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Original Research Paper

Simultaneous Saccharification and Fermentation of Cassava Waste for Ethanol Production

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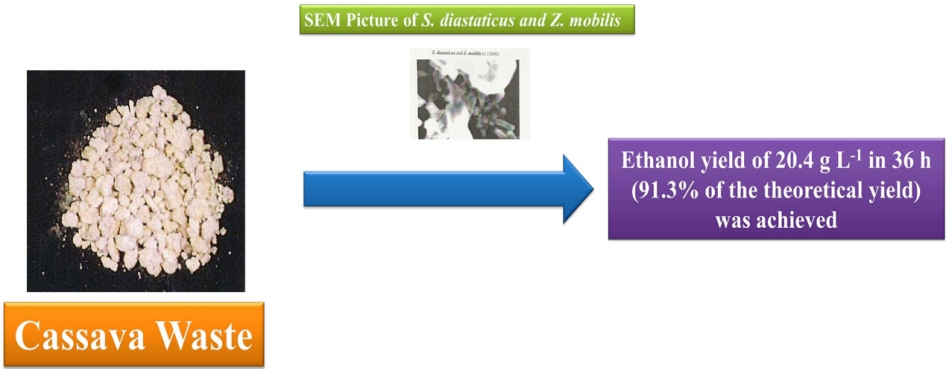
HIGHLIGHTS

- *SSF showed significantly higher ethanol yield than the separate enzymatic saccharification and fermentation.*
- *Microbial saccharification and fermentation of cassava waste using S. diastaticus and Z. mobilis led to improved ethanol production.*
- *Ethanol yield of 20.4 g L^{-1} in 36 h (91.3% of the theoretical yield) was achieved.*

GRAPHICAL ABSTRACT



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ABSTRACT

The efficiency of enzymatic and microbial saccharification of cassava waste for ethanol production was investigated and the effective parameters were optimized. The mixture of amylase and amyloglucosidase (AMG) resulted in a significantly higher rate of saccharification (79.6%) than the amylase alone (68.7%). Simultaneous saccharification and fermentation (SSF) yielded 6.2 g L^{-1} ethanol representing 64.5% of the theoretical yield. Saccharification and fermentation using pure and co-cultures of fungal isolates including *Rhizopus stolonifer*, *Aspergillus terreus*, *Saccharomyces diastaticus* and *Zymomonas mobilis* revealed that the co-culture system involving *S. diastaticus* and *Z. mobilis* was highly suitable for the bio-conversion of cassava waste into ethanol, resulting in 20.4 g L^{-1} in 36 h (91.3% of the theoretical yield).

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1. Introduction

The conventional energy resources could hardly meet the increasing energy demands (Zhu et al., 2005), and hence, biofuels such bioethanol have turned into promising alternatives to the fossil fuels used in the transportation sector. Ethanol has tremendous applications in chemical, pharmaceutical, and food industries in the form of raw material, solvent, and fuel (Alvira et al., 2010; Ohgren et al., 2006). An important issue regarding the bioethanol production is the process economy. Research efforts have been focused on designing economically viable processes capable of sustainably producing high amounts of fuel bioethanol. The cost effectiveness of bioethanol production through hydrolysis of starchy substrates by using enzymatic/microbial processes has been proved commercially viable (Baras et al., 2002, Kim and Dale, 2002).

In India, cassava (*Manihot esculenta* Crantz) is grown largely over 3.9 million hectares producing 60×10^6 million tones of tubers annually. Cassava is an industrial crop for the production of sago, vermicelli, and starch, whereas each ton of cassava tuber processed for sago and starch, yields half a ton of fibrous residue as waste. Cassava waste contains starch (50% zdry weight), cellulose, hemicellulose, and ashes in the extractable materials. Using cassava waste for ethanol production offers huge opportunities owing to the enormous availability of this inexpensive raw material in the cassava growing countries (Hermiati et. al., 2012). Lots of emphasis has been given to screen feasible bioprocess methodologies for efficient conversion of cassava waste to fuel ethanol. Since, starch derived from any plant sources is a complex molecule, therefore, it require various hydrolytic enzymes to be converted into simple fermentable sugars. A number of strategies have been adopted for the construction of starch-utilizing systems, which include the addition of amylolytic enzymes in culture broth, as well as mixed-culture fermentation.

