Enzyme Activities and Substrate Degradation by Fungal Isolates on Cassava Waste During Solid State Fermentation

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The growth and bioconversion potential of selected strains growing on cassava waste substrate during solid state fermentation were assessed. *Rhizopus stolonifer* showed the highest and the fastest utilization of starch and cellulose in the cassava waste substrate. It showed 70% starch utilization and 81% cellulose utilization within eight days. The release of reducing sugars indicating the substrate saccharification or degradation potential of the organisms reached the highest value of 406.5 mg/g by *R. stolonifer* on cassava waste during the eighth day of fermentation. The protein content was gradually increased (89.4 mg/g) on the eighth day of fermentation in cassava waste by *R. stolonifer*. The cellulase and amylase activity is higher in *R. stolonifer* than *A. niger* and *P. chrysosporium*. The molecular mass of purified amylase and cellulase seemed to be 75 KDa, 85 KDa respectively.

KEYWORDS: Amylase activity, Cassava waste, *R. stolonifer*, *A. niger* and *P. chrysosporium*, SDS-PAGE

Advances in industrial biotechnology offer potential economic utilization of agro-industrial residues, particularly those originating from tropical regions. In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues such as sugarcane bagasse, sugar beet pulp, coffee pulp/husk, apple pomace etc. Several processes have been developed that utilize these raw materials for the production of bulk chemicals and value-added fine products such as ethanol, Single cell protein, mushrooms, enzymes, organic acids, amino acids and biologically active secondary metabolites etc. Application of agro-industrial residues in bioprocesses on the one hand provides alternative substrates and on the other helps in solving pollution problems, which their disposal may otherwise cause havoc to vegetation. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new avenues have opened up for their utilization (Pandey *et al.*, 2001).

Cassava (*Manihot esculenta* crantz) also known as manioc, yucca or tapioca, is a native of tropical South America and it is a major root crop grown in more than 80 countries in the humid tropics (Philipos, 1983). In India, cassava is raised in 3~9 million hectares to produce 58~60 lakh million tons of tubers. In Tamil Nadu, it figures prominently as an industrial crop for the production of sago, vermicelli and starch. Tamil Nadu is in top slot in respect of processing tapioca into sago and starch. It also meets 85% of country's domestic and industrial demand for cassava products.

The solid waste of sago industry is called as tippi. Sago and starch are produced either from peeled or unpeeled tubers. Cassava root has an average composition of 60~65% moisture, 30~35% Carbohydrate, 0.2~0.6% other extractives, 1~2% crude protein and comparatively low content of vitamins and minerals (Balagopalan, 1988; Carta *et al.*, 1997; Panday *et al.*, 2001). If protein content could be increased by microbial biomass it could be used as animal feed, consequently the capital cost of animal feed could be reduced. In recent years, there has been increasing interest in the use of solid state fermentation processes as alternative to submerged fermentation, because it has lower energy requirements, produce less waste water and partly because of environmental concerns regarding the disposal of solid wastes (Ramesh and Lonsane, 1990). Thus solid state fermentation is considered to be used in this study for biomass protein production from a cheap substrate (Cassava and its waste) because investment cost is lower. Although many report demonstrated that biomass production by solid substrate the protein yield was still not high enough thus modification conditions of fermentation may be conducted to increase product yield by using solid state fermentation (Pothiraj *et al.*, 2006).

The aim of this study is to produce a monogastric animal feed containing both starch (for its calorific value) and protein. *Rhizopus* was chosen because it has been used in the Indonesian diet for many centuries (Wang and Hesseltine, 1982; Raimbault *et al.*, 1985). This fungus contains a relatively high protein content of high biological value (Waliszewska *et al.*, 1983) and exhibits significant protein productivity with cassava (Ramos-Valdivia *et al.*, 1983;

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Sukara and Doelle, 1988) as substrate.

Materials and Methods

Substrate. Fresh cassava waste (tippi) was collected from Varalakshmi sago industry, Namagiri, Salem Dt, India. It was sun dried, coarsely ground to uniform size (5mm) and stored in gunny bags and was used within one month after procurement.

Organisms. *Aspergillus niger*, *Phanerochaete chrysosporium*, and *Rhizopus stolonifer* were used in the solid state fermentation studies of tippi. The three organisms were previously isolated by primary selection from a sample of naturally contaminated tippi by their morphology and colony characteristics. Pure cultures of *Trichoderma viride*, *Trichoderma harzianum* and *Trichoderma reesi* procured from National Chemical Laboratory, Pune were used in the preliminary experiments. The organisms were maintained on PDA slants stored at 4°C. The slants were freshly made once a month.

