Comparative production of cellulases by mutants of *Penicillium janthinellum* NCIM 1171 and its application in hydrolysis of Avicel and cellulose.

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Abstract

Mutants of *Penicillium janthinellum* NCIM 1171 were evaluated for cellulase production using both submerged fermentation (SmF) and solid state fermentation (SSF). Mutant EU2D-21 gave highest yields of cellulases in both SmF and SSF. Hydrolysis of Avicel and cellulose were compared using SmF and SSF derived enzyme preparations obtained from EU2D-21. Surprisingly, the use of SSF derived preparation gave less hydrolysis compared to SmF derived enzymes. This may be due to inactivation of $\beta$-glucosidase at 50 °C in SSF derived enzyme preparations. SmF derived enzyme preparations contained both thermostable and thermosensitive $\beta$-glucosidases whereas SSF derived enzyme preparations contained predominantly thermosensitive $\beta$-glucosidase. This is the first report on less thermostability of SSF derived $\beta$-glucosidase which is the main reason for getting less hydrolysis.

**Key words:** Mutant EU2D-21, submerged fermentation, solid state fermentation, $\beta$-glucosidase thermostability.
1. Introduction

Lignocellulosic substances are abundantly available sources of renewable biopolymer for the production of biofuels or other bio-based products. The bioconversion of cellulosic materials has been receiving a great attention in recent years. Hydrolysis of lignocellulosic materials by cellulases and hemicellulases could be the most efficient method for the release of fermentable sugars (Lynd et al., 2002) Cellulases are the key enzymes required for the degradation of lignocellulosic polysaccharides into the simple monomeric sugars that are converted through microbial fermentation processes to biofuels or other value added products. Development of large scale bioconversion process would alleviate shortages of food and animal feeds, solve modern waste disposal problems and also diminish the dependence on fossil fuels by providing an energy source in the form of glucose. Much work has been done on the production of cellulases from different microorganisms (Depaula et al., 1999; Solomon et al., 2000). The application of these enzymes for biofuel production is hindered by the high cost of enzyme production and the suitability of pretreatment of biomass which makes it amenable to cellulase attack.

Cellulose is degraded by synergistic action of three types of enzymes in the cellulase complex: exo-1, 4-β-d-glucanase (EC 3.2.1.91), endo-1, 4-β-d-glucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). Among fungi, Trichoderma and Aspergillus have been extensively studied particularly due to their ability to secrete cellulose degrading enzymes. We have reported the hyper-production of β-glucosidase (Gokhale et al., 1984) and β-xylosidase (Gokhale et al., 1986) by Aspergillus niger NCIM 1207
isolated in our laboratory. However, the search for an efficient and possibly better source of cellulase continues due to the low activity of $\beta$-glucosidase in *Trichoderma* which limits the rate and extent of hydrolysis. Thus, the conversion of waste cellulose to glucose is not yet commercially feasible. Hence, efforts are needed to produce cellulases at affordable cost which can be used for hydrolyzing biomass to monomers with high economical potential. *P. janthinellum* NCIM 1171 was identified as cellulase producer which produces cellulases using bagasse as carbon source (Adsul et al., 2004) and for its application in bagasse hydrolysis (Adsul et al., 2005). Mutants of *P. janthinellum* NCIM 1171 were isolated that are capable of producing enhanced level of cellulases (Adsul et al., 2007). The present communication describes the comparative enzyme production by the wild type *P. janthinellum* NCIM 1171 and its mutant, EMS-UV-8 and EU2D-21 using different substrates like Avicel, cellulose powder, solka floc and tissue paper under submerged and solid state fermentation conditions. The hydrolysis of Avicel and cellulose powder using mutant EU2D-21 enzyme preparations produced by solid state and submerged fermentation conditions is also described.

