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Cellulose Utilization and Protein Productivity of Some Cellulolytic Fungal Co-cultures

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Protein productivity by the cellulolytic fungi, *Trichoderma viride* (MTCC 800), *Chaetomium globosum* and *Aspergillus terreus* was compared in co-culture and mixed culture fermentations of cashewnut bran. Co-cultures were more effective in substrate saccharification, which ranged between 85~88% compared to the 62~67% saccharification shown by the monocultures. Maximum saccharification was induced by *T. viride* and *C. globosum* co-culture resulting in the highest 34% release of reducing sugars. The maximum 16.4% biomass protein and the highest protein productivity (0.58%) were shown by *T. viride* and *A. terreus* co-culture. *A. terreus* performed better in co-culture in the presence of *T. viride* rather than with *C. globosum*. Among the cellulolytic enzymes, FPase (Filter Paper Cellulase) activity was significantly higher in all the co- cultures and in the mixed culture than in their respective monocultures. Mixed culture fermentation involving all the three fungi was not effective in increasing the per cent saccharification or the biomass protein content over the co-cultures.

KEYWORDS: ***Aspergillus terreus*, *Chaetomium globosum*, CMCase, FPase, β-glucosidase, Trichoderma viride**

Large quantities of cellulosic or lignocellulosic agro-industrial wastes and crop residues are made available every year in many tropical countries, posing severe environmental pollution problems. Efficient and controlled biodegradation of these materials by fungi or bacteria leads to a number of processes of great economic importance (Ray *et al.*, 1993).

To improve the conversion of cellulosic biomass to chemicals and fuels, many hyper cellulolytic strains have been used either as pure cultures or as mixed cultures with fermenting organisms (Lezinou *et al.*, 1995; Tanaka *et al.*, 1986). The use of mixed cultures of lignocellulolytic or cellulolytic microorganisms looks promising in increasing the protein content compared to pure cultures (Rabala *et al.*, 1994) and many of them have been reported to be more efficient in degrading lignocellulosic substrates and in producing high activity enzymes than the monocultures (Arora, 1995; Puniya and Singh, 1995).

One important aspect of cellulose research using SSF (Simultaneous Saccharification Fermentation) has been on co-culturing of two cultures together for enhanced enzyme production. A co-culture of *Aspergillus ellipticus* and *A. fumigatus* resulted in improved hydrolytic and β-glucosidase activities as compared to the occasions when they were used separately (Tengerdy, 1996).

Synergism between individual components of cellulase from different origins on substrates such as cotton (Sadama and Patil, 1985) and different paper products (van Wyk, 1998), has been applied with varying degrees of success. Solid state fermentation has been found to be the more appropriate system than submerged fermentation for protein enrichment and cellulase production from lignocellulose (Elshafei *et al.*, 1990; Illanes *et al.*, 1998; Pandey *et al.*, 2001).

Cashewnut is one of the major cash crops in India. Cashewnut bran represents 1.5% of the dry weight of whole nut. Large quantities of cashewnut bran are disposed off from cashewnut processing industries. The present investigation reports the potential of cellulose utilization and protein productivity of three cellulolytic fungi during co-culture and mixed culture fermentation of cashewnut bran.

Materials and Methods

Trichoderma viride (MTCC 800) strain was procured from National Chemical Laboratory, Pune, India. *Aspergillus terreus* and *Chaetomium globosum* were isolated from naturally contaminated cassava waste and from the effluent of a paper mill, respectively by enrichment culture technique. All the three fungi were maintained on potato dextrose agar slant, stored at 4°C and were subcultured once a month.

The fermentation processes were carried out in 250 m*l* Erlenmeyer flasks with cashewnut bran (20 g) containing 70% moisture. The flasks were plugged with cotton and sterilized at 121°C and 15 pounds pressure for 15 min. Two agar blocks (7 mm each) from actively growing 7 day-old plates of fungal pure cultures were inoculated into each flask for monoculture fermentation. For co-cul-

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ture fermentations, a single agar block from each of the two fungi in the three permutation and combination was inoculated into each flask. In mixed culture studies, a single agar block from each of the three fungal cultures, *T. viride*, *A. terreus* and *C. globosum* was inoculated into each conical flask.