Conversion of both cellulosic and starchy materials in a single process can be achieved by co-culturing two or more compatible microorganisms with the ability to utilize the materials. Fungal co-culturing offers a means to improve hydrolysis of residues, and also to enhance biomass utilization which would minimize the need for additional enzymes in the bioconversion process. Co-cultures have many advantages compared to their monocultures, including improved productivity, adaptability, and substrate utilization. Moreover, fermentation technologies utilizing strains of *Zymomonas mobilis* instead of the traditional yeast have been proposed by a number of authors, as these strains have been shown to ferment under fully anaerobic conditions with faster specific rates of glucose uptake and ethanol production close to the theoretical yield. The aim of the present work was to evaluate the efficiency of enzymatic and microbial liquefaction and saccharification of cassava waste for cost-effective production of ethanol using *Z. mobilis*.

2. Materials and methods

2.1. Materials and chemicals

Raw cassava waste (also known as thippi) was obtained from the Varalaxmi Sago Industry, Namagiri, Salem, Tamilnadu, India. It was sun dried, coarsely ground to uniform size (5 mm), stored in gunny bags, and used within 1 month after procurement. Enzymes including α-amylase (E.C3.2.1.1), amyloglucosidase (AMG)(E.C. 3.2.1.3), and cellulase (E.C.3.2.1.4) used in the experiments were kindly gifted by Novo Nordisk (India).

2.2. Fungal isolates from cassava waste

The primary isolation of fungi from cassava waste dispersed soil was done by serial dilution technique using potato dextrose agar medium at pH 6.5-7.0. The plates were maintained under aerobic conditions at 30 °C for 48 h. The isolates were identified based on their morphological and microscopical characteristics (Alexopolous et al., 1996). The fungal isolates were pre-cultured on 2% Potato Dextrose Agar (PDA) medium for 14 d. *Saccharomyces diastaticus* (NCIM 3314) and *Z. mobilis* (NCIM B806) were obtained from the National Chemical Lab, Pune, India. The rich medium used for *Z. mobilis* consisted of 2% glucose, 0.2% KH_2PO_4, 1% yeast extract, and 2% agar at pH 5.6-6.4. MGYP broth medium was used for the cultivation of *Saccharomyces diastaticus.*

2.3. Analysis of cassava waste composition

The composition of cassava waste was analyzed for the various parameters including starch (Arditti and Dunn, 1969), cellulose (Updegraff, 1969), hemicellulose (Deschatelets and Yu, 1986), lignin (Chesson, 1978), reducing sugars (Miller, 1959), and protein (Lowry et al., 1951).

2.4. Enzyme liquefaction and saccharification

2.4.1. Liquefaction using α-amylase

Optimization of the enzyme concentration for liquefaction of cassava waste was done following the method of Amutha and Gunasekaran (1994) using various concentrations of α-amylase (1-15 mg g^{-1} biomass). The optimal values of biomass concentration (50-150 g) and incubation time (12, 24, 36, 48, and 60 h) were obtained by step-wise experiments where the specified parameters were changed by keeping all other parameters constant. The pH of the reaction mixture in all the optimization experiments was kept constant at 6.0. The amount of the reducing sugars released was analyzed by DNS method (Miller, 1959).

2.4.2. Saccharification using AMG

The liquefied slurry obtained through the liquefaction was subjected to saccharification with various concentration of AMG (1-5 mg g^{-1} biomass) at 55°C and pH 4.5. The optimal values of various parameters such as biomass concentration (50 – 150g) and incubation time (12, 24, 36, 48, and 60 h) were obtained as mentioned above. The amount of reducing sugars released was also analyzed as described earlier.

2.4.3. Saccharification using enzyme mixture

Cellulase (0.15 g g^{-1} of cassava waste) was added along with AMG, and the saccharification was carried out under the same conditions presented earlier and the liberated reducing sugar was estimated.

2.5. Enzymatic saccharification and microbial fermentation

The liquefied slurry obtained was subjected to saccharification with optimum concentration of AMG (2 mg g^{-1} biomass) while *Z. mobilis* was also inoculated to the liquefied cassava waste at the time of AMG addition. Simultaneous saccharification and fermentation (SSF) was carried out at room temperature (30±2 °C) as described by Amutha and Gunasekaran (1994).

Batch fermentations involving free cells of *Z. mobilis* were also performed using the production medium developed from separate enzymatic liquefaction and saccharification. Inoculum was prepared in the rich medium and after 24 h of incubation, 1 ml of the culture was added to 50 ml of the production medium. The flasks were incubated at 30°C with 120 rpm for 48 h. Aliquots were taken every 12 h and analyzed.