2. **Methods**

2.1 **Chemicals**

Avicel PH 101 was obtained from Fluka AG, Switzerland. Solka Floc SW44 was purchased from Brown Co., Berlin, NH. Tissue paper purchased locally. Cellulose powder, p-nitrophenyl-$\beta$-D-glucopyranoside (pNPG), 4-methylumbelliferyl-$\beta$-D-glucoside, carboxymethylcellulose (CMC), xylan (oat spelts), 3, 5-dinitrosalysilic acid were obtained from Sigma-Aldrich co.st. Louis, USA. Phenyl Methane Sulfonyl Flouride (PMSF), Yeast extract and peptone were obtained from Himedia Laboratories Pvt.
limited Mumbai, India. All the other chemicals were of analytical grade and were obtained locally.

2.2 Microorganism, culture media and enzyme production

*P. janthinellum* NCIM 1171 mutant EMS-UV-8, EU2D-21, EU-1 and *A. niger* NCIM 1207 were obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India. The wild type and the mutants were maintained on Potato Dextrose Agar (PDA) and sub cultured once in every three months.

Submerged fermentation (SmF) was carried out in 250 ml Erlenmeyer flask with 70 ml of fermentation medium containing 1% (w/v) different cellulosic substrates and 2.5% (w/v) wheat bran as described earlier (Adsul et al., 2007). Solid state fermentation (SSF) was carried out as described earlier (Adsul et al., 2009). Enzyme was produced in 250 ml Erlenmeyer flask containing 4 g of wheat bran, 1 g of different cellulosic substrates and 8 ml of production medium to moistened the substrate. Filter paper cellulase (FPase), endoglucanase, xylanase and β-glucosidase activities were determined as reported earlier (Adsul et al., 2007). The reducing sugar was estimated as glucose or xylose by the DNS method (Fischer and Stein, 1961).

2.3 Enzymatic hydrolysis

The enzyme preparations for hydrolysis of Avicel and cellulose powder were derived from the growth of mutants in shake flask culture containing basal medium with cellulose (1%) and wheat bran (2.5%) as mentioned in section 2. The hydrolysis of both Avicel and cellulose powder were carried out in 100 ml conical flask containing 50 ml citrate buffer (50 mM, pH 4.5), 2.5 g Avicel or cellulose powder, 5 mg sodium azide and 10 filter paper units (FPU) per g of substrate. In case of hydrolysis using solid state...
enzyme preparations, we added same units of *Aspergillus niger*, β-glucosidase for
hydrolysis. This mixture was incubated at 50 °C with shaking at 150 rpm. The samples
were analyzed for the reducing sugars. Residual activities of all enzymes were estimated
in the hydrolysis mixtures during hydrolysis to determine inactivation of the enzymes at
50 °C.

2.4  *Native polyacrylamide gel electrophoresis and zymogram of β-glucosidase*

Crude enzyme preparations (protein 100 µg) were fractionated by native
polyacrylamide gel electrophoresis (PAGE) using 10% acrylamide as resolving gel and
4% stacking gel (Laemmli, 1970). The β-glucosidase activity in the gel was detected by
developing zymogram against 10mM 4-methylumbelliferyl-β-D-glucoside (Sigma) as
substrate prepared in sodium citrate buffer (50 mM, pH 4.5). Upon completion of
electrophoresis, the gel was immersed in substrate solution for 45 min at 50 °C in the
dark. The β-glucosidase bands in the gel were visualized under UV light using Gel
Documentation system (Syngene). The enzymes from the gel corresponding to zymogram
bands were eluted using 50 mM citrate buffer, pH 4.5 and thermostability was determined
by incubating the enzymes at 50 °C. The residual activity was estimated under standard
assay conditions.

3.  **Results and Discussion**

3.1  *Production of enzyme by SmF & SSF*

The wild strain and selected mutants were evaluated for extra-cellular production
of cellulases and xylanases in shake flask containing optimized basal medium with
different substrates such as Avicel, cellulose powder, solka floc and tissue paper (1%) and
wheat bran (2.5%) as substrates (Table 1). Both the mutants, EMS-UV-8 and EU2D-21,
showed highest activities of FPase and CMCase. FPase activity of mutant strain was two times higher than the parent strain. No mutant produced as high β-glucosidase activity as that of wild strain. The mutant EU2D-21 produced highest FPase (3.49 IU/ml), CMCase (94.4 IU/ml) in Avicel.

