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Assessment of the Effects of Intermittent Mixing On Solid-State Fermentation for Biomass-Degrading Enzymes Production by Different Fungal Strains

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ABSTRACT

The use of solid-state fermentation (SSF) for the production of industrial enzymes has received increasing attention over the years. However, the implementation of large-scale SSF processes requires an understanding of the effects of mixing on microorganism growth and product formation. This paper describes a systematic comparison of the effects of intermittent mixing on SSF, in terms of the production of biomass-degrading enzymes (endoglucanase, β-glucosidase, and xylanase) by different fungal strains (*Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*). It was found that when *A. niger* was cultivated using wheat bran as the sole substrate, mixing had a negative effect on the production of enzymes. However, this effect was minimized when the cultivation was carried out using a combination of wheat bran and sugarcane bagasse (3:1 on a dry mass basis) as substrate. In addition, use of the combined substrate enabled improvement of the overall enzymatic production by *A. niger*. Considering all the fungi tested, the enzyme most negatively affected by mixing was β-glucosidase. It is therefore important that special attention be given to β-glucosidase during bioprocess development for biomass-degrading enzymes production under SSF in large-scale bioreactors. These findings can contribute to selection of the most appropriate cultivation conditions for implementation of large-scale SSF processes.

Keyword: *Cellulases; xylanases; Trichoderma reesei; Aspergillus niger; Aspergillus oryzae; solid-state fermentation; sugarcane bagasse; agitation*

INTRODUCTION

In the biomass to ethanol conversion process, the costs of the enzymatic cocktails used in the saccharification step have a major impact on overall economic viability [1]. Therefore, there is a need to improve the efficiency of production of the enzymes (such as cellulases and xylanases) that break down plant cell wall components. These enzymes are produced by a wide variety of microorganisms (bacteria and fungi); however, the aerobic fungi especially are known for their high growth and protein secretion rates [2]. In fact, most commercial (hemi)cellulolytic enzymatic preparations are produced using filamentous fungi of the genera *Trichoderma* and *Aspergillus* [3].

In terms of the microbial cultivation process, the use of solid-state fermentation (SSF) for industrial enzyme production has received increasing attention over the past years due to the inherent advantages of this cultivation system [4, 5]. In SSF, the cultivation employs a solid substrate with sufficient moisture only for maintenance of growth and metabolism of the microorganism (in other words, there is no free water) [6-10]. Many studies have described the use of SSF to produce enzymes involved in biomass deconstruction [5, 11-16]. SSF is particularly advantageous for enzyme production by filamentous fungi, since it simulates the natural habitat of these microorganisms. From the environmental perspective, the benefit of SSF is related to the use of agro-industrial residues as solid substrate, acting as sources of both carbon and energy [17].

Despite the advantages of SSF, its large-scale industrial implementation has been held back, mainly due to operational difficulties involving the different process variables, especially those concerning heat and mass transfer [18-20]. There is, therefore, a need for studies focusing on the influence of SSF process variables on the development of large-scale SSF bioreactors. One of the main operational problems found in SSF bioreactors is the aggregation of the fungal mycelium during growth, leading to channeling and restricted heat and mass transfer [21]. This problem can be addressed using mixing, either continuously or intermittently. However, an important consideration is that such mixing can cause physical damage to the fungal mycelium, hence hindering the growth of some microorganisms. Studies of the effects of mixing on the growth of microorganisms are therefore required during the development of bioprocesses employing SSF, in order to

evaluate the sensitivity of a particular microorganism to mixing, as well as to understand the effect on production of the metabolites of interest. Nava et al. [22] studied the effect of intermittent mixing on *Aspergillus tamari* growth and pectin methylesterase production under SSF with coffee pulp. It was found that there was no damage to the mycelium at the mixing frequencies tested. More recently, Flodman and Noureddini [23] evaluated the effect of intermittent mixing on cellulase production by *Trichoderma reesei* cultivated under SSF with wet corn distillers grain, and observed a 5 to 10% decrease in cellulase production, compared to static cultivation. Sukumprasertsri [24] used a fuzzy logic controller to keep the temperature within an optimal range during *Aspergillus oryzae* cultivation with soybean meal in a rotating drum SSF bioreactor. It was found that for optimal growth and enzyme production, the rotational speed should be slower at the beginning, and then increased during the process in order to compensate for the temperature variations.