2.6. Microbial saccharification of cassava waste and ethanol fermentation

Microbial saccharification and fermentation were carried out using pure and mixed cultures of *S. diastaticus* or the fungal isolates (isolated from cassava waste disposed soil) as described by Zabala et al., (1994). Erlenmeyer flasks (250 ml) containing 5 g cassava waste in 50 ml distilled water were autoclaved at 121°C for 15 min. Spore suspensions were prepared by repeated washings of 7 d old cultures of the fungal isolates in PDA plates with distilled water. Late-log phase culture of *S. diastaticus* in MGYP broth or the spore suspensions were used as inoculum (10% v/v) at a constant OD of 0.5 for the yeast and 1.3 for the fungi. The flasks were incubated at room temperature (30±2 °C). The mixed fermentation was started with *S. diastaticus* or the fungal isolates as pure cultures, and then late-log phase culture of *Z. mobilis* (10%v/v) was inoculated either simultaneously or at 12 h intervals. Aliquots of the fermented substrates

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were taken at every 12 h and analyzed. All experiments were carried out in triplicate, and the data are the mean values with standard deviations of <3%.

2.7. Analytical Methods

Biomass composition was determined according to Doelle and Greenfield (1985). The supernatant obtained by centrifugation of the culture broth at 6,000 ×g was used for reducing sugars (Miller, 1959) and ethanol (Caputi et al., 1968) analyses. The kinetic parameters of ethanol fermentation were calculated according to Abate et al. (1996).

3. Results and discussion

3.1. Strain isolation and identification

Two phenotypically different fungal colonies were obtained from cassava waste. Based on the morphology and microscopical characters, the two isolates were identified as *Rhizopus stolonifer* and *Aspergillus terreus* (Pothiraj et al., 2006 and 2007).

3.2. Cassava waste composition

Cassava waste was found to contain 52% starch, 13.4% cellulose, 9.38% hemicellulose, 11% lignin, 1.14% free reducing sugars, and 2.88% protein (dry weight basis).

3.3. Optimization of liquefaction and saccharification of cassava waste

The degree of liquefaction and saccharification of cassava waste depends on various factors such as enzyme concentration, incubation time, and substrate concentration. For optimizing the conditions of cassava waste liquefaction, different concentrations of commercial α-amylase (1-15 mg g^{-1} cassava waste) were added to cassava waste (10% w/v) and incubated for 1.5 h at 75°C. The maximum concentration of the reducing sugars obtained was at 18.70 g/l using 11.25 mg/g enzyme (Fig. 1).

Further increase in the concentration of the enzyme did not result in a corresponding increase in the concentration of the reducing sugars in the liquefied slurry. Hence, the enzyme concentration of 11.25 mg/g cassava waste was considered as optimal for liquefaction.

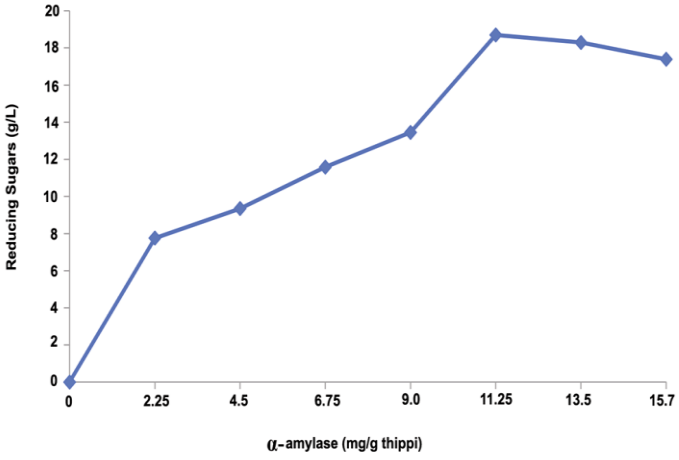

Fig.1. Influence of varying concentrations of α-amylase on liquefaction of cassava waste.

Optimization of the liquefaction time was carried out using the optimum concentration of the enzyme (11.25 mg/g cassava waste). The results showed that the release of the reducing sugars was highest (18.9 g/l) during the first 90 min; thus, this was chosen as the optimum time required for liquefaction (Fig. 2).

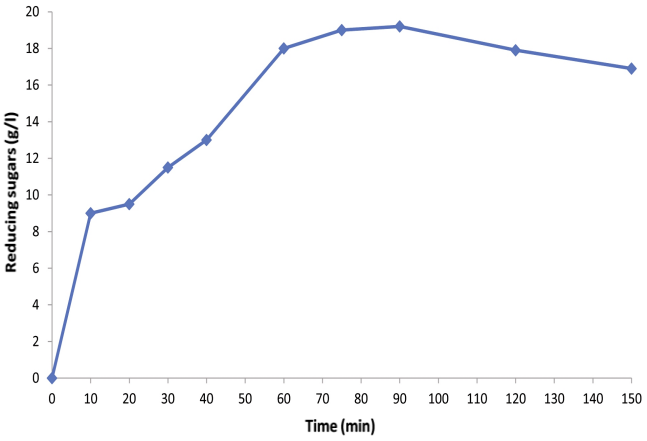

Fig.2. Influence of increase in time on liquefaction of cassava waste by α-amylase.

