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

Sridevi Jagavati · Vimala Rodhe Adivikatla · Nirupama Paritala · Venkateswar Rao Linga*

Department of Microbiology, Osmania University, Hyderabad-500 007, India

Corresponding author: * vrlinga@gmail.com

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**ABSTRACT**

The demand for cellulates is increasing globally because of its potential in the production of cellulosic bioethanol. The major applications of cellulates are in the textile and detergent industries. Cellulates 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 cellulate producers. Fungi of the genus *Trichoderma* produce relatively large quantities of endo-β-glucanase (E.C.3.2.1.4) and exo-β-glucanase (E.C.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* cellulate preparations are supplemented with *Aspergillus* β-glucosidase, for cellulate saccharification on an industrial scale. The present investigation aims to demonstrate cellulate 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., cellulate, 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 cellulate producers (Bhat 2000; Elad 2000). Currently, most commercial cellulates, including β-glucosidases are produced by *Trichoderma* spp. and *Aspergillus* spp. (Kirk et al. 2002; Cherry and Fidantsef 2003). Potential applications of cellulates 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, protein 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 lignocellulosic substrates (Zaldivar et al. 2001; Sun and Cheng 2002).

Cellulose is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Acharya et al. 2008; Chellappandi 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 [celllobiohydrolase (EC. 3.2.1.91)], which hydrolyzes celllobiose from either non-reducing or reducing end, generally from the crystalline parts of cellulose; β-glucosidase [celliohioase (EC. 3.2.1.21)], which releases glucose from celllobiose and short chain celloligosaccharides (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 (Tako 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* cellulate system which is deficient in cellobiase, results in the accumulation of the disaccharide cellobiose, 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* cellulate preparations, supplemented with *Aspergillus* β-glucosidase, are...
considered most often for efficient hydrolysis of cellulose to produce fermentable sugars on an industrial scale (Recezy et al. 1998). Due to low production of \( \beta \)-glucosidase by Trichoderma sp., many approaches were suggested to improve degradation of cellulotic 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 submerged 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\(_4\)SO\(_4\): 1.4, CaCl\(_2\): 2H\(_2\)O: 0.4, KH\(_2\)PO\(_4\): 2, MgSO\(_4\).7H\(_2\)O: 0.3, peptone: 1, Tween-80: 0.2, FeSO\(_4\).7H\(_2\)O: 0.005, MnSO\(_4\).7H\(_2\)O: 0.016, ZnSO\(_4\).7H\(_2\)O: 0.014, CoCl\(_2\)-6H\(_2\)O: 0.2, PSAC: 10, agar: 17.5, Triton-X 100: 1 ml (Mandels et al. 1974; Montencourt 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 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.

2. Submerged fermentation

Fermentation medium for cellulase production was prepared with composition (g/l): KH\(_2\)PO\(_4\): 1; CaCl\(_2\): 0.3; Urea: 0.3; MgSO\(_4\): 0.3; (NH\(_4\))\(_2\)SO\(_4\): 1.4; Peptone: 5; FeSO\(_4\).7H\(_2\)O: 0.005; MnSO\(_4\).H\(_2\)O: 0.006; ZnSO\(_4\).7H\(_2\)O: 0.0014; CoCl\(_2\): 0.002; Tween-80: 1; Na\(_2\)HPO\(_4\): 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.

**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 ml filterate 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. \( \beta \)-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 1 ml inoculum of each organism (monoculture and co-culture) to 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.

**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 Vyasa (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).

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
the other producing more levels of \( \beta \)-glucosidase (Aspergillus \( sp \).). All the components of cellulase are produced under the 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 explored for further research to establish increased production/application of cellulases by cost-effective methods.

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**REFERENCES**


Erikson KE, Patterson B (1975) Extra cellulosic enzyme system produced by the fungus \( \text{Sporotrichichum palustrivale} \). Biotechnology and Bioengineering 20, 317-322.


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

<table>
<thead>
<tr>
<th>Mean</th>
<th>Standard deviation</th>
<th>Standard error mean</th>
<th>Lower</th>
<th>Upper</th>
<th>( t )</th>
<th>df</th>
<th>Sig (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td>2.41889</td>
<td>2.28754</td>
<td>0.53918</td>
<td>1.28132</td>
<td>3.55646</td>
<td>4.486</td>
<td>17</td>
</tr>
<tr>
<td>Pair 2</td>
<td>2.63000</td>
<td>2.91083</td>
<td>0.86680</td>
<td>1.18248</td>
<td>4.07752</td>
<td>3.833</td>
<td>17</td>
</tr>
<tr>
<td>Pair 3</td>
<td>0.21111</td>
<td>0.78245</td>
<td>0.18442</td>
<td>-0.17709</td>
<td>0.60021</td>
<td>1.145</td>
<td>17</td>
</tr>
</tbody>
</table>

**Pair 1:** T1A1 TRIM (T1: \( \text{Trichoderma sp.} \), A1: \( \text{Aspergillus sp.} \); TRIM: \( \text{Trichoderma monocolure} \)).

**Pair 2:** T1A1 ASP-M (T1: \( \text{Trichoderma sp.} \), A1: \( \text{Aspergillus monocolure} \)).

**Pair 3:** TRIM ASP-M (TRIM: \( \text{Trichoderma sp. monocolure} \); ASP-M: \( \text{Aspergillus monocolure} \)).