detected with an iodine solution according to the method described by Morrison and Laignelet (1983). All hydrolyses proceeded until the iodine test showed complete conversion of all amylase in the feedstock sample. Sugar and ethanol analyses were done with calibration curves using high performance liquid chromatography

Table 1. Compositional analysis (wt% dry basis) of cassava used in this study.

Component	Unpeeled cassava (starch and peels)	Cassava starch	Cassava peel
Moisture	9.5	10.4	9.2
Protein	2.5	2.6	5.1
Starch	81.4	82.0	67.0
Fat	0.6	0.5	1.1
Ash	2.5	2.5	7
Crude fiber	3.5	2.0	10.6

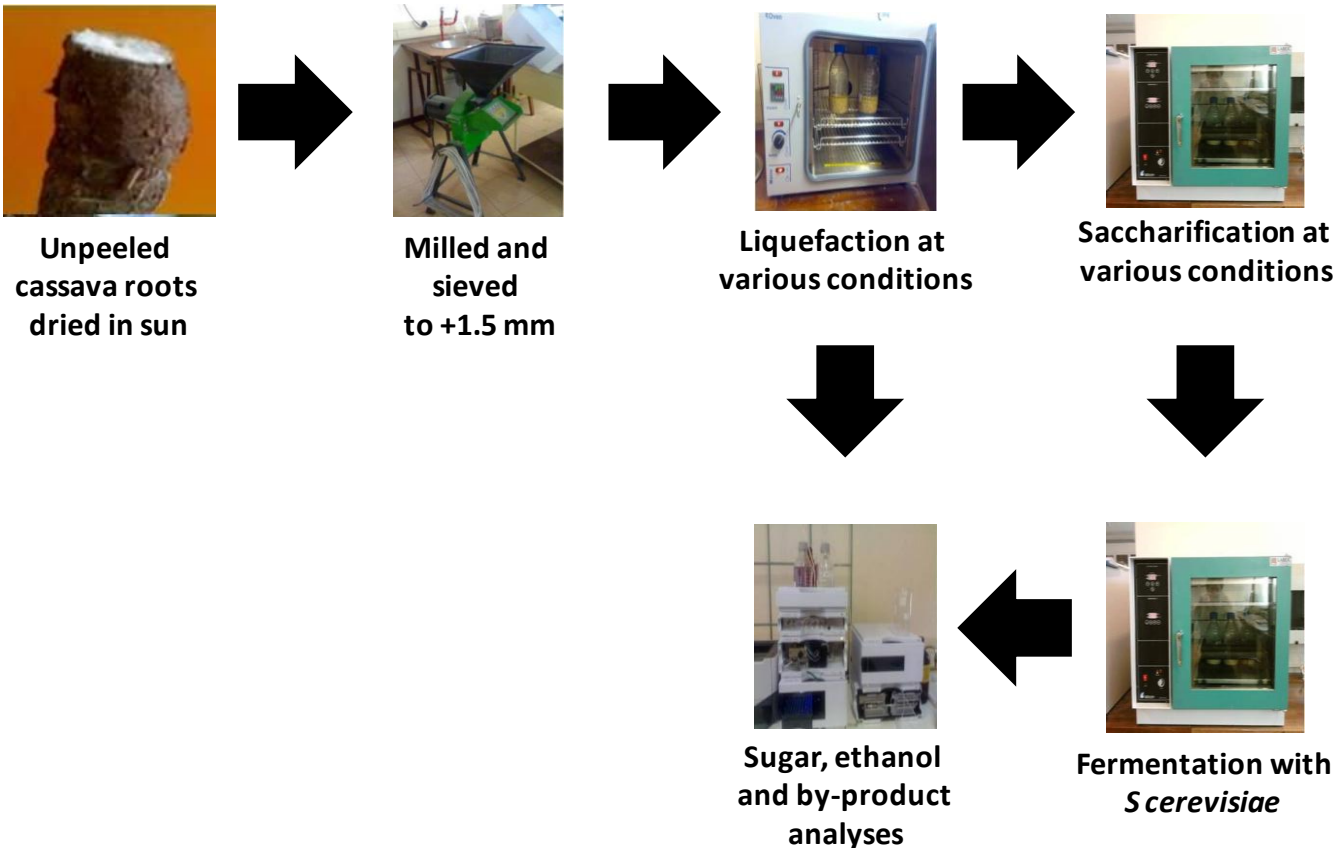

Figure 1. Experimental procedure for converting unpeeled cassava roots to ethanol.