In order to optimize the concentration of AMG for saccharification, various AMG concentrations (1-10 mg g^{-1} cassava waste) were used with the liquefied slurry.

This experiment was carried under optimum conditions for AMG activity i.e. pH 4.5 and 55°C. It was found out that during a constant saccharification time (22 h), the maximum concentration of reducing sugars (39.63 g/l) could be obtained at an enzyme concentration of 5 mg/g cassava waste (Fig. 3), and beyond which no appreciable increase in the reducing sugars was obtained. Therefore, this AMG concentration was chosen as the optimum concentration for further experiments.

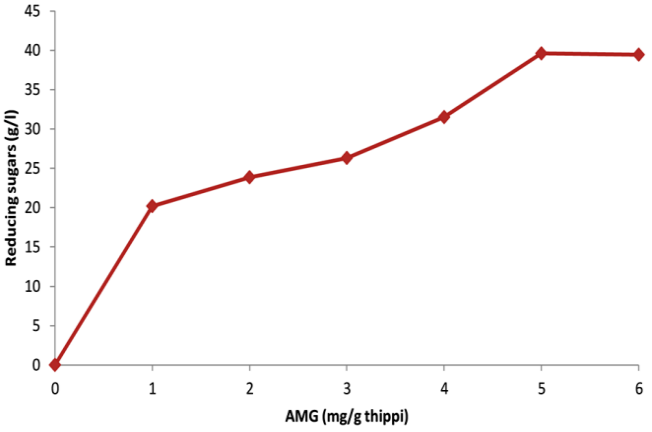

Fig.3. Influence of varying concentrations of AMG on saccharification of cassava waste.

The saccharification time of the liquefied slurry was optimized by using the obtained optimum concentration of AMG (5 mg/g cassava waste). The results obtained revealed that maximum saccharification (39.56 g/l reducing sugars) could be obtained within 22 h (Fig. 4). There was no significant increase in the reducing sugar concentration beyond 22 h of saccharification. Hence, this period was considered optimal for saccharification.

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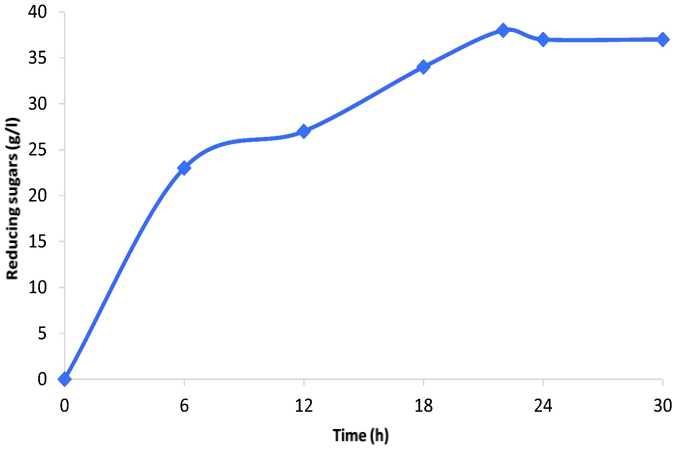

Fig.4. Influence of increase in time on saccharificationof cassava waste by amyloglucosidase.

3.4. Enzymatic saccharification and fermentation

The reducing sugars yield after the liquefaction and saccharification was measured at 39.5 g/l from 100 g/l substrate concentration, representing 76% saccharification (**Fig. 5**). While by using the combination of cellulase and AMG, a higher yield of reducing sugars (46 g/l), representing 79.6% saccharification was achieved. Arasaratnam and Balasubramaniam (1993) obtained a similar glucose yield on corn flour by using a two enzyme hydrolysis method. Simillarly, Mojovic et al. (2006) also reported a high conversion rate (dextrose equivalent 92.1%) of corn meal by using the same method.

