Batch and repeated batch cellulase production by mixed cultures of Trichoderma reesei and Aspergillus niger or Aspergillus phoenicis

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ABSTRACT

Trichoderma reesei was co-cultured with either Aspergillus niger or Aspergillus phoenicis in batch and repeated batch submerged fermentation to assess the efficiency of mixed cultures for cellulase production. In batch cultivation, biomass production of T. reesei - A. niger system was lower than that of T. reesei - A. phoenicis system. Both mixed culture combinations attained 1.6-fold higher FPA titres than their mono-cultures, indicating synergistic interactions. Cellulase productivities of both mixed cultures were 2- to 3-fold higher than that of their mono-cultures. In repeated batch operation, mixed cultures were stable for three 72-h cycles producing 1.4- to 1.8-fold higher FPA levels than mono-cultures, being T. reesei - A. niger the best combination. Mixed fungi cultures are efficient systems for cellulase production attaining higher yields and productivities than mono-cultures. Complex interactions occur in these systems but synergism seems to be the more important. The results of the present study are of commercial and biological interest. All productivity parameters revealed that mixed fungi cultures may be used for the production of cellulase in repeated batch or fed-batch operations.

Keywords: Aspergillus niger, Aspergillus phoenicis, cellulase, mixed fungi, repeated batch, synergism, Trichoderma reesei.

INTRODUCTION

The availability of energy is of great concern today and will be more serious in the coming decades. Based on increasing worldwide demand and consequent decreasing availability of petroleum over the middle- to long-term, rising prices are expected [1]. The production of biofuels from lignocellulosic biomass resources has the potential to increase biofuel production capacity while minimizing the negative environmental and social impacts because lignocellulosic resources do not compete directly with food production, or with land that may be needed for food production. Production of ethanol from lignocellulose must occur at high rate, in good yield, and to concentrations that are economically recoverable. While readily achieved with starch, these goals are much more difficult with cellulose and hemicellulose [2].

Lignocellulose biomass consists of three types of polymers, cellulose, hemicellulose and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent crosslinkages. The rigid and complex molecular polymeric structure of cellulosic biomass makes lignocellulose highly resistant to chemical attack, solubilisation and bioconversion. Physical or chemical pretreatment procedures which break down the lignocellulosic structures and thereby enhance the enzymatic accessibility are required for the conversion of biomass into biofuels [3, 4]. The enzymatic hydrolysis of cellulose materials involves synergistic actions of cellulases as well as xylanases and other enzymes [5, 6]. Cellulases are relatively costly enzymes, and a significant reduction in cost will be important for their commercial use.

Most industrial cellulases are produced by fungi in submerged fermentation. Trichoderma reesei is the most important fungal species used for cellulase production although it produces low levels of β-glucosidase [7]. Some
Aspergillus species are also important cellulase producers with higher levels of β-glucosidase than T. reesei [8]. It has been demonstrated that mixed cultivation can complement the metabolic capabilities of microorganisms leading to secretion products with improved profiles or better capabilities for bioconversion of substrates [9-11]. Enhanced production of several enzymes has been obtained with fungal mixed cultures [12-16]. The aim of this work was to assess the efficiency of Trichoderma reesei mixed cultures with either Aspergillus niger or Aspergillus phoenicis for cellulase production in submerged fermentation systems.

MATERIALS AND METHODS

Microorganisms and inoculum
The strains Trichoderma reesei LM-UC4, Aspergillus phoenicis QM329 and Aspergillus niger ATCC 10864 were used in this work [13, 17]. Stock cultures were maintained on potato dextrose agar slants. Spores were washed from 5-day agar-slant cultures with 10 ml of 0.1% (v/v) Tween 80 solution, counted in a Neubauer chamber and diluted to give 1 x 10⁶ spores per ml. This suspension was used as inoculum.

