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Cellulase Production by Co-Culture of *Trichoderma* sp. and *Aspergillus* sp. under Submerged Fermentation

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ABSTRACT

The demand for cellulases is increasing globally because of its potential in the production of cellulosic bioethanol. The major applications of cellulases are in the textile and detergent industries. Cellulases have most promising application in the bioconversion of renewable lignocellulosic biomass into fermentable sugars which can be fermented to ethanol by yeasts. Species of fungi like *Trichoderma* and *Aspergillus* are known to be cellulase producers. Fungi of the genus *Trichoderma* produce relatively large quantities of endo-β-glucanase (E.C.3.2.1.4) and exo-β-glucanase (EC.3.2.1.91), but only low levels of β-glucosidase (EC.3.2.1.21), while those of the genus *Aspergillus* produce relatively large quantities of endo-β-glucanase and β-glucosidase with low levels of exo-β-glucanase production. Furthermore, the β-glucosidases of *T. reesei* are subject to product (glucose) inhibition, whereas, those of *Aspergillus* species are more glucose-tolerant. Most often, *T. reesei* cellulase preparations are supplemented with *Aspergillus* β-glucosidase, for cellulose saccharification on an industrial scale. The present investigation aims to demonstrate cellulase production by co-culture of *Trichoderma* sp. and *Aspergillus* sp. isolated from a degrading wood source. The results showed an increased filter paper activity of 0.46 U/ml for co-culture of *Trichoderma* sp. and *Aspergillus* sp. in the ratio of 1:1, when compared to individual filter paper activities of 0.24 and 0.20 U/ml for *Trichoderma* sp. and *Aspergillus* sp., respectively. There was also an increase in CMCase and β-glucosidase activities of co-culture, when compared to their monoculture counterparts. The maximum CMCase activity was 13.46 U/ml for co-culture (1:1), when compared to CMCase activities of monocultures, i.e., 8.01 U/ml for *Trichoderma* sp. and 6.87 U/ml for *Aspergillus* sp. Maximum β-glucosidase activity of 2.02 U/ml was shown by co-culture (1:1). The monocultures of *Trichoderma* sp. and *Aspergillus* sp. showed much lower levels of β-glucosidase i.e., 0.43 U/ml and 0.98 U/ml respectively.

Keywords: *Aspergillus* sp., cellulase, co-culture, submerged fermentation, *Trichoderma* sp.
Abbreviations: CMCase, carboxymethyl cellulase; **Fpase**, filter paperase; **PASC**, phosphoric acid swollen cellulose; **PDA**, potato dextrose agar

INTRODUCTION

Cellulose is the most abundant renewable carbon resource on earth. It is synthesized mainly by plants and together with hemicelluloses, lignin and pectin, constitutes most of the plant cell wall material. Large amount of cellulose formed annually is degraded by bacteria and fungi to provide themselves with carbon and energy source and for recycling carbon back into the ecosystem. The efficient degradation of cellulose is a complex process involving the synergistic action of a number of cellulolytic enzymes (Eveleigh 2009).

Microorganisms of the genera *Trichoderma* and *Aspergillus* are known to be potential cellulase producers (Bhat 2000; Elad 2000). Currently, most commercial cellulases, including β-glucosidases are produced by *Trichoderma* spp. and *Aspergillus* spp. (Kirk *et al*. 2002; Cherry and Fidantsef 2003). Potential applications of cellulases are in food, animal feed (Ogel *et al*. 2001), textile (Galante *et al*. 1998; Nierstrasz and Warmoeskerken 2003; Ikeda *et al*. 2006) waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Beguin and Anbert 1993; Tarek and Nagwa 2007). In addition, the increasing concerns about the depletion, shortage of fossil fuels and air pollution caused by incomplete combustion of fossil fuel have also led to specific focus on production of cellulosic bioethanol from renewable lingocellulosic substrates (Zaldivar *et al*. 2001; Sun and Cheng 2002).

Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Acharya *et al*. 2008; Chellapandi and Jani 2008). The cellulase system in fungi comprises three hydrolytic enzymes acting synergistically (Lynd *et al*. 2002), endo-1,4-β-D-glucanase [carboxymethyl cellulase (EC. 3.2.1.4)], which cleaves β-linkage randomly in the amorphous parts of cellulose; exo-1,4-β-D-glucanase [cellobiohydrolase (EC. 3.2.1.91)], which hydrolyzes cellobiose from either non reducing or reducing end, generally from the crystalline parts of cellulose; β-glucosidase [cellobiase (EC. 3.2.1.21)], releases glucose from cellobiose and short chain cellooligosaccharides (Rajoka *et al*. 2004; Gray *et al.* 2006; Wilson 2009). The yield of desired enzyme can be increased by establishing the methods of fungal cultivation and optimizing the fermentation conditions (Takao *et al*. 1985).

Fungi of the genus *Trichoderma* produce relatively large quantities of endo-β-glucanase and exo-β-glucanase, but only low levels of β-glucosidase, while those of the genus *Aspergillus* produce relatively large quantities of endo-β-glucanase and β-glucosidase (Reczey *et al.* 1998; Murray *et al.* 2004) with low levels of exo-β- glucanase production. *T. reesei* cellulase system which is deficient in cellobiase, results in the accumulation of the disaccharide cellobiose, which causes repression (Zaldivar *et al.* 2001; Lockington *et al.* 2002). Furthermore, the β-glucosidases of *T. reesei* are subject to product (glucose) inhibition, whereas, those of *Aspergillus* species are more glucose tolerant (Yan and Lin 1997; Gunata and Vallier 1999; Decker *et al.* 2000). The levels of *T. reesei* β-glucosidase are presumably sufficient for growth on cellulose, but not sufficient for extensive *in vitro* saccharification of cellulose. *T. reesei* cellulase preparations, supplemented with *Aspergillus* β-glucosidase, are

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considered most often for efficient hydrolysis of cellulose to produce fermentable sugars on an industrial scale (Reczey *et al*. 1998). Due to low production of β-glucosidase by *Trichoderma* sp. many approaches were suggested to improve degradation of cellulosic material (Kovac *et al.* 2009). In order to enhance cellulase activity and to overcome feedback inhibition and catabolite repression mixed cultures are generally used. The present investigation aims to demonstrate cellulase production in co-culture and monoculture conditions under sumberged fermentation using phosphoric acid swollen cellulose (PASC), by *Trichoderma* sp. and *Aspergillus* sp. isolated from degrading wood source.

MATERIALS AND METHODS

Screening of cellulase producing microorganisms

Different environmental samples like degrading wood, soil were collected from the premises of Osmania University campus. Samples were transported to the laboratory and stored at 4°C. A 10-fold serial dilution was performed and the samples were inoculated by spread plate method, onto potato dextrose agar (PDA) plates for the isolation of fungi. After incubation at 28°C for 120 h, colonies with different morphological forms were picked and sub-cultured onto PDA slants to obtain pure cultures. Stock cultures were maintained on PDA agar at 4°C for subsequent use as inoculum.

The selection of hypercellulase producers was performed on phosphoric-acid-swollen cellulose (PASC) containing Mandel's medium agar plates. Mandel's Medium Composition (g/l): urea: 0.3, NH_4SO_4: 1.4, $CaCl_2 \cdot 2H_2O$: 0.4, KH_2PO_4: 2, $MgSO_4 \cdot 7H_2O$: 0.3, peptone: 1, Tween-80: 0.2, $FeSO_4 \cdot 7H_2O$: 0.005, $MnSO_4 \cdot 7H_2O$: 0.016, $ZnSO_4 \cdot 7H_2O$: 0.014, $CoCl_2 \cdot 6H_2O$: 0.2, PSAC: 10, agar: 17.5, Triton-X 100: 1 ml (Mandels *et al*. 1974; Montenecourt *et al.* 1977). PASC was prepared by soaking cellulose powder (HiMedia, Mumbai) in 1% phosphoric acid overnight (Tansey 1971). The plates were inoculated with pure cultures and incubated at 28°C for 120 h and compared with a known cellulase producer *Trichoderma reesei* NCIM 992. On the 5th day of incubation, the plates were flooded with Gram's Iodine and left for 5 min., at room temperature (Kasana *et al*. 2008). Zones of hydrolysis were observed around the colonies developed on the plates (**Fig. 1**). Based on the diameter of zones of hydrolysis, two fungal strains (*Trichoderma* sp. and *Aspergillus* sp.) were selected, isolated and cultured onto fresh PDA slants. The fungi *Trichoderma* sp. and *Aspergillus* sp. in our studies were identified based on their cultural characteristics and microscopic morphology (Gilman 1975).

