Production of Endoglucanase by *Trichoderma* for Control of Phytopathogenic Fungus *Sclerotium Rolfsii*

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**Abstract:** The three clones of *Trichoderma* were cloned in the yeast expression vector pYES2/CT namely, β-1, 6-endoglucanase from *Trichoderma harzianum* (pYESH2) and *Trichoderma virens* (pYESV1) and β-1, 4-endoglucanase from *Trichoderma reesei* (pYESR1). These were screened for glucanase activity in yeast host *Saccharomyces cerevisiae* strain INVSc1. Out of these organisms, a strain identified as *T. reesei* Rifai secreted highest activities. *In vitro* production of endoglucanase by *T. reesei*, a mycoparasite of phytopathogenic fungi, was examined under inducible conditions of GAL1 promoter of yeast vector pYES2/CT. Enzyme production was significantly influenced by the 2% galactose incorporated into the medium and was stimulated by acidic pH from 5.5 to 6.0. Galactose (2%) addition along with SC-U medium enhanced the production of these enzymes. Growth of *S. rolfsii* was significantly inhibited (based on the radial diameter of *S. rolfsii*) by enzyme preparations from *Trichoderma*. *T. reesei* has shown three times more, *T. harzianum* and *T. virens* has shown twice the activity when compared to control INVSc1 having pYES2/CT vector as a control.

**Key words:** *Trichoderma*, endoglucanase, *Saccharomyces cerevisiae*, INVSc1, antagonism, *Sclerotium rolfsii*, inhibition

**INTRODUCTION**

Some species of *Trichoderma* have been described as biological control agents against several fungal plant pathogens [15]. The degradation and further assimilation of phytopathogenic fungi, namely, mycoparasitism, has been proposed as the major mechanism accounting for the antagonistic activity of *Trichoderma* species against fungal pathogens [4]. From recent work, it appears that *Trichoderma* mycoparasitism is a complex process involving several successive steps [5]. Initially, the mycoparasite grows directly towards its host and often coils around it or attaches to it by forming hook-like structures and appressoria [6]. Following these interactions, *Trichoderma* spp. sometimes penetrates the host mycelium, apparently by partially degrading its cell walls [15].

The plant pathogen basidiomycete *S. rolfsii* causes stem and pod rots which are major constraints to groundnut production in many groundnut-growing regions: e.g. in the USA southern blight of peanuts is a problem in all peanut-producing states and has to be controlled primarily by the use of fungicides. Furthermore, the fungus causes disease in over 500 plant species. Recently, the fungus has also been found in Europe on different hosts including juglans and sunflowers [3, 9].

With a view to establish that the mechanism in the process of parasitism of *S. rolfsii* by a newly isolated strains of *Trichoderma* involves the release of hydrolytic enzymes by the latter, the objectives of this study was to assess the role of these enzymes in parasitism and in antagonism. *Saccharomyces cerevisiae* was used as a tool for expression of the enzyme.

**MATERIAL AND METHODS**

**Cloning of Glucanase Genes:** The glucanase genes were cloned into the vector pYES2/CT (Invitrogen Catsbad, CA) for inducible expression in Brewer’s yeast (Figure 1.). The positive clones obtained were used for endoglucanase expression in INVSc1 Brewer’s yeast cells (MATα his 3Δ1 leu2 trp1-289 ura3-52/MATα his 3Δ1 leu2 trp1-289 ura3-52/ura3-52 Invitrogen). These clones were transformed into *Saccharomyces cerevisiae* INVSc1 using Li acetate method [8]. As a control, yeast cells were also
transformed with the vector pYES2/CT. The positive clone were isolated and maintained on SC-U medium with 2% glucose.