Solid state fermentation of Cassava waste (Tippi). Solid state fermentation of cassava waste was carried out in 250 ml Erlenmeyer flask. Twenty grams (20 g) of cassava waste was taken in individual flask and 40ml of distilled water was added to give a 70% of moisture content. The flasks were plugged with cotton and autoclaved at 121°C for 15 minutes. Agar blocks (8 mm disc) removed from the plates containing seven days old cultures of fungi were used as inoculum for solid state fermentation experiments. A single block was aseptically inoculated into the individual flask containing the substrate. Three replicates were maintained for each organism. The flasks were incubated at room temperature for ten days. Bio-converted tippi samples were withdrawn at intervals of two days, oven dried at 55°C and were analyzed for starch, cellulose reducing sugar, mycelial protein, and pH. The activities of the enzyme amylase and cellulase were assayed using fresh fermented sample without drying during the course of fermentation.

Assessment of amylase activity in starch agar medium. The starch agar plates were prepared and were inoculated with *R. stolonifer*, *A. niger* and *P. chrysosporium*. The plates were incubated at 35°C for 3 days. In order to detect amylase activity, plates were flooded with iodine solution for few min. This procedure revealed distinct hydrolysis region i.e. clearing zone formation around the colony which indicated the amylase producing ability of the strains.

Analytical procedure. Starch was estimated by the method of Arditti and Dunn (1969). To detect the crude enzyme activity of double-layer plate assay procedure was

used to reveal distinct hydrolysis regions (Po-Jui Chen *et al.*, 2004). Cellulose was estimated by the method of updegraff (1969). Reducing sugar was estimated by DNS (Dinitro Salicylic acid) method (Miller, 1959). Protein content of tippi and fermented sample was estimated by Lowry *et al.* (1951). The activity of the enzyme amylase and cellulase was estimated by the method Ray *et al.* (1993).

Effect of pH and temperature for amylase activity. The effect of pH on amylase activity was determined by vary-

Table 1. Composition of cassava wastes (tippi) from sago industry using intact cassava tubers

Parameters	(% dry wt)
Starch	55.8
Cellulose	14.5
Reducing Sugar	1.21
Protein	3.13

Results are mean $\pm$ SE of three replicates.

Rhizopus stolonifer

Phanerochaete chrysosporium

Plate 1. Photomicrographs of selected fungi for cassava waste bioconversion.

ing the pH of the buffer system between 4 and 9 and the temperature stability of amylase in the culture filtrate was studied between 30°C and 70°C with 10°C increments.

The molecular weight of amylase and cellulase enzyme was determined by SDS-PAGE electrophoresis by the modified method of Bollag and Edlesein (Coral *et al.*, 2002).

Results and Discussion

Unfermented Cassava waste (or) Tippi was found to have by dry weight 55.8% starch, 14.5% cellulose, 1.21% free reducing sugars and 3.13% protein (Table 1). The organisms were isolated from naturally contaminated cassava waste (Tippi) The isolated organisms *A. niger*, *P. chrysosporium* and *R. stolonifer* were identified in pure culture by their morphology and colony characteristics (Plate 1, 2 and 3).

Preliminary experiment. The potential of the laboratory isolates *A. niger*, *P. chrysosporium* and *R. stolonifer* and that of the pure cultures of *Trichoderma viride*, *Trichoderma reesei*, *Trichoderma harizianum* and *Penicillium* sp. in utilizing cassava waste substrate was assessed by the content of reducing sugars produced by their hydrolytic action on the 6th day of solid state fermentation. The laboratory isolates *R. stolonifer*, *A. niger* and *P. chrysosporium* showed better performance than the pure cultures. The maximum amount of reducing sugar

Aspergillus niger

Plate 2. Photomicrographs of selected fungi for cassava waste bioconversion.

I : *Rhizopus stolonifer*
II : *Phanerochaete chrysosporium*
III : *Aspergillus niger*

Plate 3. Morphological characteristics of selected fungi on PDA plate.

Table 2. Reducing sugar content (mg/g) of cassava waste (tippi) on the 6th of solid state fermentation with different fungi

Organisms	Reducing sugar (mg/g)
<i>Rhizopus stolonifer</i>	304.3 ± 8.7
<i>Aspergillus niger</i>	207.2 ± 7.6
<i>Aspergillus terreus</i>	191.4 ± 4.9
<i>Trichoderma viride</i>	173.6 ± 5.4
<i>Trichoderma harizianum</i>	168.5 ± 5.9
<i>Trichoderma reesei</i>	184.2 ± 6.8
<i>Penicillium</i> sp.	162.7 ± 7.1

Results are mean ± SE of three replicates.