The maximum ethanol concentration achieved by the enzymatic saccharification and fermentation was observed after 36 h with a productivity of 9.3 g/l (45.6 % of the theoretical yield). While the maximum ethanol concentration of 13.6 g/l, representing 57.7 % of the theoretical yield, was observed after 36 h of fermentation with *Z. mobilis* when cellulase was used additionally with AMG for the saccharification of cassava waste (**Table 1**). These results are in agreement with those of Dabas et al. (1997) who obtained 6-6.4% (v/v) ethanol within 36 h at 30°C from 25% wheat mash saccharified by a combination of α-amylase and amyloglucosidase and fermented by *S. cerevisiae*. In a different study, Mojovic et al. (2006) achieved 78.5 % of
The theoretical yield of ethanol and the highest volumetric productivity of 1.6 g/l/h utilizing 17.5 % corn meal in 48 h using *S. cerevisiae*. The lower product yield obtained in the current study may be due to the different experimental conditions applied and the fermenting organism used.

3.5. Simultaneous saccharification and fermentation

To reduce the complete process time and achieve beneficial energy savings as suggested by Mojovic et al. (2006), the fermenting organism *Z. mobilis* was inoculated along with AMG and both saccharification and fermentation were carried out at room temperature (30°C). Volumetric ethanol productivity by *Z. mobilis* was higher in AMG enzyme saccharification (0.38g l^{-1} h^{-1}) while specific ethanol productivity was higher in amylase enzyme saccharification (0.171 g l^{-1} h^{-1}). Simultaneous enzyme saccharification and fermentation performed better in terms of ethanol conversion and theoretical yield than the two separate enzymatic saccharification and fermentation. Though the ethanol yield was relatively low (6.2 g/l), the highest ethanol conversion of 33.2%, representing 64.5% of the theoretical yield was obtained in this SSF process. It was also efficient in saving time by reducing the time of the complete process by 10 h as the highest product yield was obtained after 49.5 h (**Table 1**).

Table 1.
Effect of the type of enzyme saccharification on the alcoholic fermentation of cassava waste by *Z. mobilis.*

Organisms	Sugar concentration after saccharificaton or liquefaction (g/l)	Saccharification (%)	Fermentation time (h)	Ethanol (g/l)	Biomass (g/l)	Volumetric ethanol productivity (g $l^{-1}h^{-1}$)	Specific ethanol productivity (g $l^{-1}h^{-1}$)	Ethanol conversion (%)	Percent of the theoretical yield (%)
Enzymatic saccharification using AMG									
Z. mobilis	39.73	68.76	36	9.26	1.52	0.26	0.171	23.3	45.6
Enzymatic saccharification using cellulose and AMG									
Z. mobilis	46	79.61	36	13.57	2.36	0.38	0.161	29.5	57.7
Simultaneous saccharification and fermentation									
Z. mobilis	18.7	32.36	48	6.2	2.1	0.13	0.061	33.2	64.5

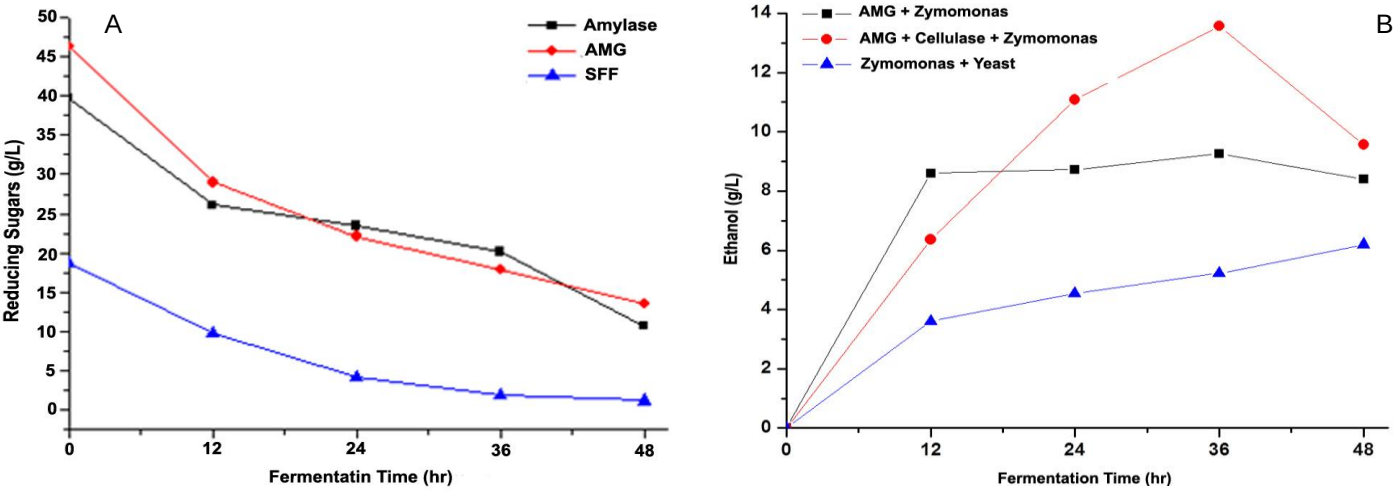

Fig. 5. A: Utilization of reducing sugar during enzymatic saccharification (amylase; AMG and SSF). B: Ethanol production during enzymatic saccharification (amylase + AMG) and simultaneous saccharification (amylase + AMG + Zymomonas mobilis).