(HPLC) with a Shodex column fitted to a refractive index detector. Water and acetonitrile mixtures were used as mobile phase and samples were prepared for analysis according to standard procedures.

RESULTS AND DISCUSSION

Liquefaction and saccharification

Influence of temperature

Cassava slurries were subjected to liquefaction (pH 6, biomass loading of 20 wt%, Termamyl ® SC loading of 7 $\mu L.g^{-1}$) and saccharification (pH 4.5, Spiritzyme Fuel loading of 7 $\mu L.g^{-1}$, Celluclast® 1.5 L loading of 4 $\mu L.g^{-1}$) at different temperatures and the glucose concentration was measured over time. The influence of varying liquefaction and saccharification temperatures on the glucose yield (gram glucose per gram milled cassava) after 60 min of liquefaction and 2 h of saccharification is presented in Figures 1 and 2, respectively.

From Figure 1 and 2, it can be seen that temperature had a significant influence on the glucose yield during liquefaction, but that the influence during saccharification was smaller. The highest glucose yield was obtained at a temperature of 95°C for liquefaction and a temperature of

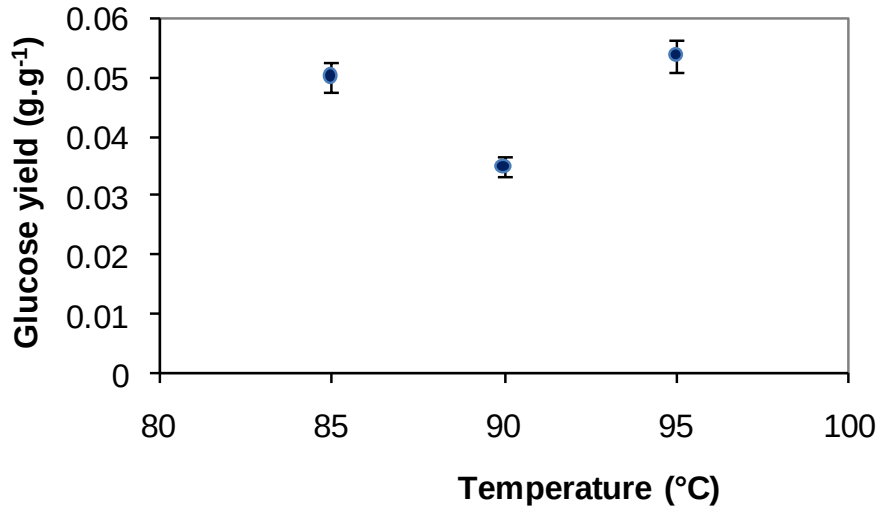

Figure 2a. Influence of temperature in glucose yield during liquefaction of milled cassava.

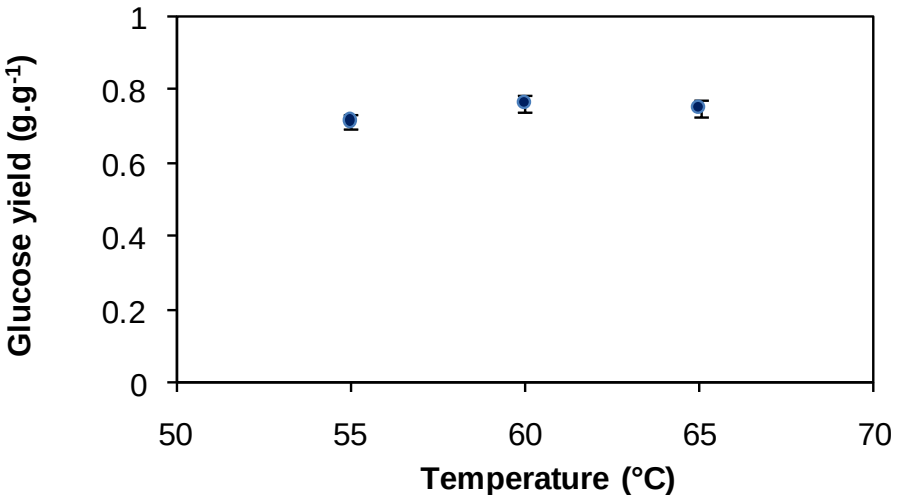

Figure 2b. Influence of temperature on glucose yield during saccharification of milled cassava

55°C for saccharification. Starch swells initially when heated in water (called gelatinization) and thus the enzymes need to diffuse through the swelled starch granules to get to active sites to liquefy the starch. Water starts to boil at approximately 90 to 92 °C at Potchefstroom. At 85°C, it is thus fair to assume that the starch was not swelled completely and the enzymes would thus have a shorter route to travel to active sites than at 90°C when the starch granules is fully cooked and swelled. At a temperature of 95°C, the starch is also completely swelled and cooked, but now the enzymes have sufficient energy to diffuse faster than at 90°C. This would explain the low glucose yield at 90°C observed during liquefaction. Temperature did not have a significant influence on the glucose yield during saccharification, but the highest glucose yield (0.75±0.02 $g.g^{-1}$) was recorded at a pH of 5.5.