**Induction for Expression by Galactose:** For induction, a single colony of INVSc1 containing recombinant pYES2/CT (pYESH2, pYESR1 and pYESV1) along with control (pYES2/CT without gene) was inoculated into 50ml of appropriate SC selective medium containing 2% glucose. It was grown overnight at 30°C with shaking at 200 rpm. Next day, the cells were harvested at 4°C at the rate of 13,000 rpm for 1 minute. The supernatant was discarded and the cells were resuspended in 200 ml of SC-U liquid medium containing 2% raffinose along with 10mg/lit adenine. The mixture was kept at 30°C with shaking at the rate of 200 rpm for 3 days. After 3 days of shaking the cells were centrifuged at 13,000 rpm for 1 minute at 4°C. The supernatant was discarded and the cells were resuspended in 50 ml of induction medium having 2% galactose. The mixture was kept at 30°C with shaking at the rate of 200 rpm for 3 days. In all the cases pH was maintained from 5.5 to 6.0. The samples were centrifuged at 4°C for 1 minute at the rate of 13,000 rpm. The supernatant and the cell pellets were stored at -20°C for further use.

**Biochemical Methods:** Glucanase activity was determined by using supernatant and cell lysates. A general protocol for small-scale preparation of cell lysates using acid-washed glassbeads was followed [1]. To prepare the cell lysates frozen cells were used. The frozen cell pellet was resuspended in 500 µl of breaking buffer (10 mM Tris-HCl, pH8.0, 1mM Na2EDTA, 100 mM NaCl, and 0.1% [w/v] SDS). The cells were centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 200 µl of breaking buffer. An equal volume of acid washed glass beads were added. The mixture was vortexed for 30 seconds, followed by 30 seconds on ice. This was repeated six times for a total of six minutes to lyse the cells. The mixture was centrifuged for 10 minutes at 13,000 rpm at 4°C. The supernatant was removed and transferred to a fresh centrifuge tube.

Protein determination was done by the method described by the Lowry. 50 µl each of supernatant and lysed product from induced cells of all the clones (pYESH2, pYESR1, pYESV1 and pYES2/CT) was taken and total protein content was determined using spectrophotometer at 660nm (Table 1).

The endoglucanase activity was assayed as laminarase and CMCase activity with laminarin and CMC as substrate in 50mM acetate buffer (pH=4.8) at 45°C for 30 minutes. The amount of released sugar was measured by 3', 5'- dinitrosalicylic acid method described by Katatny et al., 2000. One unit of enzyme activity was defined as the amount of enzyme that released 1µmol of glucose equivalent per minute.

**Table 1:** Total protein content in 50µl of supernatant and lysed product of different *Trichoderma* species

<table>
<thead>
<tr>
<th>Species</th>
<th>Supernatant</th>
<th>Lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>INVSc1 with pYES2/CT</td>
<td>61</td>
<td>23</td>
</tr>
<tr>
<td>pYESH2-Y</td>
<td>75</td>
<td>34</td>
</tr>
<tr>
<td>pYESR1-Y</td>
<td>67</td>
<td>23</td>
</tr>
<tr>
<td>pYESV1-Y</td>
<td>47</td>
<td>25</td>
</tr>
</tbody>
</table>

The endoglucanase activity was assayed as laminarase and CMCase activity with laminarin and CMC as substrate in 50mM acetate buffer (pH=4.8) at 45°C for 30 minutes. The amount of released sugar was measured by 3', 5'- dinitrosalicylic acid method described by Katatny *et al.*, 2000. (Table 2. and Figure 2.).

Finally, to assess the antifungal activity of expressed endoglucanase the galactose induced lysates of pYESH2, pYESR1 and pYESV1 along with the yeast control pYES2/CT were tested against *Sclerotium rolfsii* obtained from Department of Pathology, UAS, Dharwad. The culture filtrate of both the lysate and supernatant of each clone having 10 µg of protein was spread on the PDA plates and air-dried for 10 minutes. *S. rolfsii* was inoculated in the centre of agar plates using 5-mm mycelial discs and incubated at 30 °C for 3 days. The radial diameter of the colonies was measured at right angles every day, for six replicate plates per treatment, measuring daily growth rate (Table 3. and Figure 3.).

Further, sclerotial bodies of *Sclerotium rolfsii* were dipped in the supernatant of pYESH2, pYESR1, pYESV1 and pYES2/CT (control) having equal volume of protein (10 µg) for 10 minutes. Then they were placed on PDA plates for germination. These plates were incubated at 30°C for 3 days and again the radial diameter of the colonies was measured at right angles every day, for six replicate plates per treatment, measuring daily growth rate (Table 4. and Figure 4.).