Culture medium and inoculation procedure
One hundred twenty-five ml erlenmeyer flasks were used for batch cultures. The culture medium contained per liter: 2 g KH₂PO₄; 1.4 g (NH₄)₂SO₄; 0.3 g urea; 0.3 g CaCl₂·2H₂O; 0.3 g MgSO₄·7H₂O; 1 g peptone; 2 ml Tween 80; 5 mg FeSO₄·7H₂O; 1.6 mg MnSO₄·2H₂O; 1.4 mg ZnSO₄·7H₂O; 2 mg CoCl₂·6H₂O; and 10 g lactose. The initial pH was 5.5.

For single cultures, each flask containing 30 ml culture medium was inoculated with a 3% (v/v) of the above spore suspension and was incubated at 28°C in a shaker bath at 175 rpm.

For mixed fungal cultures, each flask containing 30 ml culture medium was inoculated with 1.5% (v/v) of the T. reesei spore suspension and 1.5% (v/v) of either A. niger or A. phoenicis spore suspension. All inoculated flasks were incubated at 28 °C in a shaker bath at 175 rpm. Four replicates were taken in all cases.

Repeated batch cultures
For repeated batch cultivation, 250 ml flasks containing 70 ml culture medium were inoculated as above and incubated at 28 °C in a shaker bath at 175 rpm. After 72 h growth a repeated batch production of enzyme was initiated. The liquid part of each flask was decanted and replaced by 70 ml fresh medium. This operation was repeated every 72 h. Four replicates were taken in all cases.

Analytical procedures
Fungal biomass was determined by measuring its dry cell weight. For this, the entire content of a flask was filtered through pre-weighed filter paper (Whatman No. 1) under suction. The retained mycelium was washed three times with distilled water and the filter paper was dried at 80ºC for a constant weight. The filtrates collected were stored frozen for later analysis of enzyme activity, soluble protein and lactose concentration.

Cellulase (filter paper activity, FPA) activities were measured as previously reported [18]. One enzyme unit (U) is defined as the amount of enzyme that releases 1 µmol glucose equivalents per min. Reducing sugars were determined colorimetrically using the dinitrosalicylic acid reagent [19] and soluble protein was determined by the Lowry method using bovine serum albumin as standard [20].

Statistical analysis
Data were analysed by Statistical Analysis System (Version 8.1) software (SAS Institute, Inc., Cary, NC, USA). Analysis of variance (ANOVA) by the General Linear Models procedure and Duncan’s multiple range tests were used to find significant differences between treatments at sampling times.

RESULTS

Single and mixed submerged cultures presented pellet morphology, being pellets of T. reesei smaller than the others. Time course growth kinetics of single and mixed submerged cultures are shown in Fig. 1a. Both A. phoenicis and A. niger produced lower final biomass than T. reesei (0.622 ± 0.002 and 0.835 ± 0.002 vs 2.310 ± 0.156 g l⁻¹, respectively). Single and mixed Aspergillus cultures had lower lactose consumption than T. reesei single cultures (Fig. 1b). The lower biomass production of both Aspergillus species single cultures may be related to their lower lactose consumption. Final biomass production of T. reesei and A. niger mixed cultures (1.45 ± 0.01 g l⁻¹) was lower than that of T. reesei and A. phoenicis mixed cultures (2.76 ± 0.05 g l⁻¹) indicating that the latter is a better partner combination. In addition, since both partner strains were inoculated with similar spore concentrations in the mixed

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cultures so that the total spore concentration was equal to that of single cultures, the couple T. reesei - A. niger showed a clear competitive interaction.

The evolution over time of FPA titres for single and mixed cultures is shown in Fig. 2. Both mixed culture combinations attained about 1.6-fold higher FPA titres than the combined titres of their respective single cultures, indicating that synergistic interactions occurred in those cultures. Interestingly, although correlations between FPA titres and secreted protein were low in single cultures of T. reesei, A. niger and A. phoenicis (R² = 0.229, 0.698 and 0.414, respectively), the opposite was found for T. reesei - A. niger and T. reesei - A. phoenicis mixed cultures (R² = 0.991 and 0.803, respectively). Table 1 shows production parameters at the maximal FPA production time. At this point, both FPA and secreted protein levels were significantly different among all culture systems being FPA and protein produced by T. reesei - A. niger much higher than that attained by the other cultures. Enzyme yields based on biomass, lactose consumption and secreted protein were generally higher for mixed cultures. Likewise, cellulase specific and volumetric productivities of both mixed cultures were, respectively, 2- and 3-fold higher than the averages of their corresponding single cultures.