Influence of pH

Cassava slurries were subjected to liquefaction (temperature of 95°C, biomass loading of 20 wt%, Termamyl® SC loading of 7 $\mu L.g^{-1}$) and saccharification (temperature of 55°C, Spiritzyme Fuel ® loading of 7 $\mu L.g^{-1}$, Celluclast® 1.5 L loading of 4 $\mu L.g^{-1}$) at different pH levels and the glucose concentration was monitored over time. The pH was adjusted to the desired level by using either sulfuric acid (H_2SO_4) or calcium hydroxide

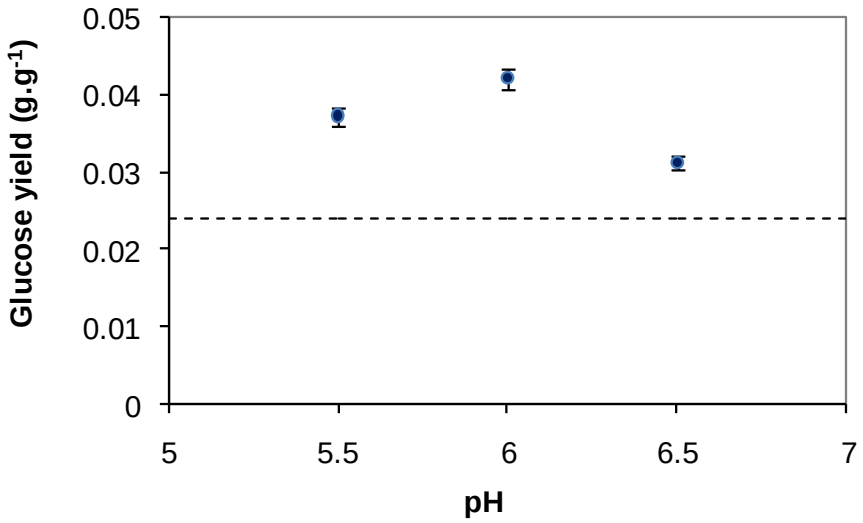

Figure 3. Influence of pH on glucose yield during liquefaction (• - experimental results, ----- control sample).

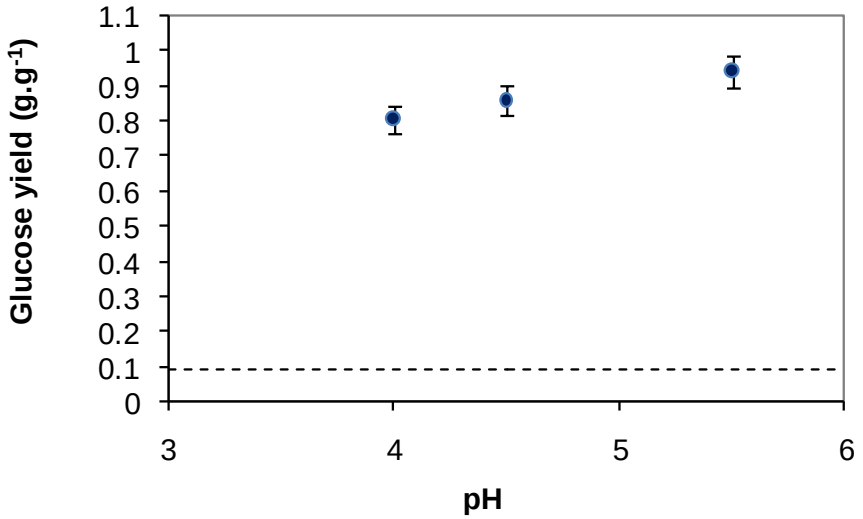

Figure 4. Influence of pH on glucose yield during saccharification (•, experimental results; -----, control sample).

$(Ca(OH)_2)$. The pH of the control sample was not adjusted and no enzymes were added to the control sample. The influence of pH on the final glucose concentration during liquefaction and saccharification is shown in Figures 3 and 4, respectively.