(304.3 mg/g) produced by *R. stolonifer* on the 6th day fermentation (Table 2). The organisms were used for the solid state bioconversion of cassava waste and they were chosen from a preliminary experiment. The present investigations were initiated with the view to study the feasibility of bioconversion of tippi into useful products.

Starch. Cassava waste contained 558 mg/g starch. Among the three cultures, *R. stolonifer* showed the highest and fastest utilization of starch. In the solid state fermentation of cassava wastes by *R. stolonifer* resulted 74.7% utilization of starch on the 10th day while *A. niger* and *P. chrysosporium* resulted in 69.7% and 67.38% utilization of starch on the same period. Fig. 1 shows that *R. stolonifer* had utilized 74.7% starch on the 10th day of fer-

Fig. 1. Percent utilization of starch in cassava waste during fermentation with *Rhizopus stolonifer*, *Aspergillus niger* and *Phanerochaete chrysosporium*.

Fig. 2. Percent utilization of cellulose in cassava waste during fermentation with *Rhizopus stolonifer*, *Aspergillus niger* and *Phanerochaete chrysosporium*.

mentation, which was comparatively higher than that utilized by *P. chrysosporium* and *A. niger*.

Cellulose. Cassava waste contained 145 mg/g cellulose. *R. stolonifer* showed the highest and fastest utilization of cellulose. In the solid state fermentation of cassava waste by *R. stolonifer* resulted in 51.24% utilization of cellulose on the 2nd day while *A. niger* and *P. chrysosporium* resulted in 38.27% and 24.13% utilization of cellulose on the same period. Fig. 2 shows that *R. stolonifer* had utilized 82.48% cellulose on the 10th day of fermentation,

which was comparatively higher than that utilized by *P. chrysosporium* and *A. niger*.

Reducing sugar. The initial reducing sugar content of cassava waste was 12.1 mg/g. The reducing sugar content was gradually increased during the course of fermentation period. In the solid state fermentation of cassava waste by *R. stolonifer* produced 406.5 mg/g of reducing sugar on the 10th day. Table 3 shows that *R. stolonifer* had produced 32.59 fold increase over the control value on the 8th day of fermentation, which was comparatively higher

Table 3. Reducing sugar content (mg/g) of cassava waste during solid state fermentation with various fungi

Name of the organisms	Period of fermentation (days)					
	0	2	4	6	8	10
<i>Rhizopus stolonifer</i>	12.1 ± 0.9	114.5 ± 3.5	186.7 ± 6.5	288.2 ± 6.5	306.5 ± 9.7	302.1 ± 9.3
<i>Aspergillus niger</i>	–	106.8 ± 5.3	168.7 ± 5.7	268.5 ± 6.5	324.6 ± 8.4	333.5 ± 7.5
<i>Phanerochaete chrysosporium</i>	–	232.3 ± 4.4	294.6 ± 8.7	361.7 ± 8.1	406.5 ± 7.9	384.5 ± 10.3

Results are mean ± SE of three replicates.

Table 4. Mycelial protein (mg/g) of cassava waste during solid state fermentation with various fungi

Name of the organisms	Period of fermentation (days)					
	0	2	4	6	8	10
<i>Rhizopus stolonifer</i>	31.3 ± 1.2	58.38 ± 1.58	70.1 ± 2.1	79.8 ± 1.93	89.4 ± 2.52	81.5 ± 2.12
<i>Aspergillus niger</i>	–	44.8 ± 1.8	58.2 ± 2.1	68.7 ± 2.4	78.3 ± 2.8	78.4 ± 2.7
<i>Phanerochaete chrysosporium</i>	–	38.4 ± 1.6	44.6 ± 1.3	55.3 ± 1.9	68.1 ± 2.9	80.7 ± 3.1

Results are mean ± SE of three replicates.

than that *P. chrysosporium*, and *A. niger*. Ofuya and Nwa-jiuba (1990) had shown that the yield of reducing sugars was positively correlated with the progress of solid state fermentation process.

Protein. The initial protein content of cassava waste was 31.3 mg/g. The protein content was gradually increased during fermentation period. In the solid state fermentation of cassava waste by *R. stolonifer* produced 89.4 mg/g of protein on the 8th day of fermentation. Table 4 shows that *R. stolonifer* had produced 1.85 fold increase over the control value on the 8th day of fermentation, which was comparatively higher than *P. chrysosporium* and *A. niger*. *P. chrysosporium* gradually increased the protein content during the course of fermentation. Maximum protein content 80.7 mg/g was produced on the final day which was nearly the same amount to *R. stolonifer*. Soccol and Krieger (1998) screened different *Rhizopus* strains capable of attacking raw bagasse for obtaining high level protein flour to be used for human or animal feeding and reported that different species exhibited different levels of development.