Mutants were evaluated for cellulase production in SSF, and it was found that both mutants produced enhanced levels of FPase, CMCase and xylanase and β-glucosidase in comparison to the wild strain during growth on wheat bran and different substrates (Table 2). Tissue paper and solka floc also proved to be a suitable substrates for cellulase production by mutant strains. Mutant EU2D-21 produced the highest FPase (67.8 IU/g), CMCase (3558 IU/g), β-glucosidase (149 IU/g) when grown on 1 g of cellulose and 4 g of wheat bran. We have used tissue paper as the cheap source for the production of cellulases which yielded cellulase activities comparable to the values obtained for cellulose or Avicel. The comparison of cellulase activities produced by mutant EU2D-21 in SSF with those reported in the literature demonstrated the superiority of the mutant to the other fungal strains with respect to CMCase and β-glucosidase production.

*Trichoderma reesei* has been most extensively studied for its ability to produce extra-cellular cellulolytic enzymes. These strains have been mutated and also genetically modified to obtain improved strains which have been used for the commercial production of cellulases (Szengyel et al., 2000). However attempts to use these enzymatic systems from *Trichoderma* mutants for the degradation of cellulosic waste have not been successful for several reasons such as low enzymatic yields, low specific activities and end product inhibition. We have reported earlier the production of enhanced level of
cellulases by mutants of *P. janthinellum* in submerged fermentation (Adsul et al., 2007). And in solid state fermentation (Adsul et al., 2009) using only pure cellulose as substrate. This work demonstrated that we can also use Avicel, solka floc and tissue paper as substrates for cellulase production. Thus, tissue paper as cheap source can also be used for effective cellulase production.

3.2 *Hydrolysis of Avicel and cellulose powder*

The enzyme preparation of mutant, EU2D-21, produced both under submerged and solid state fermentation, have been used to hydrolyze Avicel and cellulose powder at 5% concentration. Yield of hydrolysis was higher (60-62%) with submerged enzyme preparation (Table 3). Enzyme preparation produced by solid state fermentation gave comparatively less (20-25%) hydrolysis yield. The addition of PMSF along with solid state enzyme preparation during hydrolysis did not give much difference in hydrolysis indicating no role of proteases in inactivation of β-glucosidase. However, the addition of β-glucosidase from *A. niger* NCIM 1207 gave hydrolysis equal to that of submerged enzyme.

Inactivation profile of all enzymes during hydrolysis demonstrated that FPase and CMCase of both solid state and submerged enzyme preparations exhibited good thermostability retaining more than 90% of their activity at 50 °C even after 60 h (data not shown). However, β-glucosidase of solid state enzyme preparation lost its 90% of its original activity within 8 h at 50 °C. In comparison, β-glucosidase in submerged enzyme preparation retained about 80% of its activity at 50 °C even after 60 h (Fig. 1). These results concluded that the decreased yield of hydrolysis by SSF enzyme preparations could be because of less thermostability of β-glucosidase at 50 °C. Usually SSF derived
enzymes are more thermostable than SmF enzymes (Archana et al., 1997). This study also suggests that SSF derived β-glucosidase could be different from SmF derived β-glucosidase. Such reports on inactivation of β-glucosidase present in SSF derived cellulase preparations are not available so far.

3.3  Zymogram staining and thermostability of β-glucosidase

The enzyme preparations derived from submerged fermentation showed two β-glucosidases, BGL1 and BGL2. While SSF derived preparations contained predominantly BGL2 and significantly less amount of BGL1 (Fig. 2). The thermostability studies revealed that BGL1 is more thermostable than BGL2 (Fig. 3). The less hydrolysis of Avicel and cellulose by SSF derived enzyme preparations could be attributed to the significantly low levels of thermostable BGL1. Production of such β-glucosidases exhibiting differential thermostability is not reported so far.