Cellulase enzyme production

1. Inoculum preparation

The two selected fungal strains were maintained as stock cultures on PDA slants by growing at 28°C for 5 days and stored at 4°C for regular subculturing. For preparation of fungal inocula, about 2 ml of sterile distilled water containing 0.1% tween-80 was added to 120 h old PDA slants of each fungus and the spores were dislodged into the liquid with the help of an inoculation loop. The spore suspensions of *Trichoderma* sp. and *Aspergillus* sp. containing ~10^6 spores/ml were aseptically inoculated into 100 ml of inoculum media in two separate 250 ml flasks, with media composition (g/l): Corn steep liquor [CSL]: 26.8; KH_2PO_4: 2; $CaCl_2$: 0.3; $MgSO_4 \cdot 7H_2O$: 0.3; $FeSO_4 \cdot 7H_2O$: 0.005; $ZnSO_4 \cdot 7H_2O$: 0.0015; $MnSO_4.H_2O$: 0.0014; $CoCl_2$: 0.002; Cellulose: 20; $(NH_4)_2SO_4$: 1.4; Urea: 0.3; peptone: 0.1; Tween-80: 0.3%. The flasks were kept in a shaker incubator at 150 rpm, 28°C for 72 h for inoculum development before they were used for the submerged fermentation process.

2. Submerged fermentation

Fermentation medium for cellulase production was prepared with composition (g/l): KH_2PO_4: 1; $CaCl_2$: 0.3; Urea: 0.3; $MgSO_4$: 0.3; $(NH_4)_2SO_4$: 1.4; Peptone: 5; $FeSO_4 \cdot 7H_2O$: 0.005; $MnSO_4 \cdot H_2O$: 0.006; $ZnSO_4 \cdot 7H_2O$: 0.0014; $CoCl_2$: 0.002; Tween-80: 1; Na_2HPO_4: 1; Yeast extract: 1; Glucose: 1; PASC: 10. A 5% (v/v) inoculum of each organism (monoculture and co-culture) was transferred to 250 ml flask separately containing 100 ml of the fermentation medium. Five sets of fermentations were carried out with monocultures of *Trichoderma* sp. and *Aspergillus* sp. and co-cultures of both *Trichoderma* sp. and *Aspergillus* sp. in different ratios of 1:1, 1:2, 2:1 for 144 h (6 days) at 150 rpm, 28°C.

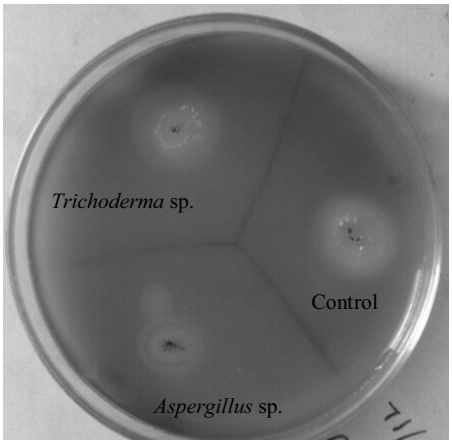

Fig. 1 ***Trichoderma sp. and Aspergillus sp.*** **on Mandel's medium plate with PASC, showing zones of hydrolysis, comparable to a known cellulase producer,** ***Trichoderma reesei*** **NCIM 992 standard strain, used as control.** (n = 3)