Among the lignocellulosic materials that can be used as carbon sources for enzyme production under SSF, sugarcane bagasse is especially attractive in Brazil, where it is readily available in large amounts at the sugarcane mills [25]. Furthermore, a major motivation for using sugarcane bagasse as the carbon source in (hemi)cellulolytic enzyme production is related to the possibility of obtaining an enzymatic cocktail that is more efficient for degradation of this specific lignocellulosic material [26]. However, sugarcane bagasse itself does not possess all the nutritional properties necessary for good microbial growth, and is particularly deficient in terms of protein sources [13]. On the other hand, wheat bran can meet this nutritional requirement quite well, so it is of interest to evaluate the feasibility of using a combination of sugarcane bagasse and wheat bran as substrate for SSF.

The aim of this study was to evaluate the effects of intermittent mixing during SSF in terms of the production of biomass-degrading enzymes (endoglucanase, β-glucosidase, and xylanase) by different fungal strains (*Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*), using a combination of sugarcane bagasse and wheat bran as substrate.

2. MATERIALS AND METHODS

2.1 Microorganisms

The microorganisms used in this study were a mutant strain of *Aspergillus niger* (*A. niger* 3T5B8) and a wild-type strain of *Aspergillus oryzae* (*A. oryzae* P6B2), both from the Embrapa Food Technology Collection (Rio de Janeiro, Brazil), and a strain of *Trichoderma reesei* (*T. reesei* Rut-C30) from the American Type Culture Collection. Stock cultures were stored at 4 °C on potato dextrose agar (PDA) slants. The cultures were revitalized and maintained on PDA slants at 32 °C for 5 days, prior to inoculation.

2.2 Solid-state fermentation (SSF)

All SSF cultivations were carried out in 500 mL Erlenmeyer flasks for a total period of 48 h. The solid medium was sterilized by autoclaving at 121 °C for 20 min before inoculation. A spore suspension volume corresponding to 10^7 conidia per g of dry solid medium was inoculated into the solid medium by gently stirring with a glass rod until a uniform mixture was obtained. All cultivations were carried out at 32 °C, and the substrate moisture levels were adjusted to 60% with 0.9% (w/v) ammonium sulfate solution in 0.1 mol/L HCl. In a preliminary set of cultivations, wheat bran (20 g) was used as the sole solid substrate for *A. niger*, and the first mixing event was carried out at 24 h after inoculation. Mixing was subsequently performed every 2 h during the next 10 h of cultivation. In the next experiments, cultivations were carried out using a combination of wheat bran (15 g) and sugarcane bagasse (5 g) (3:1 on a dry mass basis) as substrate for *A. niger*, *A. oryzae*, and *T. reesei*. Two different mixing strategies (MS) were compared in this set of cultivations, with the first mixing event at 2 h after inoculation (MS-1) or at 12 h after inoculation (MS-2). In both cases, mixing was performed every 2 h during the following 10 h of cultivation. The mixing was carried out using a device equipped with a flat paddle, at a speed of ~10 rpm. Control experiments under static conditions were carried out for all sets of cultivations. After a 48 h cultivation period, the enzymes were extracted and analyzed as described in the following Sections. All cultivation experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

2.3 Enzyme extraction

After the 48 h cultivation period, the enzymes were extracted by adding a sufficient volume of 0.05 mol/L sodium citrate buffer, at pH 4.8, to achieve a solid/liquid ratio of 1:10 (w/v). The suspension was stirred at 120 rpm for 30 min at room temperature, and the crude enzymatic solution was recovered by filtration followed by centrifugation for 20 min at 2,500 *g* and 4 °C. The enzymatic extracts were stored at -18 °C prior to the analyses.

2.4 Analytical measurements

The activity of endoglucanase was determined according to the procedure recommended by [27]. The activity of xylanase was measured according to the methodology described by [28]. In the present work, one activity unit corresponds to 1 μmol of reducing sugar released per min per mL, under the reaction conditions. Quantification of the reducing groups employed the dinitrosalicylic acid (DNS) method [29]. The β-glucosidase activity was determined using cellobiose (Sigma, St. Louis, USA) as substrate, according to the procedure recommended by [27], with quantification of the sugars released using an enzymatic kit for glucose measurement (Doles, Goiânia, Brazil). The results were expressed as activity units per mass of initial dry solid substrate (IU/g). Total protein was measured using the Bradford method [30].

3. RESULTS AND DISCUSSION

3.1 Effect of mixing on SSF of wheat bran with *Aspergillus niger*

Table 1 presents the results for biomass-degrading enzymes production after 48 h by *Aspergillus niger* cultivated in wheat bran under both static and mixed SSF. In this preliminary set of cultivations, the effect of mixing the medium was also evaluated from the percentage differences between the activity values obtained for static and mixed SSF (Table 1). A negative effect of mixing was observed for the total protein produced, as well as for all of the enzymes analyzed (endoglucanse,

β-glucosidase, and xylanase). Overall, enzyme production was up to 28% lower in the cultivations subjected to mixing.