In conclusion, *P. janthinellum* mutant EU2D-21 produced two β-glucosidases under submerged fermentation conditions. One of the β-glucosidase (BGL1) is more thermostable than other one (BGL2). SSF derived cellulase preparation contained predominantly less thermostable species of β-glucosidase which could be the cause of less hydrolysis of Avicel and cellulose by SSF derived cellulase preparation. Production of such β-glucosidases exhibiting differential thermostability is not reported so far.
References


### Table 1

Comparison of enzyme activities of *P. janthinellum* NCIM 1171 and its mutant using different substrates under submerged fermentation as described in Section 2

<table>
<thead>
<tr>
<th>Medium + carbon source</th>
<th>Strain</th>
<th>Enzyme activities (IU/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FPase</td>
</tr>
<tr>
<td>BM+WB(2.5%)+ Avicel(1%)</td>
<td>Parent</td>
<td>1.21±0.10</td>
</tr>
<tr>
<td></td>
<td>EMS-UV-8</td>
<td>3.24±0.21</td>
</tr>
<tr>
<td></td>
<td>EU-2D-21</td>
<td>3.49±0.20</td>
</tr>
<tr>
<td>BM+WB(2.5%)+ Cellulose(1%)</td>
<td>Parent</td>
<td>1.10±0.11</td>
</tr>
<tr>
<td></td>
<td>EMS-UV-8</td>
<td>3.13±0.21</td>
</tr>
<tr>
<td></td>
<td>EU-2D-21</td>
<td>3.44±0.22</td>
</tr>
<tr>
<td>BM+WB(2.5%)+ Solka Floc(1%)</td>
<td>Parent</td>
<td>1.00±0.06</td>
</tr>
<tr>
<td></td>
<td>EMS-UV-8</td>
<td>2.23±0.11</td>
</tr>
<tr>
<td></td>
<td>EU-2D-21</td>
<td>2.99±0.20</td>
</tr>
<tr>
<td>BM+WB(2.5%)+ Tissue Paper(1%)</td>
<td>Parent</td>
<td>0.40±0.02</td>
</tr>
<tr>
<td></td>
<td>EMS-UV-8</td>
<td>2.19±0.10</td>
</tr>
<tr>
<td></td>
<td>EU-2D-21</td>
<td>2.89±0.20</td>
</tr>
</tbody>
</table>

*The cultures were grown in basal medium containing different cellulosic substrates (1%) and wheat bran (2.5%) as described in section 2. The enzyme activities were estimated after 8 days of incubation. The values given are the average of three independent experiments.*
Table 2

Comparison of enzyme activities of *P. janthinellum* NCIM 1171 and its mutant using different substrates under solid state fermentation as described in Section 2