As a result, the recombinant strains obtained showed the high quantity of reducing sugar production, indicating that the *Trichoderma* enzyme was secreted in active forms by the yeasts. The control strain, *S. cerevisiae* INVSc1 transformed with vector pYES2/CT, showed less endoglucanase activity comparatively (Table1 and 2). Though the total crude protein was high in pYESH2, amount of reducing sugars released was relatively low. This may be because of different
Table 2: Reducing sugar released per 50µl of protein by different clones

<table>
<thead>
<tr>
<th>Species</th>
<th>Laminarin Supematant</th>
<th>Laminarin Lysate</th>
<th>Carboxymethyl Cellulase Supematant</th>
<th>Carboxymethyl Cellulase Lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>INVSc1 with pYES2/CT</td>
<td>360</td>
<td>161</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pYESH2-Y</td>
<td>687</td>
<td>630</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pYESR1-Y</td>
<td>-</td>
<td>-</td>
<td>908</td>
<td>803</td>
</tr>
<tr>
<td>pYESV1-Y</td>
<td>520</td>
<td>169</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

![Diagram](image1.png)

![Diagram](image2.png)
Construct map of (a) pYESH2 containing full length β-1, 6-endoglucanase gene from T. Lalics (b) pYESR1 containing full length β-1, 4-endoglucanase gene from and (c) pYESV1 containing full length β-1, 6-endoglucanase gene from in pYES2/CT yeast vector.

Sugar conversion assay of pYESR1

<table>
<thead>
<tr>
<th>Recombinants</th>
<th>Rate of growth (dm in cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYES2/CT</td>
<td>8 cm</td>
</tr>
<tr>
<td>pYESH2-Y</td>
<td>3.5 cm</td>
</tr>
<tr>
<td>pYESR1-Y</td>
<td>2.5 cm</td>
</tr>
<tr>
<td>pYESV1-Y</td>
<td>3.5 cm</td>
</tr>
</tbody>
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Glucanase activity was observed even in the control (yeast with pYES2/CT). This is because S. cerevisiae contains a wide range of endo- and exo-1, 3-β-glucanases and β-1, 6-glucanases and about 15 genes are known to encode polypeptides with glucanase or related enzymatic activities. Some of these glucanases have roles during cell separation, while others exhibit transglycosylase activity and may contribute to total protein content, percentage of glucanase may be high in the total crude protein from pYESR1. Similar observations were made earlier. It can be concluded that, as different species may differ in their potential to degrade glucan, the glucanase enzyme secreted by T. reesei may be having more potential to degrade carboxymethyl cellulose compared to glucanase enzyme secreted by other Trichoderma species. Therefore, it may be necessary to purify glucanase from these culture filtrates in future to have clear information on glucan degrading potentiality of these species.
Fig. 3: Antifungal activity of a) pYESR1, b) pYESH2 and c) pYESV1 against T. Lalics

Fig. 4: Spore germination test of (a) pYESR1 (b) pYESH2 (c) pYESV1
be involved in extending and rearranging 1, 3-β-glucan and 1, 6-β-glucan chains, and cross linking these polymers to other wall components. The antifungal activity, an effective inhibition of Sclerotium rolfsii was mediated by β-1, 4-glucanase from T. reesei alone (Figure.3). These results are in agreement with data from experiments in which some plant chitinases, with antifungal activity, inhibited the growth of only some phytopathogenic fungi when combined with β-1, 4-glucanase [11,13]. In other parasitic systems, such as that used by entomopathogenic fungi, a conjunction of proteases with chitinases is required during the penetration of insect cuticles [16]. Inhibition of S. rolfsii depended on the carbon source used and pH maintained. Previously, a purified endochitinase has shown antifungal activity; however, strong synergism was observed with chitinase, β-1, 3-glucanase and also with mycotoxic metabolites [7]. Future investigations will focus on the roles of mycotoxic substances secreted by the new T. harzianum isolate to study the synergisms between these compounds and enzymes in the biocontrol process.

REFERENCES