In order to test the stability of the mixed culture systems, repeated batch cultivations were performed under the same conditions for 72 h each cycle. Cellulase production is shown in Fig. 3. The difference in production levels of FPA in the first cycle compared to that reported above may be due to the difference in the size of the flasks used since for the repeated batch experiments larger flasks were used to provide greater surface for air transfer. There was a slight decrease in the amount of FPA produced in the third cycle in all cases but this fell sharply in the next cycle (not shown). However, T. reesei - A. niger mixed culture produced about 1.8-fold higher FPA levels than the average of its corresponding single cultures, while for T. reesei - A. phoenicis mixed culture this was about 1.4-fold. Also, FPA volumetric productivity followed this pattern being that of T. reesei - A. niger mixed culture higher than the others (Fig. 4a). On the other hand, as shown in Fig. 4b, FPA specific productivity decreased linearly until the third cycle (from 29.9 to 3.9 U g⁻¹ h⁻¹, R² = 0.933, for T. reesei - A. niger mixed culture, and from 49.4 to 9.1 U g⁻¹ h⁻¹, R² = 0.838, for T. reesei - A. phoenicis mixed culture). Interestingly, biomass concentration increased linearly until the third cycle (from 1.0 to 8.6 g l⁻¹, R² = 0.991, for T. reesei - A. niger mixed culture, and from 0.3 to 2.3 g l⁻¹, R² = 0.998, for T. reesei - A. phoenicis mixed culture). This may explain the sharp drop in the production of FPA after the third cycle due to severe mass transfer limitations imposed by fungal overgrowth.

**DISCUSSION**

In nature, microbial populations live forming dynamic mixed communities subject to positive, negative or neutral interactions among their members [21, 22]. Resembling nature, ancient oriental fermented foods are elaborated by interactions among their members [21, 22]. Resembling nature, ancient oriental fermented foods are elaborated by Trichoderma reesei can be grown in co-culture with some Aspergillus species for cellulase production and cellulose hydrolysis both in submerged fermentation and in solid-state fermentation [13-15, 18].

In the present study, T. reesei was co-cultured with either A. niger or A. phoenicis in submerged fermentation under cellulase-promoting conditions by using lactose as the sole carbon source. Although lactose metabolism is slow in these fungi, it is one of the best and cheapest sources for cellulase production [23]. As expected, T. reesei grew better and produced 2.7- to 3.7-fold more biomass than both Aspergillus species in single culture and this is in agreement with its better lactose consumption (Fig. 1). Mixed cultures of T. reesei - A. phoenicis produced 1.9-fold more biomass than T. reesei - A. niger mixed cultures and about 20% more biomass than single cultures of T. reesei. From the point of view of nutrient transformation into biomass, the couple T. reesei - A. phoenicis developed a positive interaction while the opposite was found for T. reesei - A. niger [24]. The low biomass produced in submerged fermentation by the latter mixed culture system is in disagreement with other works although different strains and culture conditions were used [15]. However, in solid-state fermentation, mixed cultures of the same strains gave similar results being T. reesei - A. phoenicis the best partners in terms of biomass production [13, 17]. It is unclear what type of interactions occurred in these mixed culture systems since lactose is not easily metabolized by any of the strains.