All the flasks were incubated as static cultures at room temperature for 25 days. Composition of cashewnut bran was analyzed for cellulose, reducing sugars and protein. The contents of the flasks were removed periodically at an interval often days from the 5th day of SSF (Simultaneous Saccharification Fermentation) and were analyzed for cellulose (Updegraff, 1969) reducing sugars (Maheswari *et al.*, 1993) and protein (Lowry *et al.*, 1951). The activities of enzymes, CMCase (Carboxymethylcellulase), FPase (Filter Paper Cellulase) and β-glucosidase (Ray *et al.*, 1993) were assayed at an interval of five days. All the three cellulolytic enzyme activities were expressed in International Unit (IU/m*l*). One International Unit is defined as the micromoles of glucose liberated by 1 m*l* of enzyme in 1 min. The experiments were carried out with three replicate samples.

Results and Discussion

Cashewnut bran contained 32.5% cellulose, 4% protein and 3.3% free reducing sugars. The increase in the protein content (from 4% to 12.1%) and the per cent saccharification (66% and 67% respectively) of the substrate were nearly equal in the monocultures of *T. viride* and *C. globosum* on the 25th day of solid state fermentation of cashewnut bran. *A. terreus* monoculture increased the substrate protein content by 6.8% and it could saccharify only 62% of the substrate (Table 1).

Co-cultures involving these organisms were more effective than their respective monocultures, showing 85~88% saccharification in the same period. Among the co-cultures, *A. terreus* and *C. globosum* co-culture was the least effective producing 12.4% biomass protein by saccharifying 85% of the substrate in 25 days (Table 1). The maximum 88% saccharification was observed in co-cultures involving *T. viride* and *C. globosum*, resulting in the release of the highest 34% reducing sugars, while the maximum biomass protein was produced in *T. viride* and *A. terreus* co-culture (16.4%). In the mixed culture fermentation, more reducing sugars were produced than the co-culture of *T. viride* and *A. terreus*, but its biomass production was less than that of the latter. Similarly, accumulation of reducing sugars has been observed to inhibit cell density or biomass (Tabassum *et al.*, 1990). Mixed cultures involving all the three fungi could neither improve the biomass protein nor induce better saccharification than the co-cultures.

Protein productivity (g protein produced/g cellulose consumed) of *T. viride*, *C. globosum* and *A. terreus* monocultures was 0.38, 0.37 and 0.34, respectively (Table 1). These results are in conformity with those of Zabala *et al.* (1994) who reported a protein productivity value of 0.33 for *T. reesei* and those of Puniya and Singh (1995) who observed a 4.67% protein enrichment in wheat straw using *Phaenerochaete chrysosporium* and *Azotoacter chroococcum*.

Co-cultures involving *T. viride* as one of the partners showed more synergistic growth (protein productivity 0.55

Table 1. Cellulose, reducing sugars and protein (%) of cashewnut bran during simultaneous saccharification fermentation with pure, co cultures and mixed culture of *Trichoderma viride*, *Chaetomium globosum* and *Aspergillus terreus*

S. No	Organisms	Cellulose (%)					Reducing sugars (%)					Proteins (%)				
		Fermentation period (Days)														
		0	5	15	25	a	0	5	15	25		0	5	15	25	b
1	*Trichoderma viride*	32.5 ±2.8	23.8 ±1.9	15.2 ±1.1	11.0 ±0.9	66 ±5.5	3.2 ±0.2	4.3 ±0.07	14.3 ±1.0	33.7 ±2.0		4.0 ±0.3	6.3 ±0.2	7.8 ±0.3	12.1 ±0.9	0.38 ±0.02
2	*Aspergillus terreus*		25.4 ±1.9	18.1 ±1.0	12.2 ±0.9	63 ±6.0		4.8 ±0.1	13.8 ±0.9	27.9 ±2.1			5.9 ±0.1	7.5 ±0.3	10.8 ±0.8	0.33 ±0.03
3	*Chaetomium globosum*		21.7 ±1.2	14.3 ±1.0	10.7 ±0.7	67 ±5.5		5.3 ±0.1	15.4 ±1.1	31.4 ±2.2			6.3 ±0.2	8.1 ±0.5	12.1 ±0.8	0.37 ±0.02
4	Coculture 1		15.3 ±1.0	7.2 ±0.2	3.9 ±0.1	88 ±6.9		8.7 ±0.8	21.5 ±1.2	34.0 ±2.0			5.0 ±0.1	10.8 ±0.1	19.6 ±1.2	0.55 ±0.04
5	Coculture 2		18.7 ±1.5	8.2 ±0.4	4.8 ±0.08	85 ±7.9		7.9 ±0.01	20.9 ±1.1	32.0 ±2.0			4.4 ±0.07	9.7 ±0.3	16.4 ±1.1	0.45 ±0.4
6	Coculture 3		16.3 ±1.1	7.6 ±0.3	4.4 ±0.9	87 ±8.0		8.7 ±0.4	22.1 ±1.3	32.4 ±1.9			5.2 ±0.2	12.8 ±0.1	20.4 ±1.3	0.58 ±0.4
7	Mixed culture		14.9 ±0.9	8.5 ±0.4	4.5 ±0.1	86 ±7.5		16.2 ±1.0	23.5 ±1.4	33.3 ±2.4			8.9 ±0.7	13.2 ±1.3	19.6 ±1.2	0.56 ±0.5