<table>
<thead>
<tr>
<th>Medium + carbon source</th>
<th>Strain</th>
<th>Enzyme activities (IU/g)*</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FPase</td>
<td>CMCase</td>
<td>Xylanase</td>
<td>β-Glucosidase</td>
<td></td>
</tr>
<tr>
<td>BM+WB(4g)+ Avicel(1g)</td>
<td>Parent</td>
<td>11.1±2.10</td>
<td>688±2.99</td>
<td>221±10.0</td>
<td>88.9±9.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EMS-UV-8</td>
<td>54.4±5.21</td>
<td>3255±34.2</td>
<td>3000±33.2</td>
<td>141±11.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EU-2D-21</td>
<td>59.9±5.20</td>
<td>3421±36.7</td>
<td>3250±34.2</td>
<td>143.2±12.2</td>
<td></td>
</tr>
<tr>
<td>BM+WB(4g)+ Cellulose(1g)</td>
<td>Parent</td>
<td>16.7±1.10</td>
<td>710±25.5</td>
<td>231±15.5</td>
<td>98.8±10.4</td>
<td></td>
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<tr>
<td></td>
<td>EMS-UV-8</td>
<td>65.1±7.11</td>
<td>3300±38.8</td>
<td>2990±40.2</td>
<td>144±8.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EU-2D-21</td>
<td>67.8±3.50</td>
<td>3558±36.6</td>
<td>3100±42.2</td>
<td>149±9.81</td>
<td></td>
</tr>
<tr>
<td>BM+WB(4g)+ Solka Floc(1g)</td>
<td>Parent</td>
<td>8.90±0.06</td>
<td>591±16.0</td>
<td>185±10.0</td>
<td>81±8.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EMS-UV-8</td>
<td>40.0±4.50</td>
<td>2562±28.2</td>
<td>2875±36.2</td>
<td>151±9.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EU-2D-21</td>
<td>42.2±4.20</td>
<td>2775±30.0</td>
<td>3000±40.1</td>
<td>139±8.80</td>
<td></td>
</tr>
<tr>
<td>BM+WB(4g)+ Tissue Paper(1g)</td>
<td>Parent</td>
<td>7.40±1.02</td>
<td>510±12.0</td>
<td>120±10.0</td>
<td>49.2±5.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EMS-UV-8</td>
<td>36.9±4.50</td>
<td>2000±22.5</td>
<td>2331±30.5</td>
<td>111±7.51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EU-2D-21</td>
<td>38.0±4.80</td>
<td>2131±24.5</td>
<td>2451±32.1</td>
<td>100±7.00</td>
<td></td>
</tr>
</tbody>
</table>

*The cultures were grown in basal medium containing different cellulosic substrates (1 g) and wheat bran (4 g) as described in section 2. The enzyme activities were estimated after 4 days of incubation. The values given are the average of three independent experiments.*
Table 3

Comparison of hydrolysis between Avicel (5%) and cellulose (5%) using enzyme preparations of mutant EU2D-21 (10FPU/g of substrate) at 50 °C for 60 h.

<table>
<thead>
<tr>
<th>Enzyme(10FPU/g of substrate)</th>
<th>Substrate</th>
<th>Avicel</th>
<th>Cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residual Sugar (mg/ml)</td>
<td>Hydrolysis (%)</td>
<td>Residual Sugar (mg/ml)</td>
</tr>
<tr>
<td>SSF Enzyme (10U/g Fpase+21 U/g β-Glucosidase)</td>
<td>600±12</td>
<td>24±0.50</td>
<td>535±11</td>
</tr>
<tr>
<td>SmF Enzyme (10U/g Fpase+12.5 U/g β-Glucosidase)</td>
<td>1506±20</td>
<td>60.2±0.81</td>
<td>1533±22</td>
</tr>
<tr>
<td>SSF(10U/g Fpase+21 U/g β-Glucosidase) + β-glucosidase (12.5 U/g) from A.niger NCIM 1207</td>
<td>1500±18</td>
<td>60±0.73</td>
<td>1458±16</td>
</tr>
<tr>
<td>SSF Enzyme (10U/g Fpase+21 U/g β-Glucosidase) +PMSF</td>
<td>675±16</td>
<td>27±0.81</td>
<td>688±18</td>
</tr>
</tbody>
</table>

*The values of hydrolysis given in table are the average of three independent experiments.*
Fig. 1. Effect of temperature on stability of β-glucosidase produced by submerged fermentation (▲) and solid state fermentation conditions (Δ) at 50 °C for 60 h.
Fig 2. Zymogram staining of β-glucosidase (BGL) produced by mutant EU2D-21 in submerged fermentation (SmF) and solid state fermentation (SSF).
Fig. 3. Effect of temperature on stability of *P. janthimellum* mutant EU2D-21 β-glucosidases ( ■, BGL1-SmF; □, BGL2- SmF; ▲, BGL1-SSF; ∆, BGL2-SSF )