EXPLORING FIBROUS BYPRODUCTS AS SUBSTRATES TO GENERATE FIBROLYTIC ENZYMES

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ABSTRACT
This research was focused to identify suitable fibrous byproducts to grow Trichoderma viridae and optimize the conditions for the production of fibrolytic enzymes viz. cellulase and xylanase. An experiment was carried out on agro-industrial fibrous byproducts viz, coconut coir pith, saw dust, groundnut shells, paddy husk and sugarcane bagasse to select best substrates for Trichoderma viridae. The selection was based on nine measurements on a score card that gave weightage to parameters such as physical growth of Trichoderma viridae, absence of contamination, spore count on the 10th day, cellulase and xylanase activity of enzyme extract on 10th day. Among the agro-industrial fibrous byproducts paddy husk inoculated at the rate of 10^6 spores / 5 gram in 10 days of incubation at 30°C at pH 5 and 70 per cent moisture showed good physical growth of Trichoderma viridae, with no contamination, had higher spore count and was the only substrate that evinced activity of cellulase and xylanase.

Key words: Fibrolytic enzymes, Fibrous byproducts, Paddy husk, Trichoderma viridae.

INTRODUCTION
High cost of production of plant cell-wall hydrolyzing enzymes is a limiting factor in their commercial production and industrial applications (Spano et al., 1978). One area currently considered as cost-reduction strategy is the use of waste plant materials as carbon sources for the production of the enzymes. Solid state fermentation using lingo-cellulosic wastes as substrates can reduce the cost of cellulase production (Xia and Cen, 1999). It was in this line that locally available fibrous byproducts were screened for their suitability in production of cellulase and xylanase using solid state fermentation with Trichoderma viridae.

MATERIALS AND METHODS
Trichoderma viridae was selected as inoculum source because it has served as a model for fungal lingo-cellulosic degradation (Ooshima et al., 1983) and does not produce any deleterious effect like Aspergillus niger. Freeze dried cultures of Trichoderma viridae pers IMI 304060 (MTCC catalogue no.800) were procured from Microbial type culture collection and gene bank, Institute of Microbial Technology, Chandigarh, India and stored at 4°C.

The freeze dried culture of Trichoderma viridae was subcultured on potato dextrose agar slants (Aberkane et al., 2002) and incubated at 30°C (Singh and Gupta, 2008). Inoculum suspensions were prepared from fresh, mature (7 day old) cultures as per the procedure adopted by Aberkane et al. (2002). The colonies were covered with 5 ml of distilled sterile water containing 1% Tween 20. Then, the conidia were rubbed carefully with a sterile cotton swab and transferred to a sterile tube; the resulting suspensions were homogenized for 15 seconds with a gyratory vortex mixer at 2000 rpm. Appropriate dilutions were performed in order to get the desired concentration for counting in a cell-counting haemocytometer. All inocula preparations were checked for the presence of hyphae or clumps by a previous examination in the cell-counting haemocytometer chamber. Upon detection of significant number of hyphae (>5% of fungal structures), 5 ml suspension was transferred to a sterile syringe attached to a sterile filter with a pore diameter of 11 im (Millipore), filtered and collected in a sterile tube. This step removed hyphae and yielded a suspension composed of spores. If clumps
were detected, the inoculum was shaken again in the gyratory vortex mixer at 2000 rpm for a further 15 seconds. This step was repeated as many times as necessary if clumps were visualized again.

The final inoculum size was adjusted to a range of $10^6$ spores/ml by microscopic enumeration with a cell-counting haemocytometer (Sharma, 1989).

Three samples each of agro-industrial fibrous byproducts viz., coconut coir pith, saw dust, groundnut shells, paddy husk and sugarcane bagasse were collected as test substrates from their respective agro-industries located in Tamil Nadu. The collected fibrous byproducts were sun dried, coarsely ground to uniform size (0.5 mm) in a vortexer (Ojumo et al., 2003) and stored in glass containers. These fibrous byproducts will be hereinafter referred to as 'substrates'.

Initial moisture content of substrates was estimated (AOAC, 2000) and known quantities of sterile water considering the initial moisture content of the substrate and inocula water content, were added to obtain the desired substrate moisture levels (Pang et al., 2006). The desired moisture level in this experiment was chosen as 70%.