Potential for starch hydrolysis. The starch hydrolysis potential of the selected organisms were assessed by their growth in starchy substrate medium and they were described by Anadu *et al.* (1988). *R. stolonifer*, *A. niger* and *P. chrysosporium* produced zone formation surrounding the edge of colony. The zone producing organisms indicate their potential for starch utilization (Plate 4).

Amylases. Activity of extracellular saccharifying amylase enzyme of *R. stolonifer*, *A. niger* and *P. chrysosporium* were measured by assaying the crude enzyme filtrate of cassava waste infused with fungal cultures for a period of days of definite intervals.

I : *Rhizopus stolonifer*
 II : *Aspergillus niger*
 III : *Phanerochaete chrysosporium*

Plate 4. Starch utilization of selected fungi in starch agar plate by measurement of zone formation.

Amylase enzyme activity of the three organisms as shown in Table 5 clearly demonstrated a difference to their pattern of saccharification. The highest amylase activity (6.1 IU/ml) was observed on 8th day in *R. stolonifer* mediated fermentation, while the amylase of *A. niger* and *P. chrysosporium* showed the maximum activity 4.1 IU/ml and 4.5 IU/ml on the same period during fermentation. Akpan *et al.* (1999); Babu and Satyanarayana (1995) reported that cheap and readily available source of carbo-

Table 5. Amylase activity (IU/ml) of cassava waste during solid state fermentation with selected fungi

Name of the organisms	Period of fermentation (Days)				
	Amylase activity (IU/ml)				
	2	4	6	8	10
<i>Rhizopus stolonifer</i>	2.4 ± 0.02	3.8 ± 0.03	4.9 ± 0.04	6.1 ± 0.04	5.9 ± 0.05
<i>Aspergillus niger</i>	1.3 ± 0.02	2.6 ± 0.04	3.3 ± 0.06	4.1 ± 0.05	4.2 ± 0.05
<i>Phanerochaete chrysosporium</i>	1.2 ± 0.02	2.3 ± 0.04	3.4 ± 0.04	4.5 ± 0.03	4.6 ± 0.05

Results are mean ± SE of three replicates.

Fig. 3. Effect of pH on the amylase activity of the *R. stolonifer*, *A. niger* and *P. chrysosporium* on 8th day of solid state fermentation. **Fig. 4.** Effect of temperature on the amylase activity of the *R. stolonifer*, *A. niger* and *P. chrysosporium* on 8th day of solid state fermentation.

hydrates and protein can be used to replace commercial and expensive supplements yeast extract or peptone to wheat, rice bran for amylase production. In this context

the use of cassava waste as a supplement to wheat bran or rice bran for amylase production seems to be a bright idea as cassava waste supports high amylase production of *R.*

stolonifer.

Effect of pH and temperature for amylase. The selected strains were allowed to grow in different pH and temperature to determine their optimum growth. At pH 6, the strains were showed heavy growth and high amylase activity. But at pH 3 and 4, the strains showed low growth and liquefaction and at pH 9 showed moderate growth and liquefaction (Fig. 3). Many workers reported heavy growth at pH 6.0 with different microorganisms (Khoo *et al.*, 1994).

The results showed that heavy growth and high amylase activity was obtained at 50°C of these strains. The growth and liquefaction of strains were found to decrease with the increase as well as decrease of temperature (Fig. 4). The maximum growth of mesophilic organisms at 40°C was reported by Abd El-Rahman (1990).

Determination of molecular weight. The molecular weight of the amylolytic enzymes of the selected strains was found to be 75 KDa (Plate 5). Similar results have been earlier reported by several worker (Abou-zeid, 1997).

Plate 5. Molecular mass determination of amylase by SDS-PAGE.

Potential for cellulose hydrolysis. The cellulose degradation potential of the selected strains were assessed by their growth and zone formation in double layer plate medium and they are described by Po-jui chen *et al.* (2004). *R. stolonifer*, *A. niger* and *P. chrysosporium* produced zone formation surrounding the edge of colony. The zone producing organisms indicate their potential for cellulose utilization (Plate 6).

Cellulase activity. Activity of extracellular saccharifying cellulase enzyme of *R. stolonifer*, *A. niger* and *P. chrysosporium* were measured by assaying the crude enzyme filtrate of cassava waste in fused with fungal culture for a period of days at definite intervals.