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The reducing sugars yield (18.7 g/l) from 100 g/l cassava waste in simultaneous saccharification experiment was lower compared to the yield in enzymatic liquefaction followed by saccharification (39.7 and 46 g/l, respectively). This was expected because in SSF, the reducing sugars were assessed after liquefaction with α-amylase and since the organisms were inoculated along with AMG, the actual yield of the reducing sugars due to saccharification could not be found as both saccharification and fermentation proceeded simultaneously. Similar results were observed by Mojovic et al., (2006) in their SSF experiments on corn meal using *S. cerevisiae* (**Table 1**).

3.6. Microbial saccharification and fermentation

Direct fermentation of cassava waste was carried out in submerged state employing the pure cultures of the yeast *S. diastaticus* or the spore suspensions of the fungi *R. stolonifer* or *A. terreus* in separate experiments.

The mixed fermentations were started as a single process with a pure culture of *S. diastatiicus, R. stolonifer* or *A. terreus. Z. mobilis* was added at different times of fermentation (including simultaneous inoculation) in order to determine the appropriate time to initiate the mixed fermentation.

3.6.1. Fermentation using S. diastaticus and Z. mobilis

The biggest increment in the reducing sugars yield was observed after 24 and 36 h of fermentation using pure culture of *S. diastaticus* and the maximum reducing sugars content of 43.8 g/l was detected at 36 h (**Table 2**). In simultaneously-inoculated mixed culture fermentation, the variations of glucose concentration during fermentation time indicated a synergism between the saccharifying and the fermenting organisms.

Table 2.
Submerged fermentation of cassava waste (100 g/l) by pure and mixed culture of *S. diastaticus* and *Z. mobilis*.

Culture	Period of fermentation (h)					
	0	12	24	36	48	60
	Reducing sugars (g/l)					
a	1.13	11.78	15.56	43.76	41.60	40.40
b	-	15.32	13.68	11.48	9.68	8.16
c	-	-	12.58	12.08	11.38	11.02
d	-	-	-	15.96	15.38	15.08
e	-	-	-	-	29.02	16.7
	Biomass (g/l)					
a	-	1.92	2.18	2.67	2.82	2.91
b	-	2.28	2.67	3.12	3.24	3.38
c	-	-	2.30	2.87	3.34	3.27
d	-	-	-	3.34	3.41	3.41
e	-	-	-	-	3.45	3.49
	Ethanol (g/l)					
a	-	8.02	10.5	17.92	17.78	17.65
b	-	11.26	14.66	20.42	20.70	20.56
c	-	-	13.88	18.04	17.96	17.88
d	-	-	-	14.68	18.34	18.16
e	-	-	-	-	18.7	18.48

Pure culture a: *S. diastaticus*
Mixed cultures b: Simultaneous inoculations of *S. diastaticus* and *Z. mobilis.*
c, d, e: *S. diastaticus* inoculated with *Z. mobilis* after 12, 24 and 36 h of incubation, respectively.

Direct fermentation of cassava waste by the pure culture of *S. diastaticus* produced the maximum amount of ethanol at 17.9 g/l at 36 h. Simultaneous co-culturing of *S. diastaticus and Z. mobilis* resulted in a higher final ethanol concentration (20.42 g/l) during the same period, and the ethanol yield remained constantly high till the end of fermentation. No significant difference in ethanol production was observed when the yeast was allowed to grow for 12 to 36 h before *Z. mobilis* was added. In the other mixed fermentations, the maximum ethanol concentrations detected ranged between these two values and were obtained either after 36 h or 48 h.

3.6.2. Fermentation using R. stolonifer and Z. mobilis

The release of reducing sugars by the pure culture of *R. stolonifer* increased slowly peaking at 42.76 g/l after 60 h fermentation. This was 24 h more to yield nearly the same reducing sugar concentration released by *S. diastaticus.*

Table 3 shows the reducing sugar, biomass, and ethanol production results using pure cultures of *R. stolonifer* and mixed fermentations of *R. stolonifer* with *Z. mobilis*. Reducing sugar levels obtained by mixed co-culture fermentation dropped continuously reaching nearly half the values obtained by using the pure culture *R. stolonifer* fermentation at 48 h. All the mixed processes showed higher values of microbial biomass than the single pure culture fermentation processes. Maximum 14% increase in biomass was obtained at 36 h fermentation.