Determination of enzyme activity

Fermented samples were withdrawn for determination of enzyme activity from 96th hour of incubation for every 24 h. Cellulase activity (FPA) was analysed on filter paper, according to Ghose (1987). One cellulase unit was defined as the amount of enzyme required for liberating 1 µmol of glucose per ml per minute per ml culture filtrate that released 1 microgram of glucose per minute. Carboxy methyl cellulase (EC.3.2.1.4) activity was determined with carboxy methyl cellulose as substrate (Ghose 1987). One unit of CMCase was defined as the amount of enzyme required for liberating 1 µmol of reducing sugar per ml per minute. β-glucosidase (EC.3.2.1.21) activity was determined by using salicin as the substrate (Jeffries 1987). One unit of enzyme corresponds to the amount of enzyme necessary to form 1 µmol of glucose per ml per minute. The reducing sugars were measured by the dinitrosalicylic acid (DNS) method according to Miller (1959).

Experimental design

The two cellulase producing organisms *Trichoderma* sp. and *Aspergillus* sp. isolated from a degrading wood source were used for cellulase production by submerged fermentation. The two organisms *Trichoderma* sp. and *Aspergillus* sp. were selected depending upon zone of hydrolysis in comparison with control (*Trichoderma reesei* NCIM 992). Determination of zones of hydrolysis was done in triplicates (n = 3). Five sets of fermentations were carried out separately in 250 ml erlenmeyer flasks containing 100 ml of the fermentation medium by transferring 5% (v/v) inoculum of *Trichoderma* sp. and *Aspergillus* sp. and co-cultures of both in different ratios of 1:1, 1:2, 2:1 for 144 h (6 days) at 150 rpm, 28°C. Fermentation experiments were carried out in six batches (n = 6).

Statistical analysis

To assess whether there was any significant difference between the monocultures and co-cultures of *Trichoderma* sp. and *Aspergillus* sp. for cellulase activity, a paired sample *t*-test was performed using SPSS (software for windows release, 17.0, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Screening and isolation of cellulolytic fungi

During the screening process, two fungi were selected i.e., *Trichoderma* sp. and *Aspergillus* sp. for detection of their potential to produce cellulase. They were inoculated along with control organism (*Trichoderma reesei* NCIM 992) for comparison, onto PASC containing Mandel's mineral agar (HiMedia). At the end of 120 h, the plates were flooded with Gram's iodine solution for observation of zone of hydrolysis and its measurement (**Fig. 1**). It is clearly evident that the zones of hydrolysis of the two isolates, *Trichoderma* sp. (1.1 cm) and *Aspergillus* sp. (1.0 cm) are significantly comparable to the control organism, *Trichoderma reesei* NCIM 992 (1.2 cm). The cellulolytic activities of the isolates were further evaluated by subjecting them to cellulase production under submerged fermentation.

Cellulase production under submerged fermentation

When shake flask fermentations by mono and co-cultures of *Trichoderma* sp. and *Aspergillus* sp. were carried out, the maximum enzymatic activity was shown by co-culture of *Trichoderma* sp. and *Aspergillus* sp. (1:1) with activities of 0.46 U/ml for Fpase (**Fig. 2**), 13.46 U/ml for CMCase (**Fig. 3**) and 2.02 U/ml for β-glucosidase (**Fig. 4**), at the end of 120 h of fermentation, which were significantly more when compared to their monoculture counterparts i.e. *Trichoderma* sp. (0.24 U/ml for FPase, 8.01 U/ml for CMCase and 0.43 U/ml for β-glucosidase) and *Aspergillus* sp. (0.20 U/ml for FPase, 6.87 U/ml for CMCase and 0.98 U/ml for β-glucosidase) (**Table 1**). The co-cultures of *Trichoderma* sp. and *Aspergillus* sp. in the ratios of 1:2 and 2:1 showed 0.27 U/ml for FPase, 7.92 U/ml for CMCase and 1.23 U/ml for β-glucosidase and 0.24 U/ml for FPase, 7.16 U/ml for CMCase and 1.01 U/ml for β-glucosidase respectively. Our results were in close comparison with those of Vyas and Vyas (2005), where the maximum cellulase activity was 0.457 U/ml with a co-culture of *Aspergillus terreus* and *Trichoderma viridae*, using ground nut shells under SSF. Ahamed and Vermette (2008) investigated production of cellulase by co-culturing *Trichoderma reesei* and *Aspergillus niger* in a bioreactor to convert cellulose substrate into soluble sugars through a synergetic action of enzyme complex simultaneously produced by these two fungi. The results of mixed culture experiments exhibited a highly significant increase in production of filter paper activity (7.1 U/ml).

