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Paper mill sludge as a potential source for cellulase production by *Trichoderma reesei* QM 9123 and *Aspergillus niger* using mixed cultivation

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Paper mill waste was tested as a possible substrate for cellulase production using mixed cultures of *Trichoderma reesei* QM 9123 and *Aspergillus niger*, a sludge isolate. The efficacy of the strains both by staggered and simultaneous cultivation were compared. Optimal cellulase production (0.8 mg/ml) along with the best utilization of substrate was observed with mixed cultivation of two strains simultaneously.

INTRODUCTION

Sludge, a waste discharged from pulp and paper mills represents a potential source of lignocellulosic materials which have been rendered accessible to enzymatic attacks by the pulping process. *Trichoderma reesei*, a potent cellulase producer has been studied extensively (Mandels, 1982). It was earlier observed by Grous *et al.* (1985) that complete hydrolysis of cellulose occurs only when *T. reesei* (Rut C-30) producing endo- and exo-glucanases, is supplemented with *Aspergillus niger*, producing cellobiose degrading β-glucosidase activity. Therefore, mixed cultivation of cellulolytic fungi has been an alternative choice for hypercellulase production. Attempts were made using mixed cultures to increase the biomass production of cellulose fermentations (Rolz & Humphrey, 1982). The present investigation was aimed at studying simultaneous and staggered inoculation effects of mixed cultures of *T. reesei* 9123 and *A. niger* on cellulase enzyme synthesis using paper mill sludge as a substrate.

MATERIALS AND METHODS

Sludge was collected from a straw paper mill at Bhopal, India. The strain of *A. niger* was isolated from the sludge using a standard dilution technique (Warcup, 1950). *T. reesei* QM 9123 was obtained from the National Collection of Industrial Microorganisms, Pune, India. Both the strains were grown on agar (1.5%) plates containing Mandel and Sternberg's (1976) cellulose media, which has a pH of 5.5, and incubated at 35°C for 4 days. The medium used for enzyme production was the same as above except for the carbon source which was replaced by the sludge. In the first experiment, *T. reesei* and *A. niger* were inoculated separately each with two mycelial discs (diam. 10 mm) from agar plates. The second experiment was based on staggered inoculation using different ratios of inoculum of the organisms, i.e. one organism was inoculated into a preinoculated culture of another organism after 24 h incubation. In the third set of experiments, two mycelial discs of each organism were inoculated at the same time

and incubated under static conditions at 35°C. The experiment was carried out using inoculum having different combinations of *T. reesei* and *A. niger* at ratios of 1:1, 1:2, 2:1 and 2:2. The ratio 2:2 would mean double the absolute volume of inoculum in each flask. After 8 days, the cultures were filtered and the filtrate analyzed for enzyme activity. The cellulase activity was measured according to Ghose *et al.* (1985) in terms of reducing sugar produced in mg/ml of culture filtrate by the DNSA method using glucose as standard. Extracellular protein was determined according to Lowry's method. The substrate utilized was measured in terms of reducing sugar in mg/ml of the culture filtrate.

RESULTS AND DISCUSSION

Mixed cultures of *T. reesei* and *A. niger* inoculated at the same time gave hypercellulase activity, endo-β-glucanase being maximum (5.2 mg/ml), followed by β-glucosidase (1.8 mg/ml) and exo-β-glucanase (0.7 mg/ml), when compared with staggered inoculation experiments (Fig. 1). In this set of experiments, when inoculation of *A. niger* was followed by that of *T. reesei*, the growth of the latter was restricted due to the strong acidic environment created by *A. niger* as suggested by Ghose *et al.* (1985), where they found a dominant population of *Aspergillus wentii* for the above mentioned reason in a culture phased by *T. reesei*. Enzyme production during staggered inoculation experiments depended on the ratio of the inoculum used (Figs 2 and 3) and was found to be a maximum at a ratio of 2:2. Out of the three enzymes secreted by the organisms, exo-β-glucanase production (0.98 mg/ml) increased substantially in the case of the *A. niger* staggered inoculation experiment, as compared with the *T. reesei* staggered inoculation experiment which yielded only 0.5 mg/ml of the enzyme. The culture containing simultaneous inocula of *A. niger* and *T. reesei* caused a significant increase in the utilization of substrate (620 mg), as compared with the *A. niger* staggered (470 mg) and *T. reesei* staggered (420 mg) inoculation cultures (Table 1). The production of extracellular protein also revealed a similar pattern. The study of the change of pH in mixed culture filtrates