A possible explanation for the negative effect of mixing on enzymatic production could involve two factors: (1) the timing of the first mixing event, which was 24 h after inoculation; and (2) the physical characteristics of the solid substrate (wheat bran). In relation to the first factor, a delay of 24 h prior to the first mixing event could be too long, because mixing at this point could cause disruption of the mycelium that has already been formed. Regarding the second factor, wheat bran is a solid substrate that forms a very sticky and agglomerated material during cultivation, leading to bed compaction. The combination of these factors may therefore have resulted in lower enzyme production by *A. niger* subjected to mixing during cultivation. Furthermore, it can be speculated that factors related to bed compaction would be even more pronounced in large-scale SSF bioreactors, leading to greater energy requirements as well as other negative consequences in terms of process efficiency.

Table 1. Influence of mixing on enzymatic production by *A. niger* cultivated under SSF using wheat bran as sole substrate.

	Static	Mixed	Percentage difference (%)
Protein (mg/mL)	0.37±0.00	0.37±0.05	-1
Endoglucanase (IU/g)	52.08±1.34	37.68±7.34	-28
β-glucosidase (IU/g)	103.56±3.42	98.10±6.35	-5
Xylanase (IU/g)	570.08±35.07	426.72±96.61	-25

Table 2. Influence of mixing on enzymatic production by *A. niger*, *A. oryzae*, and *T. reesei* cultivated under SSF using a combination of wheat bran and sugarcane bagasse (3:1 on a dry mass basis).

	Static (WM)	**Mixing - after 2 h (MS-1)**	**Mixing - after 12 h (MS-2)**
A. niger			
Protein (mg/mL)	0.19±0.00	0.15±0.01	0.20±0.00
Endoglucanase (IU/g)	73.04±4.11	76.69±6.18	76.87±3.18
β-glucosidase (IU/g)	139.53±30.74	125.58±5.65	100.11±1.94
Xylanase (IU/g)	1063.78±151.69	1168.86±5.98	823.75±39.90
A. oryzae			
Protein (mg/mL)	0.42±0.01	0.32±0.03	0.47±0.03
Endoglucanase (IU/g)	17.86±0.98	16.36±2.21	17.63±4.48
β-glucosidase (IU/g)	21.05±1.26	19.83±2.78	11.96±3.91
Xylanase (IU/g)	306.39±17.97	395.30±33.59	230.56±40.60
T. reesei			
Protein (mg/mL)	0.30±0.05	0.28±0.05	0.32±0.03
Endoglucanase (IU/g)	51.25±9.27	65.24±8.02	49.65±1.56
β-glucosidase (IU/g)	2.27±1.02	2.00±0.13	1.39±0.19
Xylanase (IU/g)	654.79±57.08	536.70±44.55	918.08±40.11

According to Nava et al. [22], the physical properties of the substrate and the variables related to mixing can determine the way in which shear stress will interfere in microorganism growth and consequent enzyme production. Alberton et al. [31] evaluated the use of a mixture of sunflower seeds and sugarcane bagasse for lipase production by *Rhizopus microsporus* under SSF, and found that there was no compaction of the substrate mixture, which could therefore be used in large-scale SSF bioreactors. Delabona et al. [16] reported that the use of wheat bran in combination with sugarcane bagasse was very effective for increasing enzyme production.

Given the above findings, the next set of cultivations using different fungal strains investigated the combination of wheat bran and sugarcane bagasse as substrate for SSF. In addition, in order to address the factor related to the timing of the first mixing event, two mixing strategies were compared, with the first mixing event at either 2 h (MS-1) or 12 h (MS-2) after inoculation. Enzymatic productivities for cultivations using these mixing strategies were compared to control cultivations carried out without mixing (WM)

3.2 Effect of mixing on SSF using a combination of wheat bran and sugarcane bagasse with different fungal strains

Table 2 presents the results for biomass-degrading enzymes production by *A. niger*, *A. oryzae*, and *T. reesei* cultivated using a combination of wheat bran and sugarcane bagasse (3:1 on a dry mass basis) under static (WM) or mixed (strategies MS-1 and MS-2) conditions. The effect of mixing the medium was also evaluated by the percent differences among the mean activity values obtained under static and mixed conditions (Figures 1, 2 and 3).