Statistical evaluation of cellulase production by co-culture of *Trichoderma* sp. and *Aspergillus* sp. under submerged fermentation

The data for zone of hydrolysis between *Trichoderma* sp., *Aspergillus* sp. and control (*Trichoderma reesei* NCIM 992) was subjected to paired or dependent sample "t" test to find the significant relationship between all the organisms. All the three organisms were subjected to different combinations of paired sample (*Trichoderma* sp.-*Aspergillus* sp., *Trichoderma* sp. control, *Aspergillus* sp. control) to find out the significant difference and correlation among them. The test yielded positive *t* values for all the three organisms indication that the zone of hydrolysis (**Table 2**) of *Trichoderma* sp. and *Aspergillus* sp., was highly comparable to the control organism *i.e., Trichoderma reesei* NCIM 992. Further, there was no significant difference in zone of hydrolysis between the control and other two organisms (*Trichoderma* sp. and *Aspergillus* sp.) i.e., $P > 0.05$. Hence we can conclude that there is a significant and positive correlation between zones of hydrolysis of *Trichoderma* sp. and *Aspergillus* sp. and control organism (**Table 2**).

For cellulase production by monoculture and coculture fermentation, there was a significant difference for co-culture fermentation in comparison with mono culture fermentation by *Trichoderma* sp. and *Aspergillus* sp. (**Table 1**, Sig – 0.000, 0.000, 0.268). We could not find any significant difference among the monocultures i.e., *Trichoderma* sp. and *Aspergillus* sp. ($P > 0.05$).

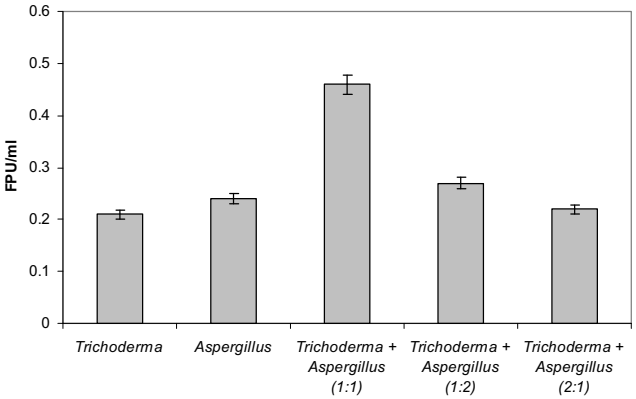

Fig. 2 Filter paper activities of mono- and co-cultures. (n = 6)

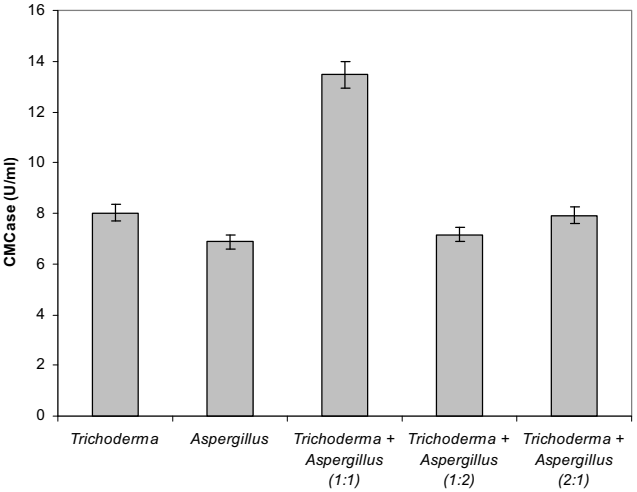

Fig. 3 CMCase activities of mono- and co-cultures. (n = 3)