From Figure 3 and 4, it can be seen that all samples showed glucose yields higher than that of the control samples, validating the activity of the enzymes added during liquefaction and saccharification. During liquefaction, pH had a significant effect on the glucose yield with the highest yield of 0.04±0.001 $g.g^{-1}$ obtained at a pH of 6. The lower glucose yields at a pH of 6.5 and 5.5 is attributed to the lower enzyme activity at these pH values as stated by the supplier's specification sheet for the Termamyl® enzyme mixture. Glucose yield did increase with an increase in pH during saccharification with the highest significant glucose yield of 0.94±0.03 $g.g^{-1}$ obtained at a pH of 5.5.

Influence of biomass loading

Two different biomass loadings were used during liquefaction (temperature of 95°C, pH of 6, Termamyl® SC loading of 7 $\mu L.g^{-1}$) and saccharification (temperature of 55°C, pH of 4.5, Spiritzyme Fuel® loading of 7 $\mu L.g^{-1}$,

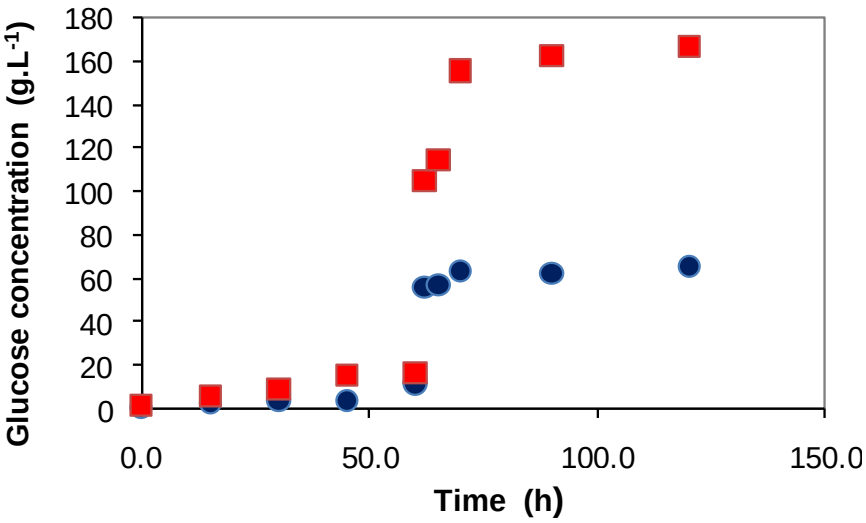

Figure 5. Influence of biomass loading on final glucose concentration after hydrolysis of unpeeled milled cassava (• - 10 wt% biomass loading ■ - 20 wt% biomass loading).

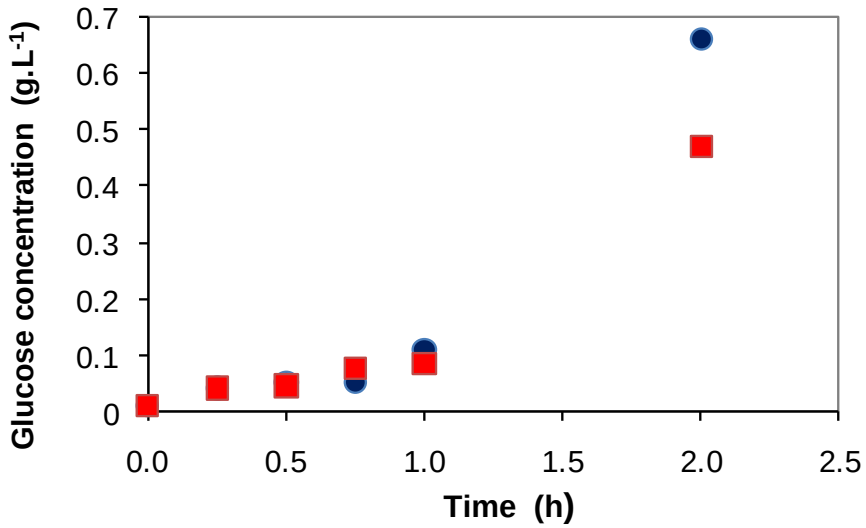

Figure 6. Influence of biomass loading on final glucose yield after hydrolysis of unpeeled milled cassava (• - 10 wt% biomass loading ■ - 20 wt% biomass loading).

Celluclast® 1.5 L loading of 4 $\mu L.g^{-1}$) that is 10 and 20 wt%. The influence of the biomass loading in the final glucose concentration and glucose yield after 60 min of liquefaction followed by 2 h of saccharification is presented in Figures 5 and 6, respectively.

