CELLULASE PRODUCTION BY A MUTANT STRAIN OF TRICHODERMA REESEI FROM BAGASSE

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ABSTRACT

Cellulase production from bagasse-containing media by the strain D1, a mutant of Trichoderma reesei QM9414, was examined in batch culture with a 5L jar fermentor. The bagasse was reduced to sizes within the range of 100 to 200 mesh and treated with 2% NaOH at 120°C for 30 min, which is optimal for cellulase production. When the strain was cultured in a medium containing glucose, lactose, corn starch hydrolysate, and bagasse supplied as carbon sources, the growth rate was rather rapid, and a high cellulase productivity was attained. Under optimal conditions, the filter paper activity (FPane) of 12.59 U/ml and carboxymethyl cellulase activity (CMCase) of 21.56 U/ml were obtained within 96h. A process for the concentration of culture filtrate 10-fold with an activity of more than 90% has been developed. The concentrated enzyme solution, stored at 25°C for 6 months, only resulted in only 22% loss of activity. The enzyme solution activity was still stable to some extent up to 60°C. The optimal reaction pH and temperature were found to be 5.0 and 50°C, respectively.

Keywords: Cellulase, bagasse

INTRODUCTION

Mutants of Trichoderma reesei are the best known source of active extracellular cellulase, and have therefore been intensively investigated to increase cellulase production by adding cellulose inducers, such as pure cellulose (Sternberg, 1976; Tangmu et al.,1981), sorbose (Kawamori et al.,1986; Schaffner and Toledo, 1991), cellobiose (Mandels and Reese, 1960; Vaheeri et al.,1979), sophorose (Sternberg and Mandels, 1979) and cellulose hydrolysate (Chen and Wayman, 1992), etc. There are comparatively few or no reports relating to cellulase production from Trichoderma reesei using bagasse. Sugarcane bagasse as an agricultural residue is cheap and quantities are enormous. After some pretreatment, bagasse could be utilized to produce cellulase at a relatively low price.

In this report, a mutant of Trichoderma reesei QM9414 was isolated that could produce high yields of cellulase from bagasse containing media. In batch cultures, the fermentation variables for maximal cellulase production, and the recovery of cellulase from a culture broth and some of its properties were investigated.

MATERIALS AND METHODS

Microorganism

The strain used throughout this study was Trichoderma reesei D1, which is a hypercellulolytic mutant resistant to 2-deoxy-glucose from Trichoderma reesei QM9414.

Media

The compositions of the media for cellulase production were:

Slant medium: potato dextrose agar, 40g; tap water, 1000 ml.
NaH$_2$P0$_4$, 7; citric acid, 5; peptone, 1; urea, 0.3; MgSO$_4$, 0.3; FeSO$_4$, 0.005; MnSO$_4$, 0.0016; ZnSO$_4$, 0.0014; and CaCl$_2$, 0.002; (pH 4.5 with HCl).

Preculture medium contained (in g/L): glucose, 10; lactose, 5; (NH$_4$)$_2$SO$_4$, 2.8; KH$_2$PO$_4$, 7; NaH$_2$P0$_4$, 7; citric acid, 5; peptone, 1; urea, 0.3; MgSO$_4$, 0.3; FeSO$_4$, 0.005; MnSO$_4$, 0.0016; ZnSO$_4$, 0.0014; CaCl$_2$, 0.002; and bagasse powder 10; (pH 3.5 with HCl).

Production medium contained (in g/L): glucose, 10; lactose, 10; NH$_4$H$_2$PO$_4$, 2.4; K$_2$SO$_4$, 0.3; urea, 0.3; citric acid, 0.006; MgSO$_4$, 0.3; FeSO$_4$, 0.0002; MnSO$_4$, 0.0016; ZnSO$_4$, 0.0014; CaCl$_2$, 0.1; catechol, 0.0055; bagasse powder 20; and the initial pH' was 4.5.