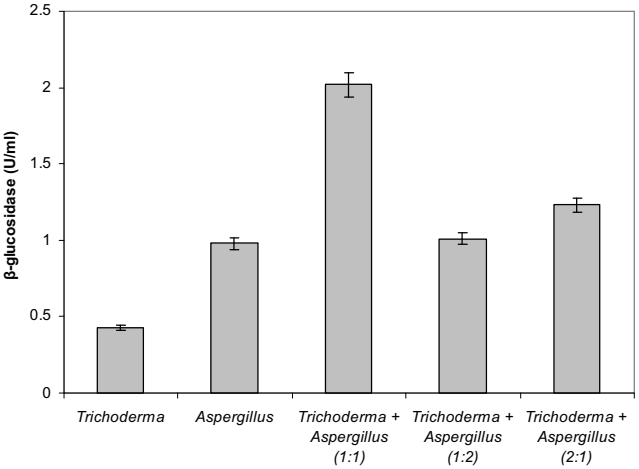

Fig. 4 β-glucosidase activities of mono- and co-cultures. (n=3)

CONCLUSION

In the present study, the co-culture of the two isolates when taken in equal ratio (1:1) produced high amount of cellulase (maximum enzyme activities of Fpase, CMCase and β-glucosidase were 0.46, 13.46 and 2.02 U/ml, respectively). This shows that both the organisms complement each other, one producing more exoglucanase (*Trichoderma* sp.) and

Table 1 Statistical evaluation (paired differences (dependent sample *t*-test) of cellulase production by monocultures and co-cultures of *Trichoderma* sp., *Aspergillus* sp.) at a 95% confidence interval of the difference.

	Mean	Standard deviation	Standard error mean	Lower	Upper	*t*	df	Sig (2-tailed)
Pair 1	2.41889	2.28754	0.53918	1.28132	3.55646	4.486	17	0.000
Pair 2	2.63000	2.91083	0.68609	1.18248	4.07752	3.833	17	0.001
Pair 3	0.21111	0.78245	0.18442	-0.17799	0.60021	1.145	17	0.268

Pair 1: T1A1 TRI-M (T1 : *Trichoderma* sp. ; A1 : *Aspergillus* sp. ; TRI-M : *Trichoderma* monoculture)
Pair 2: T1A1 ASP-M (T1 : *Trichoderma* sp. ; A1 : *Aspergillus* sp. ; ASP-M : *Aspergillus* monoculture)
Pair 3: TRI-M ASP-M (TRI-M : *Trichoderma* sp. monoculture ; ASP-M : *Aspergillus* monoculture)

Table 2 Statistical evaluation (paired differences (dependent sample *t*-test) of zones of hydrolysis of *Trichoderma* sp., *Aspergillus* sp. and control (*Trichoderma reesei* NCIM 992) at a 95% confidence interval of the difference.

	Mean	Mean differences	Standard deviation	Lower	Upper	*t*	Sig (2-tailed)
Pair 1	1.0000	0.10000	0.10000	- 0.14841	0.34841	1.732	0.225
Pair 2	1.2000	0.20000	0.30000	- 0.54524	0.94524	1.155	0.368
Pair 3	1.1000	0.10000	0.20000	- 0.39683	0.59683	0.866	0.478

** $P > 0.05$
Pair 1: *Trichoderma* sp.: *Aspergillus* sp.
Pair 2: Control (*Trichoderma reesei* NCIM 992) : *Aspergillus* sp.
Pair 3: Control (*Trichoderma reesei* NCIM 992) : *Trichoderma* sp.

the other producing more levels of β-glucosidase (*Aspergillus* sp.). All the components of cellulase are produced under same fermentation conditions and the organisms acting synergistically under optimized culture conditions for co-culturing. The enhanced production of cellulase in co-culture can be exploited for further research to establish increased production/application of cellulases by cost-effective methods.

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