Table 3.
Submerged fermentation of cassava waste (100 g/l) by pure and mixed cultures of *Rhizopus stolonifer* and *Z. mobilis.*

Culture					
	Reducing sugars (g/l)				
a	7.90	20.34	22.88	25.14	42.76
b	38.14	34.23	29.42	19.68	17.43
c	-	16.84	15.74	14.52	12.44
d	-	-	21.92	18.42	16.54
e	-	-	-	24.62	22.46
	Biomass (g/l)				
a	1.61	1.89	2.21	2.76	2.98
b	1.84	2.21	2.51	2.98	3.18
c	-	2.05	2.45	3.10	3.15
d	-	-	2.94	3.04	3.1
e	-	-	-	3.08	3.4
	Alcohol (g/l)				
a	7.56	8.44	12.4	13.02	13.62
b	9.01±	10.38	15.16	17.48	18.67
c	-	12.28	13.02	14.22	14.82
d	-	-	14.96	15.14	14.66
e	-	-	-	16.92	17.22

Pure culture a: *R. stolonifer.*
Mixed cultures b: Simultaneous inoculations of *R. stolonifer* and *Z. mobilis.*
c, d, e: *R. stolonifer* inoculated with Z. mobilis after 12, 24 and 36 h of incubation, respectively.

The maximum ethanol concentration achieved by direct fermentation of cassava waste using the pure culture of *R. stolonifer* was recorded at 13.6 g/l after 60h fermentation. Simultaneous co-culturing of *R. stolonifer* and *Z. mobilis* resulted in a higher final ethanol concentration (18.7 g/l) at the same time. The maximum final ethanol concentrations in the other mixed fermentations were obtained at either 48 or 60 h fermentation; however, none of these processes were better than the simultaneously-inoculated mixed culture system of *R. stolonifer* and *Z. mobilis*.

3.6.3. Fermentation using A. terreus and Z. mobilis

A. terreus directly fermented cassava waste into ethanol and the highest increment in the reducing sugar concentration was obtained 24 h earlier compared to the *R. stolonifer* fermentation and the reducing sugar level was maintained till the end of the fermentation period (**Table 4**). The reducing sugars left in the substrate at the end of the fermentation period were least in this simultaneous co-culture system. The residual sugars content progressively increased by time of the inoculation of *Z. mobilis* in to the fungal culture. Inoculation of *Z. mobilis* after 60 h growing of *A. terreus* resulted in the highest increase (18%) in biomass compared to the pure culture.

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Table 4.
Submerged fermentation of cassava waste (100 g/l) by pure and mixed cultures of *A. terreus* and Z. *mobilis.*

Culture	Period of fermentation (h)				
	12	24	36	48	60
	Reducing sugars (g/l)				
a	5.84	18.88	40.58	42.46	42.60
b	41.74	36.51	19.40	14.14	12.40
c	-	21.26	20.06	17.64	14.82
d	-	-	24.52	22.92	21.66
e	-	-	-	26.58	20.10
	Biomass (g/l)				
a	1.45	1.79	2.18	2.59	2.71
b	1.73	2.01	2.23	2.78	2.99
c	-	1.98	2.28	2.61	2.97
d	-	-	2.48	2.81	3.15
e	-	-	-	2.85	3.19
	Alcohol (g/l)				
a		7.80	12.60	12.68	12.82
b	10.10	16.68	18.58	18.32	18.18
c	-	10.10	10.68	12.58	14.32
d	-	-	13.02	13.78	14.44
e	-	-	-	15.14	15.64

Pure culture a: *A. terreus*
Mixed cultures b: Simultaneous inoculations of *A. terreus* and *Z. mobilis*
c, d, e: *A. terreus* inoculated with *Z. mobilis* after12, 24 and 36 h of incubation, respectively.

these organisms were capable of direct conversion of cassava waste into ethanol.

It was observed that the saccharification efficiency of *R. stolonifer* and *A. terreus* was significantly higher in the presence of the fermenting organism *Z. mobilis* than that of their respective pure cultures (**Tables 3 and 4**). These data suggested that ethanol produced during fermentation had a stimulatory effect on enzyme action.

The highest percent of the theoretical ethanol yield observed in the present investigation was 91.3% in the mixed cultures of *S. diastaticus* and *Z. mobilis*. The mixed culture systems of *R. stolonifer* with *Z. mobilis* and *A. terreus* with *Z. mobilis* led to 85.4% and 89.5% of the theoretical ethanol yield, respectively. These results clearly demonstrated that starch saccharification potential of *S. diastaticus* was higher than that of *R. stolonifer* and *A. terreus*. Similar results have been reported by Abate et al. (1996).