Does more growth mean increased production of cellulase in mixed culture systems? According to the results attained in this work it seems that there is not a simple relationship between growth and cellulase production in mixed cultures though these produced about 1.6-fold higher FPA titres than single cultures as shown in Fig. 2. Although the T. reesei - A. niger mixed culture system produced less than half of the biomass attained by the T. reesei - A. phoenicis mixed system, cellulase production was almost 30% higher than that produced by the latter. Also, yields and productivities were higher in mixed cultures, being highest in T. reesei - A. niger mixed cultures (Table 1). In this sense, it seems that important synergistic interactions were occurring in the mixed cultures even...
though competition was also occurring [22]. This synergism was also found in solid-state fermentation and was stronger when mixed cultures were under low nutrient availability [13, 18]. Likewise, it has been found that cellulase produced in submerged fermentation by mixed cultures of either \emph{T. reesei} - \emph{A. niger} or \emph{T. reesei} - \emph{A. phoenicis} was more efficient in hydrolyzing cellulose though the amount of enzyme produced was only slightly higher or lower than that produced by single cultures, indicating that the synergism was also evident in the composition of the enzyme complex [14, 25].

\textbf{Table 1 Comparison of cellulase production between single and mixed fungi submerged fermentation}

<table>
<thead>
<tr>
<th></th>
<th>\emph{T. reesei}</th>
<th>\emph{A. niger}</th>
<th>\emph{A. phoenicis}</th>
<th>\emph{T. reesei} and \emph{A. niger}</th>
<th>\emph{T. reesei} and \emph{A. phoenicis}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak of maximum FPA production (h)</td>
<td>72</td>
<td>72</td>
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<td>72</td>
</tr>
<tr>
<td>FPA (U l(^{-1}))</td>
<td>950 ± 71(^{c})</td>
<td>800 ± 141(^{d})</td>
<td>330 ± 71(^{d})</td>
<td>2850 ± 357(^{a})</td>
<td>2250 ± 212(^{b})</td>
</tr>
<tr>
<td>Dry biomass (g l(^{-1}))</td>
<td>1.5 ± 0.1(^{b})</td>
<td>0.3 ± 0.0(^{b})</td>
<td>0.5 ± 0.07(^{a})</td>
<td>0.8 ± 0.01(^{b})</td>
<td>1.8 ± 0.2(^{a})</td>
</tr>
<tr>
<td>Residual lactose (g l(^{-1}))</td>
<td>4.7 ± 0.4(^{d})</td>
<td>6.3 ± 0.0(^{b})</td>
<td>6.1 ± 0.1(^{c})</td>
<td>5.9 ± 0.6(^{c})</td>
<td>6.5 ± 0.0(^{d})</td>
</tr>
<tr>
<td>Secreted protein (g l(^{-1}))</td>
<td>0.2 ± 0.0(^{c})</td>
<td>0.2 ± 0.08(^{d})</td>
<td>0.2 ± 0.04(^{b})</td>
<td>0.7 ± 0.1(^{b})</td>
<td>0.5 ± 0.08(^{c})</td>
</tr>
<tr>
<td>(Y_{\text{FPA}}) (U g(^{-1}))</td>
<td>655 ± 103(^{d})</td>
<td>2927 ± 517(^{a})</td>
<td>608 ± 57(^{b})</td>
<td>3539 ± 408(^{a})</td>
<td>1269 ± 275(^{c})</td>
</tr>
<tr>
<td>(Y_{\text{FPSN}}) (U g(^{-1}))</td>
<td>178 ± 2(^{d})</td>
<td>216 ± 38(^{a})</td>
<td>85 ± 21(^{b})</td>
<td>708 ± 184(^{a})</td>
<td>643 ± 61(^{a})</td>
</tr>
<tr>
<td>(Y_{\text{FPA}}) (U g(^{-1}))</td>
<td>3965 ± 369(^{a})</td>
<td>3496 ± 555(^{a})</td>
<td>2189 ± 1012(^{b})</td>
<td>3882 ± 767(^{a})</td>
<td>4216 ± 226(^{a})</td>
</tr>
<tr>
<td>(q_{\text{FPA}}) (U g(^{-1}) h(^{-1}))</td>
<td>9.1 ± 1.4(^{d})</td>
<td>40.7 ± 7.2(^{b})</td>
<td>8.4 ± 0.8(^{d})</td>
<td>49.2 ± 5.7(^{a})</td>
<td>17.6 ± 3.8(^{c})</td>
</tr>
<tr>
<td>(\Gamma_{\text{FPA}}) (U l(^{-1}) h(^{-1}))</td>
<td>13.2 ± 0.9(^{a})</td>
<td>11.1 ± 1.9(^{d})</td>
<td>4.6 ± 0.9(^{d})</td>
<td>39.6 ± 4.9(^{a})</td>
<td>31.3 ± 2.9(^{b})</td>
</tr>
</tbody>
</table>