The pH considered for this experiment was 5 because optimum biomass production of Trichoderma viridae occurs at a pH range of 4.6 to 6.8 (Jackson et al., 1991). The substrate pH was initially determined (AOAC, 2000) and the pH was adjusted either by adding 1 M HCl or 1 M NaOH (Shafique et al., 2004).

Five gram of each substrate viz coconut coir pith, saw dust, groundnut shells, paddy husk and sugarcane bagasse were taken in individual flasks and were plugged with non-absorbent cotton and autoclaved at 121°C for 15 min. Inoculum containing the spore density of $10^6$ spores/ml of substrate was aseptically inoculated (one ml per 5 g substrate) into each flask containing the substrate. The flasks were incubated at 30°C for ten days in an incubator. In order to control the humidity of the incubator an additional flask filled with water was placed inside (Ustoka and Tarib, 2007). At the end of the incubation period the flasks were examined for the physical growth of Trichoderma viridae and presence or absence of contamination.

The spore count of the incubated substrates at the end of the incubation period was carried out by adding 50 ml of 0.1 M sodium acetate buffer of pH 4.8 at 30°C shaking it for one hour so that the spores are suspended in the buffer. The content was filtered in Whatman filter paper No 1 (11 µ) and filtrate was subjected to spore count. Nine microlitre of this spore suspension (filtrate) was taken and loaded into one of the counting chambers of the haemocytometer. All the spores in each of the four 0.1 mm³ corner squares were counted and their average was calculated. The spore count was calculated using the equation Spores/ml = n x 10⁴, where n is average spore count per square of the four corner squares counted. The spore count was expressed as n x 10⁶ spores per ml.

At the end of the incubation period, the fibrolytic enzymes from the respective substrates were extracted by a simple contact method (Krishna and Chandrasekaran, 1996). The fermented samples were shaken (150 rpm) with 50 ml of 0.1 M sodium acetate buffer of pH 4.8 at 30°C for one hour and filtered through Whatman filter paper No 1. The filtrates were centrifuged at 10,000 rpm (4°C) for 15 min to remove spores of the organism and supernatants were used as crude enzyme extracts for enzyme assay. The crude enzyme extracts were assayed for cellulase and xylanase activities as per dinitrosalicylic acid (DNSA) reducing sugar method (Miller, 1959). Enzyme activity was calculated based on sugar release (glucose/ xylose) and was expressed as U/ five gram of substrate.

In order to identify and select the best substrate for further optimization studies, a 10 point score card system was evolved. The weightage was given to parameters such as physical growth, absence of contamination, spore count on the 10th day, cellulase and xylanase activity of enzyme extract on 10th day. Nine measurements were made for each of substrate and weightage were recorded accordingly.

RESULTS AND DISCUSSION
Table 1 gives details of physical growth of Trichoderma viridae, presence or absence of contamination in substrates, spore count per five gram of substrate, cellulase and xylanase activity (U) per gram of substrate on 10th day after inoculation of substrates with $10^6$ spores of Trichoderma viridae.
TABLE 1. Physical growth of *Trichoderma viridae*, presence or absence of contamination, spore count (per g), cellulase and xylanase activity (U/g) on tenth day after inoculation of substrates with 10⁶ spores of *Trichoderma viridae*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coconut coir pith</th>
<th>Saw dust</th>
<th>Paddy husk</th>
<th>Sugarcane bagasse</th>
<th>Ground nut shells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical growth</td>
<td>Good</td>
<td>Poor</td>
<td>Good</td>
<td>Poor</td>
<td>Good</td>
</tr>
<tr>
<td>Contamination</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Spore count/g substrate</td>
<td>0.17 ± 0.01 x 10⁶</td>
<td>1.49 ± 0.16 x 10⁶</td>
<td>37.59 ± 0.42 x 10⁶</td>
<td>0.22 ± 0.14 x 10⁶</td>
<td>0.05 ± 0.02 x 10⁶</td>
</tr>
<tr>
<td>Cellulase activity U/g substrate</td>
<td>Nil</td>
<td>Nil</td>
<td>12.17 ± 0.02</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Xylanase activity U/g substrate</td>
<td>Nil</td>
<td>Nil</td>
<td>21.44 ± 1.22</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Mean of 9 measurements

Based on the score card system adopted, scores were given to each of the parameter studied for all the substrates and is presented in Table 2. Best substrate was selected based on the overall score secured.