The major limitation in starch or carbohydrate conversion into ethanol was the rate at which a saccharifying organism could hydrolyse the complex carbohydrates. The total time required for the complete process of starch conversion in to ethanol is also a crucial factor determining the overall economy of the process.

In this study, 6.2 g/l ethanol from 18.7 g/l reducing sugar resulted from the liquefied cassava waste was achieved after simultaneous inoculation of AMG and *Z. mobilis* at 48h and 30^{o}C. Since the saccharification was effectively performed along with the fermentation at the room temperature (30^{o}C), the energy required for a separate saccharification step by AMG (optimum temperature 5of 5^{o}C) could be saved.

Direct fermentation of cassava waste by *R. stolonfier* and *A. terreus* showed a higher ethanol concentration (13.62 g/l, 12.8 g/l, respectively) but the production was delayed for 60 h.

Direct fermentation by *S. diastaticus* was superior to the fungal fermentations in terms of product yield and total time duration. Simultaneous mixed cultures of either of these fungi with *Z. mobilis* gave 18.6 g/l ethanol yield after 60 h and 48 h, respectively. In *A. terreus* and *Z. mobilis* mixed culture system, the ethanol production was attained in a shorter fermentation period (48 h).

Table 5.
Effect of microbial saccharification on the alcoholic fermentation of cassava waste by *Z. mobilis.*

Organism	Fermentation time (h)	Maximum ethanol (g/l)	Maximum biomass (g/l)	Volumetric ethanol productivity (g l^{-1}h^{-1})	Specific ethanol productivity (g l^{-1}h^{-1})	Percent of the theoretical yield (%)
Microbial saccharification and fermentation (using AMG)						
S. diastaticus	36	17.92	2.67	0.497	0.187	80.1
S. diastaticus + *Z. mobilis*	36	20.42	3.12	0.567	0.18	91.3
Microbial saccharification and fermentation (using cellulose and AMG)						
R. stolonifer	60z	13.62	2.98	0.26	0.087	62.3
R. stolonifer + *Z. mobilis*	60	18.67	3.18	0.31	0.10	85.4
Microbial saccharification and fermentation						
A. terreus	60	12.8	2.18	0.36	0.165	61.7
A. terreus + *Z. mobilis*	48	18.58	2.23	0.52	0.233	89.5

Direct fermentation of cassava waste by the pure culture of *A. terreus* produced the maximum ethanol content of 12.8 g/l after 60 h fermentation. Simultaneous co-culturing of *A. terreus* and *Z. mobilis* resulted in a higher ethanol concentration at earlier time (16.7 g/l at 24 h). Ethanol concentrations near this value were only obtained at 60 h in the other mixed culture fermentations.

The mixed culturing of *S. diastaticus* with *Z. mobilis* proved to be the best method for converting cassava waste carbohydrates into biomass protein and ethanol (**Tables 2-5**).

The production of ethanol in the submerged fermentation of cassava waste by pure cultures of *S. diastaticus* or *A. terreus* or *R. stolonifer* indicated that

There are a few reports in the literature on the use of fungi in pure or mixed cultures for bioconversion of starchy substrates into ethanol. For instance, Dabas et al. (1997) used the hydrolytic enzymes and *A. awamori* for ethanol production from cassava starch and obtained 10% ethanol in five d at 30^{o}C**.**

The results of all the microbial saccharification and fermentation experiments showed that the simultaneous mixed cultures system involving *S. diastaticus* with *Z. mobilis* was best suited for the bioconversion of cassava waste into ethanol. This system produced the highest ethanol concentration of 20.4 g/l after 36 h fermentation from 100 g/l cassava waste representing 91.3% theoretical yield.

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4. Conclusion

In the present study, simultaneous enzymatic saccharification and fermentation showed significantly higher ethanol yield than the separate enzymatic saccharification and fermentation. The production of ethanol in the submerged fermentation of cassava waste by pure cultures of *S. diastaticus* or *A. terreus* or *R. stolonifer* indicated that these organisms were capable of direct conversion of cassava waste into ethanol. The results of the present study indicated that the microbial saccharification and fermentation of cassava waste involving *S. diastaticus* with *Z. mobilis* can be used for improved ethanol production from cassava waste or other starch residues for reducing the ethanol production cost by saving energy, the cost of pure hydrolytic enzymes and by reducing the total time of the complete process.

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