Cultivation

The spores from a 3-day-old slant were inoculated into 50 ml sterile preculture I medium in a 500ml Hinton flask. After 40h cultivation on a rotary shaker at 30° and 290rpm, 15 ml of the culture broth were transferred to a 150 ml sterile preculture II medium in a 500ml Hinton flask. This was incubated at 30° and 200 rpm for 30 hr, to serve as the inoculum for the fermentors. All the cellulase production was carried out in a 5L fermentor containing 3L production medium by the strain D1 in batch culture for 96h. The initial pH and temperature were controlled at 4.5 and 31°, and after 30h they were adjusted to 3.5 with 2N HCl solution and 29°. During fermentation, agitation and aeration at all times were held at 400 rpm and 1.5 vvm, respectively.

Analytical method

The culture broth was centrifuged at 3000 rpm for 20 min to remove mycelia and other insoluble substances. Cellulase activity from the supernatant was examined as filter paper activity (FPase) and carboxymethyl cellulase activity (CMCase) (Mandels et al., 1974).

RESULTS AND DISCUSSION

Production of cellulase in batch culture

Effect of bagasse

A comparison of cellulase production by T. reesei D1 in a 5L fermentor was made, using differently pretreated bagasse as inducer. Figure 1 demonstrates the effect of concentration of powdered bagasse, which was dried native bagasse milled and passed through a 60 mesh sieve, on cellulase production. The highest cellulase activity was obtained with 2%. After 144 h incubation, the corresponding FPase and CMCase were 4.55 U/ml and 12.53 U/ml, respectively. The effects of ball milling pretreatment of native bagasse on cellulase biosynthesis are shown in Table 1. It was observed that bagasse reduced to 100 to 200 mesh is optimal for FPase and CMCase production. Next, the inducing ability of powdered bagasse (100-200 mesh) treated under different conditions was compared. The results revealed that powdered bagasse treated with 2% NaOH for 30 min at 120° could greatly enhance cellulase production. The activities FPase and CMCase could reach 8.85 U/ml and 18.34 U/ml, respectively, within 96 hr of batch culturing. (Table 2).
Fig. 1: Effect of powdered bagasse concentration on cellulase production

Table 1. Effect of particle size of powdered bagasse on cellulase production

<table>
<thead>
<tr>
<th>Powdered bagasse (Mesh)</th>
<th>FPase (U/ml)</th>
<th>CMCCase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 200</td>
<td>6.61</td>
<td>14.75</td>
</tr>
<tr>
<td>100 – 200</td>
<td>7.06</td>
<td>16.06</td>
</tr>
<tr>
<td>60 – 100</td>
<td>5.75</td>
<td>14.11</td>
</tr>
<tr>
<td>35 – 60</td>
<td>4.34</td>
<td>12.49</td>
</tr>
</tbody>
</table>

The batch culture was carried out in 5L jar fermentor for 144 h

Table 2. Effect of treatment condition of powdered bagasse on cellulase production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temp. (°C)</th>
<th>NaOH (%)</th>
<th>Time</th>
<th>FPase (U/ml)</th>
<th>CMCCase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>120</td>
<td>8</td>
<td>30 min</td>
<td>3.10</td>
<td>9.39</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>4</td>
<td>30 min</td>
<td>4.24</td>
<td>11.66</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>2</td>
<td>30 min</td>
<td>5.85</td>
<td>14.14</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8</td>
<td>24 h</td>
<td>4.89</td>
<td>12.64</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4</td>
<td>24 h</td>
<td>4.34</td>
<td>10.96</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2</td>
<td>24 h</td>
<td>3.81</td>
<td>10.53</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td></td>
<td>4.63</td>
<td>11.87</td>
</tr>
</tbody>
</table>
The batch culture was carried out in 5L jar fermentor for 100 h

**Effect of Tween 80 concentration**

A range of Tween 80 concentrations (0.05, 0.1 and 0.2%) were tested for their effects on FPase and CMCase. The results are shown in Fig. 2. They indicate that activities of FPase and CMCase were increased with the increase of Tween 80. The mechanism of enhancement by Tween 80 is not understood, but may be related to the increased permeability of the cell membrane, allowing more rapid secretion of the enzyme which, in turn, leads to greater enzyme synthesis.