\(Y_{\text{X/S}}\) (g g\(^{-1}\)) = biomass formed (g l\(^{-1}\))/substrate consumed (g l\(^{-1}\)), \(Y_{\text{FPA/S}}\) (U g\(^{-1}\)) = FPA produced (U l\(^{-1}\))/substrate consumed (g l\(^{-1}\)), \(Y_{\text{FPA/Pr}}\) (U g\(^{-1}\)) = FPA produced (U l\(^{-1}\))/protein secreted (g l\(^{-1}\)), \(q_{\text{FPA}}\) = FPA specific productivity, \(\Gamma_{\text{FPA}}\) = FPA volumetric productivity. Values represent mean of four repetitions ± SD. Means with the same letter within a row are not significantly different (\(P < 0.0001\)). Data were evaluated by ANOVA and Duncan’s test.

\textbf{Figure 1 Biomass (a) and residual lactose (b) kinetics by single (open symbols) and mixed (closed symbols) fungi culture. (○) \emph{Trichoderma reesei} alone; (□) \emph{Aspergillus niger} alone; (△) \emph{Aspergillus phoenicis} alone; (■) \emph{T. reesei} – \emph{A. niger}; (▲) \emph{T. reesei} – \emph{A. phoenicis}. Error bars on the graphs represent four replicates.}
The reusability of the mixed fungi culture systems was examined in repeated batch operations. Repeated batch cultivation is a very useful technique because it has a better cost–benefit ratio than other cultivation methods and it has been used to increase the yields of several processes [26, 27]. Pellet morphology of the mycelia made it possible to carry out only three 72-h cycles of repeated batch culture. Both mixed culture systems produced more cellulase during the three cycles than their single cultures but due to overgrowth new cycles were not possible (Fig. 3). It seems that under the conditions of the experiments the level of competition decreased giving rise to higher biomass production in both mixed culture systems without impairing synergism for cellulase production and volumetric productivity (Fig. 4a). However, due to the high biomass concentrations attained, specific cellulase productivities decreased linearly (Fig. 4b). Also, at high biomass concentrations oxygen transfer may became the limited condition [28, 29]. Further optimization work is needed together with the use of fed-batch operations in which fungal growth can be carefully controlled [14, 15, 25].

Figure 2 Filter paper cellulase kinetics by single (open symbols) and mixed (closed symbols) fungi cultures. Symbols as in Fig. 1. Error bars on the graphs represent four replicates.

Figure 3 Filter paper cellulase production in repeated batch by single and mixed fungi cultures. *Trichoderma reesei* alone (white bars); *Aspergillus niger* alone (light grey bars); *Aspergillus phoenicis* (dark grey bars); *T. reesei – A. niger* (diagonally striped bars); *T. reesei – A. phoenicis* (horizontally striped bars). Error bars on the graphs represent four replicates.
CONCLUSION

The present study demonstrates mixed fungi cultures are efficient systems for cellulase production attaining higher yields and productivities than single cultures. Complex partner interactions occur in these systems but as long as there is a good partner choice synergism seems to be more important than competition. Industrial application of mixed fungi cultivation will be improved by using quantitative molecular tools for developing useful mathematical models that allow for the scale up of the process. Further work is being carried out with mixed fungi biofilms to improved cellulase production.

Acknowledgements

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REFERENCES


Figure 4 Cellulase volumetric (ΓFPA) and specific (qFPA) productivities in repeated batch mixed fungi cultures. T. reesei – A. niger (blue bars); T. reesei – A. phoenicis (red bars). Error bars on the graphs represent four replicates.