a : Percent saccharification.

b : Protein productivity [protein (g) produced/cellulose (g) utilized].

and 0.58) than the co-culture involving *C. globosum* and *A. terreus*. *A. terreus* monocultures with low protein productivity showed higher rate of cellulose utilization and protein productivity in co-cultures. This may be due to the increased growth of the slow growing organism such as *A. terreus* in the presence of the other member in co-cultures. Similar results were reported by Zabala *et al.* (1994) in a mixed culture of *T. reesei* and *Monosporium* sp.

The relative activities of the three cellulolytic enzymes varied with the organism employed, the combination of co-culture and the incubation period (Figs. 1, 2 and 3). Among the cellulolytic enzymes, β-glucosidase activity was found to be higher in all the cultures. The co-culture, *A. terreus* with *T. viride* exhibited the maximum β-glucosidase activity on the 25th day of incubation in cashew nut bran. Similarly other lignocellulosic substrates like wheat straw and bagasse have been reported to be the best inducers for β-glucosidase (Lakshmikant and Mathur, 1990).

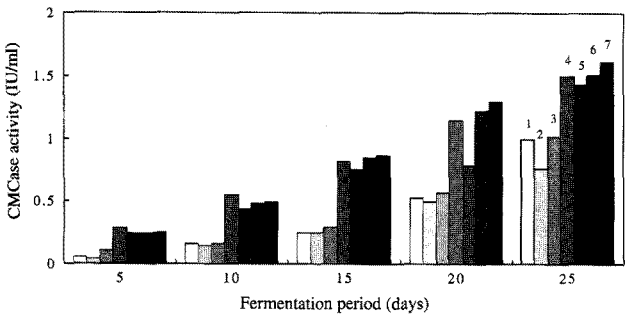

Fig. 1. Carboxymethyl-cellulase activity during simultaneous saccharification fermentation of cashew nut bran by 1. *Trichodermea viride*, 2. *Apergillus terreus*, 3. *Chaetomium globoum*, 4. *T. viride* + *C. globoum*, 5. *C. globoum* + *A. terreus*, 6. *A. terreus* + *T. viride*, 7. Mixed culture.

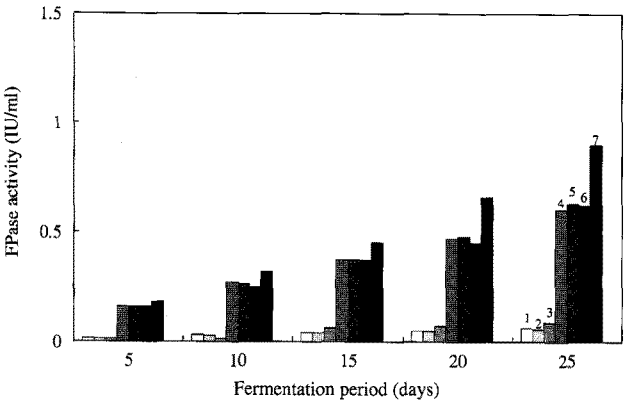

Fig. 2. Filter paper cellulase activity during simultaneous saccharification fermentation of cashew nut bran by 1. *Trichodermea viride*, 2. *Apergillus terreus*, 3. *Chaetomium globoum*, 4. *T. viride* + *C. globoum*, 5. *C. globoum* + *A. terreus*, 6. *A. terreus* + *T. viride*, 7. Mixed culture.