In terms of total protein in the extract medium, the earlier mixing adopted in MS-1 appeared to have a negative effect for all fungi, in special for *A. niger* (Figure 1) and *A. oryzae* (Figure 2). In the case of the endoglucanase enzymes, mixing had a relatively small effect for *A. niger* or *A. oryzae*. On the other hand, earlier mixing (MS-1) had a positive effect for *T. reesei* (Figure 3). Considering the xylanase enzymes, the early mixing (MS-1) had positive effects for both *A. niger* and *A. oryzae*, while later mixing (MS-2) resulted in a very positive effect for *T. reesei*. A possible explanation underlying such differential effect of mixing observed for *T. reesei* can be related to its slower kinetics of growing in comparison to *Aspergillus* strains. Differential sensitivity of each particular microorganism to mixing can also be associated with such phenomena. Nevertheless, the greatest impact of mixing was observed for the production of β-glucosidase enzymes, with negative effects for all fungal strains and mixing strategies, and decreases exceeding 40%, compared to the static cultivations.

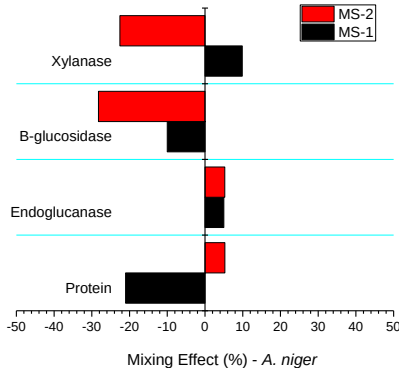

Figure 1. Effect of mixing during SSF in terms of biomass-degrading enzymes production by *A. niger* cultivated using a combination of wheat bran and sugarcane bagasse (3:1 on a dry mass basis) for the two mixing strategies (MS-1 and MS-2).

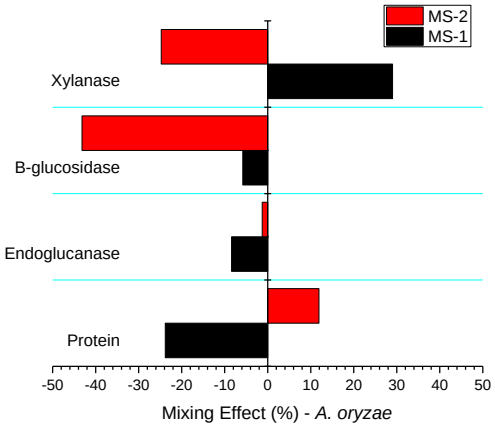

Figure 2. Effect of mixing during SSF in terms of biomass-degrading enzymes production by *A. oryzae* cultivated using a combination of wheat bran and sugarcane bagasse (3:1 on a dry mass basis) for the two mixing strategies (MS-1 and MS-2).

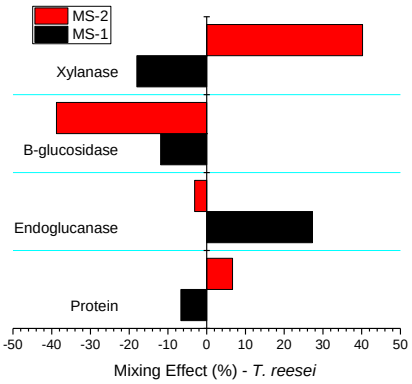

Figure 3. Effect of mixing during SSF in terms of biomass-degrading enzymes production by *T. reesei* cultivated using a combination of wheat bran and sugarcane bagasse (3:1 on a dry mass basis) for the two mixing strategies (MS-1 and MS-2).

In biomass conversion processes, cellulase enzymes act synergistically in the hydrolysis of cellulose: endoglucanases and exoglucanases or cellobiohydrolase (CBH) act directly on the cellulose fibers, while β-glucosidase hydrolyzes oligosaccharides and cellobiose to produce glucose [32, 33]. The β-glucosidase enzyme plays a vital role in the hydrolysis of lignocellulosic biomass, because it is required in order to reduce inhibition effects. Furthermore, cellobiose needs to be further degraded to glucose before it can be utilized by conventional yeasts for ethanol production [34]. It has recently been reported that β-glucosidase represents the bottleneck in the overall biomass bioconversion process, because it is a key enzyme required for complete cellulose hydrolysis [33]. Interestingly, the present findings demonstrated that β-glucosidase was the enzyme most negatively affected by mixing during fungal cultivation under SSF. Therefore, special attention should be given to β-glucosidase during bioprocess development for cellulase production under SSF in large-scale bioreactors.