Among all the agro-industrial fibrous byproducts *viz.*, coconut coir pith, saw dust, groundnut shells, paddy husk and sugarcane bagasse used as substrates, paddy husk showed good physical growth of *Trichoderma viridae*, with no contamination, had higher spore count than other substrates and was the only substrate that evinced activity of cellulase and xylanase. Thus paddy husk got an overall highest score of 10.

Cavalcante *et al.* (2007) while evaluating the use of low-cost substrates to produce *Trichoderma* spores using rice, corn bran, and wheat bran as solid substrate to grow *Trichoderma harzianum* spp., *Trichoderma viride* spp., *Trichoderma koningii* spp. and *Trichoderma polysporum* spp. with no external nutrient sources, found that high spore counts were obtained for *T. harzianum* spp. (28.30×10⁶/gds) and *T. viride* spp. (24.10×10⁶ spores/gds). Wherein in the present study low spore counts ranging between 0.05×10⁶ to 37.59×10⁶/g substrate were observed.

Similar to the results obtained in the present study Hong *et al.* (2010) also reported that coconut fibre and wood dust did not support the growth of *Trichoderma* spp. Vyas *et al.* (2005) reported that they could use groundnut shells for cellulase production using *Trichoderma viride* only after pretreatment with sodium hydroxide. Similarly, Muniswaran and Chariyulu (1994) also opined that hydrogen peroxide-pretreated coconut coir pith was found to be better for the production of cellulase enzyme by solid substrate cultivation of *Trichoderma viride*. Sugarcane bagasse though a carbon source for fungal culture and enzyme production, required chemical pretreatment for turning the cellulose in it to a more amenable form for fungal attack (Auguiar, 2001).

In contrast to present study Hong *et al.* (2010) had reported that *Trichoderma viride* did not grow well on paddy husk and therefore yielded only lower quantity of fermentable sugars.

It is evident from this experiment that the different agro-industrial byproducts used *viz.*, coconut coir pith, saw dust, groundnut shells, paddy husk and sugarcane bagasse behaved differently in their capacity to support the growth of *Trichoderma*...
TABLE 2. Mean score secured by each substrate on tenth day after inoculation of substrates with 10⁶ spores of *Trichoderma viridae*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coconut coir pith</th>
<th>Saw dust</th>
<th>Paddy husk bagasse</th>
<th>Sugarcane bagasse</th>
<th>Ground nut shells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical growth</td>
<td>1.42</td>
<td>0.79</td>
<td>2</td>
<td>0.83</td>
<td>1.17</td>
</tr>
<tr>
<td>Contamination</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Spore count / g</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Cellulase activity U / g substrate</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xylanase activity U / g substrate</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total Score</td>
<td>3.42</td>
<td>4.79</td>
<td>10</td>
<td>4.83</td>
<td>3.17</td>
</tr>
</tbody>
</table>

Mean of 9 observations

*Trichoderma viridae* and produce fibrolytic enzymes. This is because factors such as diffusion within the solid substrate, mass transfer to and from the solid substrate and the hydrophilic and hydrophobic nature of the solid particles are important factors in the adherence of the fungus to the solid substrate. These factors in turn affect enzyme production (Hong et al., 2010). The nature of solid substrate is a major factor for solid state fermentation. It not only supplies the nutrients to the culture but also serves as an anchorage for the growth of the fungus (Sharma et al., 2008).

Most of the industrial by products used viz., coconut coir pith, saw dust, groundnut shells and sugarcane bagasse were not able to support growth of *Trichoderma viridae* and produce enzymes as these substrates generally require pretreatment to make their chemical constituents more accessible and their physical structure more susceptible to mycelial penetration (Manpreet et al., 2005). Pretreatment causes the removal of lignin and hemicelluloses effectively, while at the same time loosening the structure of lignin and decreasing the crystallinity of digestible cellulose that improves the porosity characteristic of substrate which is essential for growth of *Trichoderma viridae*.

Paddy husk was found to support growth of *Trichoderma viridae* and produce enzymes even without pretreatment because structurally it is thinner and coarser providing greater aeration that is a critical factor for growth of any microorganism in solid state fermentation. Kapilan and Arasaratinam (2011) had also used paddy husk as substrate for solid state fermentation to produce xylanase from *Bacillus pumilus*.

REFERENCES