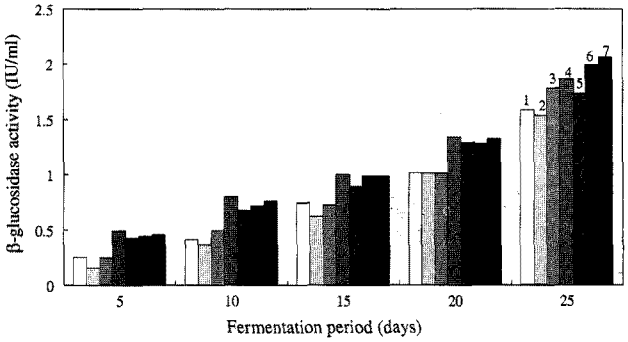

Fig. 3. β-glucosidase activity during simultaneous saccharification fermentation of cashew nut bran by 1. *Trichodermea viride*, 2. *Apergillus terreus*, 3. *Chaetomium globoum*, 4. *T. viride* + *C. globoum*, 5. *C. globoum* + *A. terreus*, 6. *A. terreus* + *T. viride*, 7. Mixed culture.

All the three fungal monocultures showed peak activities of CMCase, FPase and β-glucosidase on the 25th day of fermentation. Similarly Lakshmikant and Mathur (1990) obtained maximum cellulase activity between 16 days and 20 days of incubation using *C. globosum* on various cellulosic substrates.

While CMCase and β-glucosidase activities started dramatically increasing from the 20th and 10th day of SSF respectively, FPase activities were comparatively lower and they increased gradually throughout the experimental period in the monocultures. Even though *A. terreus* grown on corn stover in liquid culture reportedly produced more CMCase, FPase and β-glucosidase earlier than *T. viride* (3), we observed that the production of CMCase and β-glucosidase on cashew nut bran by *A. terreus* was less than that of *T. viride* and *C. globosum*. Direct comparisons of the results obtained in this study with similar results of other researchers are difficult, since many factors, including media composition and choice of substrate affect enzyme activity (Shamala and Sreekantiah, 1986). However similar very low FPase activities as against CMCase and β-glucosidase activities observed in the monocultures in this study were reported by Elshafai *et al.* (1990).

All the three enzyme activities increased in co-cultures, but interestingly FPase showed a 6 fold (in co-cultures containing *C . globosum*) to 10 fold (in co-cultures containing *T . viride* or *A . terreus*) increase in activity in co-cultures. Mixed culture fermentation involving all the three fungi was not effective in increasing the CMCase and β-glucosidase enzyme activities further but showed a 10~15 fold increase in FPase activity over the monocultures.

The increase in saccharification percentage of substrate in the co-cultures may be attributed to the significant increase in activities of all the three components of cellulase enzyme complex in the co-cultures. Canevascini and

Gattlen (1981) observed that complete cellulase production was essential for efficient cellulose degradation.

Though the cellulase activities were maximum on the 25th day of SSF in all the monocultures and co-cultures, the rate of cellulose utilization decreased after the 20th day, indicating that there was no relationship between cellulase production and substrate utilization as was observed earlier for *T. reesei* growing on wheat straw (Maheswari *et al.*, 1993).

Although *A. terreus* monoculture showed lower relative cellulase activity than the other monocultures, β-glucosidase activity of *A. terreus* and *T. viride* co-culture was higher than the other co cultures (1.99 IU/m*l*). This may explain the higher per cent saccharification (88%) induced by this co-culture. Similar synergism between cellulases of *T. viride* and *P. funiculosum* on different paper products has been reported by van Wyk (1998). The significance of β-glucosidase in the overall efficacy of a cellulase enzyme preparation was studied on a comparative basis in *Penicillium funiculosum* and *T. reesei* by Srinivasan and Laxman (1988). The ratio of β-glucosidase to FPase in the enzyme broth is a useful parameter to be considered along with FPase activity itself when reporting activities and productivities of hyper cellulolytic strains.

As there was a concerted increase in both FPase and β-glucosidase activities in the co cultures, they showed better saccharification than the monocultures and mixed culture. These results are corroborated by the views of Srinivasan and Laxman (1988) who observed that a strain with significant extracellular levels both activities would be superior than the strain with high FPase and with a loss in the β-glucosidase secretion.

Determining a specific cellulase requirement for each cellulosic material could make the process of cellulose degradation even more efficient. Ray *et al.* (1993) have emphasized the importance of such synergistic co-cultures and microbial consortia for efficient cellulose degradation and microbial protein production.

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