Biomass loading had a significant effect on the final glucose concentration. The glucose concentration more than doubled from 65±1.9 to 167±5 $g.L^{-1}$ with a doubling in the biomass loading. The enzymes that are added during liquefaction and saccharification are added per mass of biomass used and thus it was expected that more biomass should yield more glucose. From Figure 6, it is clear however that the 10 wt% biomass loading produced more glucose per gram of biomass used than was expected. At a lower biomass loading, the viscosity of the mixture is significantly lower than at a biomass loading of 20wt% and it was shown by Herrera-Gomez et al. (2002) that starch cooked in limited amounts of water results in a significant amount of agglomeration betweens starch molecules. The high state of agglomeration at 20wt% biomass loading will thus result in longer diffusion times for the enzymes to get to active sites and thus

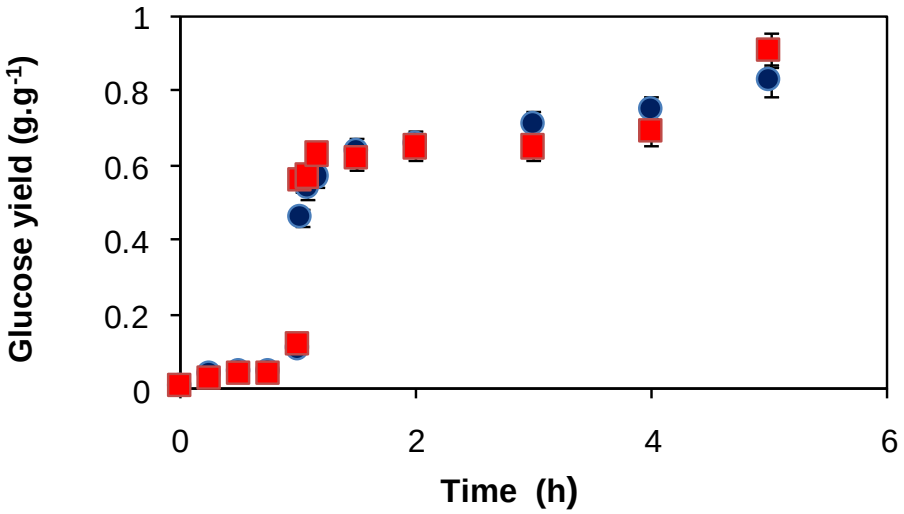

Figure 7. Glucose yield obtained from hydrolysis of unpeeled cassava roots with (■) and without (●) the addition of cellulase enzymes.

lower overall conversion to glucose in the same amount of time as for the 10wt% biomass loading.

Influence of addition of cellulase enzymes during saccharification

Unpeeled, milled cassava roots contain approximately 3.5 wt% crude fiber (Table 1). It is believed that milling of the dried cassava roots have liberated enough of the cellulose in the crude fiber component that it should be accessible to cellulase enzymes for conversion to glucose. The influence of adding cellulase enzymes (Celluclast® 1.5 L) to the hydrolysis mixtures was investigated by performing a complete hydrolysis with and without Celluclast® 1.5 L using a 10 wt% biomass loading and noting the final glucose yield. The glucose yield obtained with and without the presence of cellulase enzymes is presented in Figure 7.

Addition of Celluclast® 1.5 L did significantly improve the glucose from 0.83±0.04 to 0.91±0.05 $g.L^{-1}$. The slight increase is attributed to the conversion of the available cellulose in the crude fiber. The additional 8 wt% glucose yield gained by the addition of Celluclast® 1.5 L will results in an additional 40 L of ethanol per ton of unpeeled cassava roots, which is significant in monetary terms.

Fermentation

Liquefaction during the SHF process was done at pH 6 and 95°C using Termamyl® SC (7.5 ($\mu L.g^{-1}$) α-amylase enzymes. Saccharification was done at pH 4.5 and 55°C using Spiritzyme Fuel ® (7.5 $\mu L.g^{-1}$) and Celluclast® 1.5 L (4 $\mu L.g^{-1}$). Yeast (*S. cerevisiae*) was added to the hydrolyzate at a loading of 8 $g.L^{-1}$. During the SSF process, liquefaction was at the same conditions as for the SHF process. After liquefaction, both yeast (8 g.L-1) as well as Spiritzyme Fuel® (7.5 $\mu L.g^{-1}$) and Celluclast® 1.5 L (4 $\mu L.g^{-1}$) was added simultaneously at pH 4.5 and 30°C. The fermentation was allowed to continue for 72 h. The ethanol yield (gram ethanol per gram unpeeled cassava roots) for both fermentation processes is presented in Figure 8.