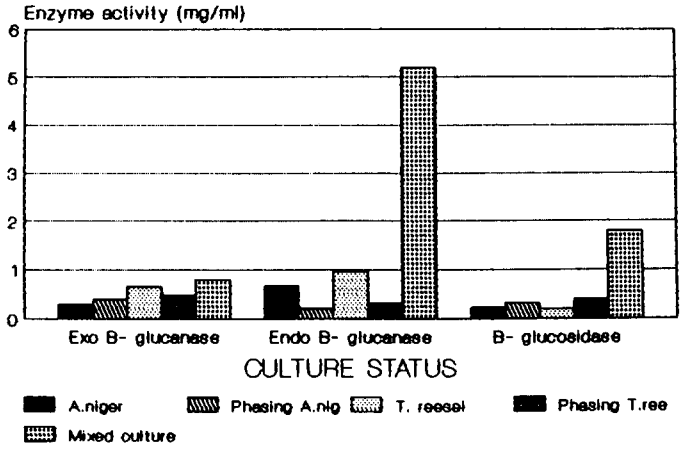

Fig. 1. Enzyme activities by cultures of *T. reesei* and *A. niger*.

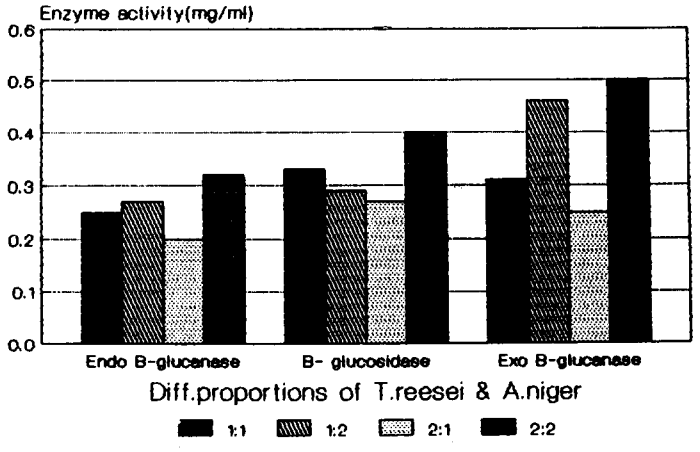

Fig. 2. Enzyme production by mixed cultures (*T. reesi* staggering).

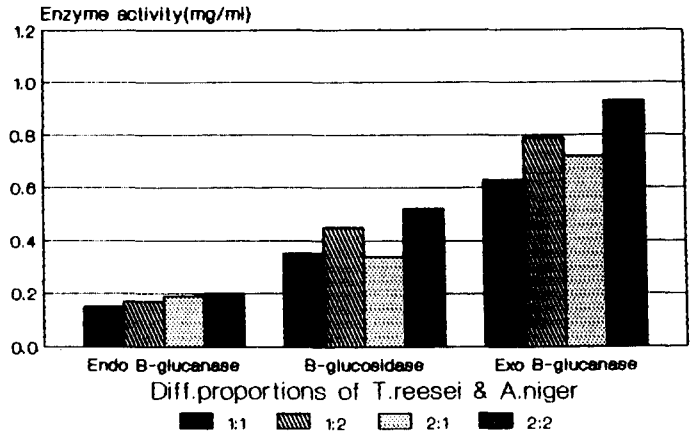

Fig. 3. Enzyme production by mixed cultures (*A. niger* staggering).

Table 1. Substrate utilization and production of extracellular protein by *T. reesei* and *A. niger* at different culture status

Culture status	Substrate utilized (mg/ml)	Extracellular protein (mg/ml)
T. reesei only	348	0·98
A. Niger only	220	0·34
Mixed culture with *A. niger* staggered	470	0·90
Mixed culture with *T. reesei* staggered	400	0·84
A. niger and *T. reesei* mixed simultaneously	620	0·95

presented interesting data. The pH of the medium in the culture filtrate on the eighth day of incubation became less acidic (6.2) when inoculation of *T. reesei* was followed by *A. niger*; however, when both the cultures were inoculated simultaneously the pH turned acidic to 4.8. This observation led to the conclusion that in mixed cultures, one organism acts as a buffering agent and controls the pH drop during sugar utilization. Thus, it perhaps protects β-glucosidase activation and relieves cellobiose inhibition resulting in a better performance by mixed cultures than the staggered inoculum cultures.

In order to enhance cellulase activity, mixed cultures have been proposed by earlier workers (Clementi *et al.*, 1985; Friedrich *et al.*, 1987). Application of mixed cultures is an alternative tool to overcome feedback inhibition and catabolic repression, since the products of one commensal act as a substrate for other commensals (Torre & Campillo, 1984). From these findings it could be concluded that mixed cultures of *T. reesei* and *A. niger* should be exploited to improve fermentations of cellulosic substrates.

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