CMCase activity. CMCase activity of the selected organisms as shown in Table 6, 7 and 8 clearly demonstrated a difference to the pattern of saccharification. The

Plate 6. Cellulose utilization of selected fungi in CMC plate by measurement of zone formation.

Table 6. Cellulase activity (IU/ml) of cassava waste during solid state fermentation with *Rhizopus stolonifer*

Parameters	Period of fermentation (Days)					
	Enzyme activity (IU/ml)					
	0	2	4	6	8	10
CMcase (IU/ml)	ND	0.44 ± 0.01	0.34 ± 0.02	0.026 ± 0.01	0.31 ± 0.03	0.46 ± 0.03
FPase (IU/ml)	ND	0.28 ± 0.01	0.20 ± 0.02	0.13 ± 0.01	0.23 ± 0.01	0.26 ± 0.02
β-Glucosidase (IU/ml)	ND	0.38 ± 0.01	0.32 ± 0.01	0.25 ± 0.01	0.38 ± 0.02	0.41 ± 0.02
Protein (%)	3.13	5.83	7.01	7.98	8.94	8.15
Cellulose utilization (%)	ND	51.24	68.68	77.37	81.57	82.48

ND, Not detected.

Results are mean ± SE of three replicates.

Table 7. Cellulase activity (IU/ml) of cassava waste during solid state fermentation with *Aspergillus niger*

Parameters	Period of fermentation (Days)					
	Enzyme activity (IU/ml)					
	0	2	4	6	8	10
CMcase (IU/ml)	ND	0.02 ± 0.011	0.03 ± 0.001	0.04 ± 0.002	0.12 ± 0.002	0.07 ± 0.002
FPase (IU/ml)	ND	0.008 ± 0.001	0.01 ± 0.001	0.02 ± 0.001	0.04 ± 0.001	0.03 ± 0.001
β-Glucosidase (IU/ml)	ND	0.06 ± 0.001	0.07 ± 0.002	0.1 ± 0.002	0.1 ± 0.001	0.08 ± 0.001
Protein (%)	3.13	4.48	5.82	6.87	7.83	7.84
Cellulose utilization (%)	ND	38.27	58.06	62.55	76.13	79.65

ND, Not detected.

Results are mean ± SE of three replicates.

Table 8. Cellulase activity (IU/ml) of cassava waste during solid state fermentation with *Phanerochaete chrysosporium*

Parameters	Period of fermentation (Days)					
	Enzyme activity (IU/ml)					
	0	2	4	6	8	10
CMcase (IU/ml)	ND	0.03 ± 0.001	0.04 ± 0.001	0.05 ± 0.001	0.1 ± 0.003	0.08 ± 0.001
FPase (IU/ml)	ND	0.001 ± 0.01	0.009 ± 0.001	0.03 ± 0.001	0.04 ± 0.001	0.03 ± 0.001
β-glucosidase (IU/ml)	ND	0.06 ± 0.001	0.07 ± 0.001	0.07 ± 0.002	0.11 ± 0.002	0.07 ± 0.001
Protein (%)	3.13	3.84	4.46	5.53	6.81	6.87
Cellulose utilization (%)	ND	24.13	40.34	54.27	68.41	73.24

ND, Not detected.

Results are mean ± SE of three replicates.

highest CMCase activity (0.31 IU/ml) was observed on 8th day in *R. stolonifer* mediated fermentation, while the CMCase of *A. niger* and *P. chrysosporium* showed the maximum activity (0.12 IU/ml and 0.1 IU/ml) respectively on the same period of solid state fermentation.

FPase activity. FPase activity of the selected organisms as shown in Table 6, 7 and 8 clearly demonstrated a difference to the pattern of saccharification. The highest FPase activity (0.13 IU/ml) was observed on 8th day in *R. stolonifer* mediated fermentation, while the FPase of *A. niger* and *P. chrysosporium* showed the maximum activity (0.04 IU/ml and 0.04 IU/ml) respectively on the same period during fermentation.

β-Glucosidase activity. β-Glucosidase activity of the selected organisms as shown in Table 6, 7 and 8 clearly demonstrated a difference to the pattern of saccharification. The highest β-glucosidase activity (0.38 IU/ml) was observed on 8th day in *R. stolonifer* mediated fermentation, while the β-glucosidase of *A. niger* and *P. chrysosporium* showed the maximum activity (0.1 IU/ml and 0.11 IU/ml) respectively on the same period during fermentation.

Determination of molecular weight. The molecular weight of cellulase was determined as 85 KDa for selected fungi (Plate 7). Several workers (Coral *et al.*,

Plate 7. Molecular mass determination of cellulase by SDS-PAGE.

2002; Habib *et al.*, 2004) have earlier reported similar results.

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