From Figure 8, it can be seen that the SSF process ultimately produced a significantly higher ethanol yield (0.53±0.03 $mL.g^{-1}$) than the SHF process (0.48±0.02 $mL.g^{-1}$). After 48 h, both processes produced approximately the same amount of ethanol (0.5±0.02 $mL.g^{-1}$). There was an increase in ethanol yield after 48 h for the SSF process, while the ethanol yield for the SHF process decreases slightly. The SHF process requires the additional of 2 h of saccharification at 55°C prior to fermentation and if that is taken into account in interpreting the above results, it is clear that the SSF process produces the same amount of ethanol than the SHF process in a shorter time (2 h less). The final ethanol yield for the SSF process translates to 530 L of ethanol per ton of unpeeled cassava roots or 2400 L ethanol per hectare.

Direct fermentation

Direct fermentation with unpeeled Cassava roots with *S. occidentallis* was done with 20wt% biomass loading. The final ethanol yield obtained was only 0.0025 $g.g^{-1}$. The final yield was too low to be economically feasible; therefore this production was not investigated further.

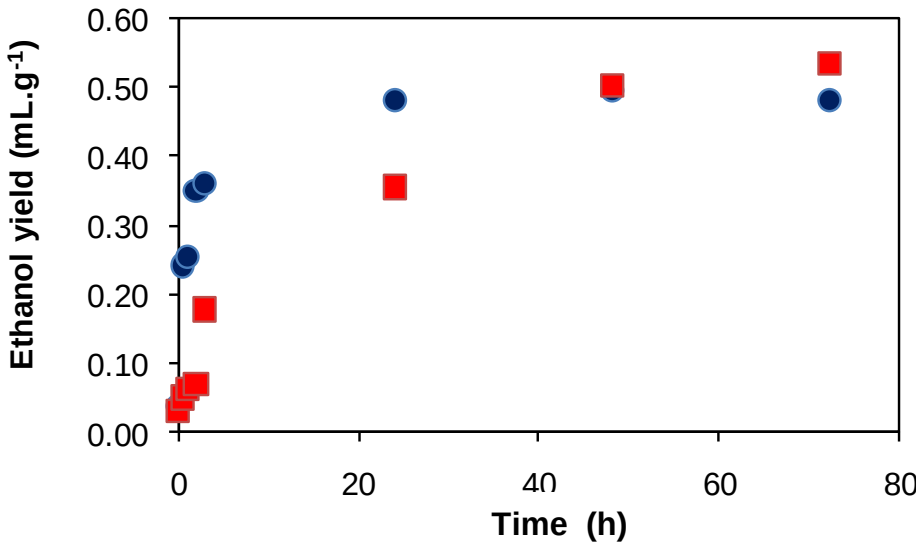

Figure 8. Ethanol yield obtained for SHF (●) and SSF (■) processes.

From this it could be concluded that direct fermentation using *S occidentallis* is not yet an economically feasible method for bio-ethanol production from Cassava starch.

Conclusions

In this study, on the optimization of the ethanol yield from cassava, it was found that temperature had a significant effect on glucose yield during liquefaction, but not saccharification. The best operating temperature was found to be 95 and 55°C for the liquefaction and saccharification step respectively. The pH during hydrolysis was found to have a significant effect on glucose yield during both liquefaction and saccharification. The optimum operating pH for liquefaction and saccharification was found to be 6 (as was recommended by the supplier) and 5.5, respectively. Biomass loading also had a significant effect on the glucose yield and glucose concentration during hydrolysis. It was found that a 10 wt% biomass loading performed significantly better than a 20 wt% biomass loading due to the agglomeration of starch molecules at the higher biomass loading. Celluclast® 1.5 L was found to increase glucose yield significantly if added during saccharification. The glucose yield increase with 8% when Celluclast® 1.5L was added to co-convert the cellulose in the unpeeled roots to glucose. Finally, the SHF and SSF process for producing ethanol from unpeeled cassava roots were compared. It was found that the SSF process can produce the same amount of ethanol in a short time than the SHF process. Ethanol yields for direct fermentation were found to be very low in this study. A final ethanol yield of 530 L of ethanol per ton of unpeeled cassava roots was obtained. This yield is high enough to produce ethanol economically from unpeeled cassava roots.

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