![Graph showing effect of Tween 80 concentration on cellulase production]

**Fig. 2**: Effect of Tween 80 concentration on cellulase production

**Effect of wheat bran extract**

Figure 3 demonstrates the effect of wheat bran extract on cellulase production of *T. reesei* D1. The addition of wheat bran extract to the production medium was very effective in cellulase secretion. The maximum FPase and CMCase activities on 1.5% (v/v) wheat bran extract were 9.51 U/ml and 19.07 U/ml, respectively.

![Graph showing effect of wheat bran extract on cellulase production]

**Fig. 3**: Effect of wheat bran extract on cellulase production
Effect of corn starch hydrolysate

It has been reported (Sternberg and Mandels, 1979) that acid-hydrolysed starch is an excellent inducer of cellulase in T. reesei. Fig. 4 illustrates the inducing ability of the corn starch hydrolysate. The results showed the corn starch hydrolysate was a powerful inducer for cellulase production. In all cases tested, addition of corn starch hydrolysate to the fermentation system increased cellulase activity in the culture filtrates. The maximum FPase and CMCase activities on 3% (v/v) corn starch hydrolysate were 12.59 U/ml and 21.56 U/ml, respectively.

![Fig. 4: Effect of corn starch hydrolysate on cellulase production](image)

Preparation of cellulase concentrate

The enzyme concentrate was made from culture filtrate by ultrafiltration or evaporation at pH 5. Table 3 shows the effect of evaporation temperature on enzyme activity. When culture filtrate was concentrated to 10-fold at 45°C with a yield of activity more than 90%, the CMCase was more stable than the FPase at various temperatures. Table 4 shows that culture filtrates were concentrated by ultrafiltration. Tests were conducted at pH 5 and 10°C under different inlet pressures in spiral-wound membrane of molecular weight cutoff 5000. Comparatively better results than the 75% and 90%, respectively, for FPase and CMCase activities were obtained at operation pressure of 3 kg/cm.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Recovery (%) FPase</th>
<th>CMCase</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>83.90</td>
<td>93.42</td>
</tr>
<tr>
<td>45</td>
<td>90.32</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>89.32</td>
<td>92.99</td>
</tr>
<tr>
<td>55</td>
<td>82.48</td>
<td>89.98</td>
</tr>
</tbody>
</table>
Table 4. Effect of transmembrane pressure on the yield of cellulase activity

<table>
<thead>
<tr>
<th>Pressure (kg/cm²)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPase</td>
</tr>
<tr>
<td>2</td>
<td>73.41</td>
</tr>
<tr>
<td>3</td>
<td>75.65</td>
</tr>
<tr>
<td>4</td>
<td>73.01</td>
</tr>
<tr>
<td>5</td>
<td>71.09</td>
</tr>
</tbody>
</table>

Properties of the cellulase concentrate

The effect of pH on the activity of cellulase concentrate was investigated at 50°. Fig. 5 shows that the optimal reactions of the pH of FPase and CMCase were the same at 5. The optimal reaction temperature of FPase and CMCase at pH 5 was found to be the same at 50° (Fig. 6). The heat stability of the FPase and CMCase in the aqueous solution at pH 5 is shown in Fig. 7. 90% and 94% of the enzyme activities of FPase and CMCase were maintained, respectively, after the reaction proceeded at 60° for 30 min. The enzyme concentrate solution was stored at 25° in pH 5 to examine stability. As shown in Fig. 8, the concentrate was stable, and 78% and 88% of the activity of FPase and CMCase were maintained, respectively, after 6 months.

Fig. 5: Influence of assay pH on cellulase activity of *T. reesei* D1
Fig. 6: Influence of assay temperature on cellulase activity of *T. reesei* D1

![Graph showing the influence of assay temperature on cellulase activity. The graph displays two curves: one for CMCase and another for FPase.](image)

Fig. 7: Thermostability of the cellulase from *T. reesei* D1

![Graph showing thermostability of the cellulase. The graph displays the relative activity of CMCase and FPase against reaction temperature.](image)
CONCLUSION