Flodman and Noureddini [23] studied the effect of mixing on cellulase production by *T. reesei* NRRL 11460 cultivated under SSF with wet corn distillers grain. It was found that mixing resulted in a 5-10% reduction in cellulase production (measured as total cellulase by filter paper activity). Deschamps et al. [35] reported that mixing caused a reduction of around 40% in cellulase production by *T. harzianum* under SSF. Therefore, although mixing during SSF cultivations can be used to prevent channeling and overheating problems in large-scale bioreactors, careful consideration should be given to the negative effects on cellulase production, especially the impacts on β-glucosidase enzymes.

Overall, it can be concluded from the results described above that mixing during SSF had more negative than positive effects on overall enzyme production. Furthermore, evaluation of the two different mixing strategies showed that starting the mixing after 12 h of cultivation (MS-2) was more deleterious to enzymatic production than starting mixing after 2 h (MS-1).

Such results can be explained by the fact that the mixing strategy after 12 h could be contributing to break the mycelium and leading to a negative effect on growth and enzyme production. In addition, mixing can also interfere with the microbial respiration rate in SSF since hyphae could be either broken or pressed between substrate particles, resulting in oxygen limitation. These findings could be very useful for the selection of mixing strategies most suitable for the implementation of large-scale SSF processes for biomass-degrading enzymes production.

3.3 Comparison of enzymatic production by the different fungal strains cultivated under SSF

Besides evaluation of the effects of mixing on SSF, analysis of the results enabled comparison of the enzymatic productivities of the different fungal strains (*A. niger*, *A. oryzae*, and *T. reesei*) when cultivated under SSF using a combination of wheat bran and sugarcane bagasse (Table 2). Higher production of all of the enzymes analyzed was achieved by *A. niger*. For example, endoglucanase production by *A. niger* was 4-fold higher than obtained using *A. oryzae*. The difference was even greater for β-glucosidase, with 60-fold higher production by *A. niger*, compared to *T. reesei*. The superiority of *A. niger* strains over *Trichoderma* strains for β-glucosidase production was expected, but the magnitude of the difference was even higher than reported by Pirota et al.[36], who found that production of β-glucosidase by *A. niger* was almost 35-fold higher than by *T. reesei*.

Trichoderma reesei is one of the most productive cellulolytic fungi, and the *T. reesei* Rut-C30 strain is one of the most studied and best characterized strains [3]. Nonetheless, the amount of β-glucosidase secreted by *Trichoderma reesei* is not sufficient for efficient biomass conversion [2], because the complete conversion of cellulose to glucose is dependent on the amount of active β-glucosidase. Overall, the present results demonstrate the considerable potential of the *A. niger* 3T5B8 strain for the production of glycosyl hydrolases. This organism showed a significantly higher enzymatic biosynthesis capacity, compared to the well-established cellulolytic strain *T. reesei* Rut-C30, in agreement with the results of Pirota et al.[36].

Another interesting finding in these sets of experiments concerns the improvement in enzymatic production when *A. niger* was cultivated under SSF using a combination of wheat bran and sugarcane bagasse (Table 2), rather than wheat bran alone (Table 1). This improvement was observed for all three enzymes, but was much more significant for the xylanase enzymes, whose activity value almost doubled. As mentioned before, wheat bran is a substrate that has a higher protein content than sugarcane bagasse [37], which is beneficial for the development of microbial growth. It was found here that enzymatic production by *A. niger* could be improved by using a combination of wheat bran and sugarcane bagasse (3:1 on a dry mass basis). This improvement could have been due to the better overall porosity of the substrate, resulting in reduced heat and mass transfer limitations, compared to wheat bran alone. Delabona et al. [16] also studied the use of wheat bran in combination with sugarcane bagasse (1:1 on a dry mass basis) for cellulase and xylanase production by *A. fumigatus*, but the activity values obtained were similar or inferior to those achieved with wheat bran alone. This indicates that the ratio of wheat bran to sugarcane bagasse is also a relevant parameter that needs to be evaluated during SSF bioprocess development.

4. CONCLUSIONS

Studies of the effects of mixing on the growth of microorganisms and enzyme production should be conducted during the development of a SSF bioprocess. It was found here that when *A. niger* was cultivated using wheat bran as the sole substrate, there was a negative effect of mixing on the production of enzymes. However, this negative effect on fungal growth and enzyme production was reduced when the cultivation was carried out using a combination of wheat bran and sugarcane bagasse as substrate. For all the fungi tested, the enzyme most affected by mixing during SSF was β-glucosidase, for both of the mixing strategies evaluated.

Conflict of interests

The authors declare they have no conflict of interests.

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