A high level of cellulase could be produced by T. reesei D1 using cheap bagasse-containing media. Native bagasse is highly resistant to enzymatic attack and microbial use. In order to enhance the inducing ability of bagasse on cellulase production, alkali and ball-milling treatments were necessary. Maximum inducing ability of the treated bagasse was found to be produced by heat treatment with 2% NaOH at 120°C for 30 min and particle size reduced to 100 to 200 mesh. It has been demonstrated that wheat bran extract and corn starch hydrolysate were powerful inducers of cellulase production by T. reesei D1. It should be noted that in this study a mixture of carbon sources, including glucose, lactose, bagasse, and corn starch hydrolysate, succeeded in shortening the lag phase, increasing the activity, and quickening the production of cellulase. The activity of enzyme concentrate had a pH optimum of 5 and was shown to be relatively stable at 50°C. The thermal stability of CMCase is much higher than FPase. The very satisfactory storage stability of cellulase concentrate at 25°C would reduce storage and handling costs. The reason for only 22% inactivation of this enzyme after six months of storage was not clear. Moreover, because the cost of enzyme concentrate produced was calculated to be NT 12.58/l (50U FPase/ml) i.e. very low, this process should be suitable for industrial application.
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REFERENCES


PRODUCCIÓN DE CELULASA MEDIANTE UNA CEPA MUTANTE DE *TRICHODERMA REESEI* A PARTIR DE BAGAZO

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RESUMEN

Se estudió la producción de celulasa a partir de un medio conteniendo bagazo empleando la cepa D1, un mutante de *Trichoderma reesei* QM 9414, mediante fermentación por lotes en un fermentador de 5 litros de capacidad.

El bagazo con un tamaño de partícula entre 100 y 200 mesh y tratado con NaOH al 2% a 121°C por 30 minutos resulta óptimo para la síntesis de celulasa. Cuando la cepa se cultiva en un medio conteniendo glucosa, lactosa, almidón de maíz hidrolizado y bagazo como fuente de carbono, la velocidad de crecimiento fue relativamente rápida y se alcanzó una alta productividad de celulasa. Bajo condiciones óptimas se alcanzó una actividad de papel de filtro (FPase) de 12,59U/ml y una actividad de carboximetil celulasa (CMCase) de 21,56U/ml a las 96 horas. Se desarrolló un proceso para la concentración del filtrado del cultivo de 10 veces con una actividad de más del 90%. Sólo hay una pérdida del 22% cuando la solución de enzima concentrada se almacenó a 25°C por 6 meses. La actividad de la solución de enzimas resultó alta aún a temperaturas de 60°C. El pH y la temperatura óptima de reacción se localizaron a 5,0 y 50°C respectivamente.

Palabras claves: Celulasa, bagazo
PRODUCTION DE CELLULASE AU MOYEN DE VARIÉTÉ MUTANTE DE TRICHODERMA REESEI DE LA BAGASSE

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RÉSUMÉ

La production de cellulase a été effectuée dans un milieu à base de bagasse avec la souche D1, un mutant de Trichoderma reesei QM9414, en culture par lots en fermenteur de 5 L. La bagasse, réduite à des tailles comprises entre 100 à 200 mesh et traitée avec du NaOH à 2% à 121°C pendant 30 min. s’est avérée la meilleure source. Quand la souche est cultivée dans un milieu contenant du glucose, du lactose, de l’hydrolysat d’amidon, de maïs et de la bagasse comme source de carbone, la croissance est assez rapide et la productivité de cellulase élevée. Dans les meilleures conditions, l’activité du papier filtre (FPase) de 12,59 U/ml et l’activité de cellulase carboxyméthyllique (CMCase) de 21,56 U/ml furent obtenues en 96 heures. Un procédé permettant de concentrer des filtrats de culture avec une augmentation d’un facteur, et une activité supérieur à 90% a été développé. Le concentré de la solution d’enzyme emmagasiné à 25°C pendant 6 mois a accusé une perte d’activité de l’ordre de 22% seulement de l’activité. L’activité de la solution enzymatique était encore stable jusqu’à un certain point à 60°C. Les meilleures conditions de pH et de température sont respectivement de 5,0 et 50°C.

Mots clés : cellulase, bagasse.