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Ethanol production from agroindustrial biomass using a crude enzyme complex produced by *Aspergillus niger*

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ABSTRACT

This study investigates ethanol production from simultaneous fermentation and saccharification (SFS) and separated hydrolysis and fermentation (SHS) using enzyme complexes produced by *Aspergillus niger* strains (ATCC 16404, ATCC 1057, ATCC 9029). The enzyme complexes were produced by solid-state fermentation (SSF) on inexpensive and readily available agroindustrial products: rice byproduct (composed of AFEX-treated rice rust and rice bran), whey and sugarcane bagasse. The ethanol was produced by *Saccharomyces cerevisiae* Y904 using whey and rice byproduct as the substrate and the enzyme complex produced by *A. niger*. The best result for solid-state fermentation (40 U/g of dry substrate, *A. niger* ATCC 16404) was obtained in a 0.5 L rotating drum bioreactor at 40 °C filled half filled with solid biomass composed of rice byproduct (86% wt/wt), whey (12% wt/wt) and $CaCl_2$ (2.0% wt/wt). The best result for ethanol fermentation (11.7 g/L of ethanol) was obtained after 12 h of SFS at pH 4.5 and 35 °C. A comparative study of ethanol production by *Trichoderma reesei* CCT 2768 and *A. niger* ATCC 16404 complexes under the same optimised SFS and SSF conditions was also performed, revealing that ethanol production by the *A. niger* enzyme complex was 2.25 times higher than that by *T. reesei*. These findings suggest that the ethanol production using crude enzymatic complexes produced by *A. niger* and agroindustrial biomass described in this paper is very promising in terms of disposal of the whey produced by cheese-making and other dairy food processing.

1. Introduction

Environmental concerns about *agro-industrial waste* have inspired a search for new methodologies for energy production and have become a leading interest of the global scientific community. In this context, Brazil is one of *the largest agro-industrial cellulosic residues* producers and the production of ethanol from biomass is expected to become increasingly important. Cellulosic materials have a potentially negative environmental impact due to waste disposal issues; however, they are also the most abundant source of fermentable sugars and many extremely cost-effective solid-state fermentations can be used to produce enzyme complexes to digest cellulosic materials. Agro-industrial residues such as wheat bran, leaves, wood dust, rice hull and bran, municipal solid waste, whey and sugarcane are suitable for use as substrates for ethanol fermentation by the enzymatic conversion of carbohydrates to fermentable sugars [1,2].

Solid-state fermentation is a process in which microorganisms grow on the surface of solid materials. In this process, the composition of the solid material can be modified by adding nutrients or water to optimise the medium or to regulate the water activity during fermentation. SSF offers some advantages over submerged fermentation, including the use of simple growth and production media comprised of agro-industrial residues, the release of negligible or considerably less effluent, simplicity, ability to handle small volumes, low energy use, and the ease of product separation [3,4].

Several types of microorganisms, including bacteria, algae and fungi, are used in solid-state fermentation, but filamentous fungi are most commonly used and the most thoroughly studied for SSF. This popularity could be due to their abilities to naturally grow on solid surfaces with lower water activity and their hyphal growth mode, which can penetrate deeper into the substrate [3,5]. Although many hydrolytic enzymes are commercially exploited and used in research to catalyse the hydrolysis of cellulosic biomass to produce ethanol, there is still no complete understanding of

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exactly how hydrolysis works. In the context of the growing interest in enhancing ethanol productivity, *Aspergillus* and *Trichoderma* fungi have been widely used for cellulosic ethanol research [1,2,6–9].

Thus, the present work evaluated the production of *Aspergillus niger* crude enzyme complexes by solid-state fermentation and the use of these crude enzyme complexes in the batch ethanol fermentation of *Saccharomyces cerevisiae* in a culture broth composed of whey, rice hulls and rice bran. The use of SSF for the production of crude enzyme complex of *Trichoderma reesei* CCT 2768 for biomass hydrolysis and ethanol production was also comparatively evaluated.

2. Material and methods

2.1. Microorganism

A. niger ATCC 16404, ATCC 1057 and ATCC 9029 *strains obtained* from the *Culture Collection* of the Fundação Tropical André Tosello (Brazil) were used for solid-state fermentations. The strains were *kept* on *slants* of potato dextrose agar (PDA) stored at 5 ± 1 °C for 20 days and routinely subcultured onto PDA with the following composition (g/L): potato (200), glucose (15). The solid-state fermentations were conducted using 1.0×10^7 to 1.0×10^8 spores/g of cell as inoculum. *T. reesei* CCT 2768 from the *Culture Collection* of the Fundação *Tropical André* Tosello (Brazil) was also used in solid-state fermentation. All ethanol fermentations were performed using *S. cerevisiae* Y904.

2.2. Solid-state fermentations

Solid-state fermentations were performed in a 0.5 L rotating drum bioreactor incubated for 3–72 h at 3 rpm to produce the enzyme complex. The solid biomass used in the fermentations was *AFEX-treated* [10] rice byproduct milled and sieved through 1.8 mm mesh, whey powder reconstituted with distilled water to give a solid concentration of 60 g/L and sugarcane bagasse milled and sieved under the same conditions as the rice byproduct. The fermentations were performed using the compositions described in Table 1.

2.3. Ethanol fermentations

The ethanol fermentations were placed in 250-ml shake flasks incubated at 35 °C ± 1 °C and agitated at 150 rpm in a rotary shaker. The initial pH value was 4.5, and the inoculum concentration was 60 g/L. The fermentations were performed in simultaneous fermentation and saccharification (SFS) and separated hydrolysis and fermentation (SHF) with 100 ml of fermentation broth containing whey and rice byproduct, yeast extract and mineral salt solution and water (Table 2).

Table 1
Composition of solid media used in solid fermentation[a,b].

Medium	Rice byproduct[a] (% wt/wt)	Whey[b] (% wt/wt)	Sugarcane bagasse (% wt/wt)	$CaCl_2$ (% wt/wt)
RW[c]	86	12	0	2
BW[d]	0	12	86	2
RB[e]	43	12	43	2

[a] Rice byproduct was composed of 50% *AFEX-treated* rice hulls and 50% rice bran (vol/vol).
[b] Whey powder was reconstituted with distilled water to a solid concentration of 60 g/L.
[c] RW: Rice byproduct + whey.
[d] BW: Bagasse + whey.
[e] RB: Rice byproduct + rice bran.

Table 2
Composition of fermentation media used in ethanol fermentation.

Medium	Rice byproduct[a] (% wt/vol)	Whey[b] (% vol/vol)	Enzymatic extract (%vol/vol)	Water (% vol/vol)	Mineral solution[c]
SRW[d]	50	50	50	0	yes
SW[e]	0	50	50	0	yes
SR[f]	50	0	50	50	yes

[a] Rice byproduct consisted of 50% *AFEX-treated* rice hulls and 50% rice bran (vol/vol).
[b] Whey powder was reconstituted with distilled water to a solid concentration of 60 g/L.
[c] KCl (5.0 g/L), NH_4Cl (1.0 g/L), KH_2PO_4 (5.0 g/L), $MgSO_4$ (1.0 g/L).
[d] SRW: Submerged fermentation medium containing mostly rice byproduct + whey (1:1 ratio).
[e] SW: Submerged fermentation medium containing mostly whey.
[f] SR: Submerged fermentation medium containing mostly rice byproduct + whey.

2.4. Extraction conditions

After the fermentation, the media was poured into Erlenmeyer flasks containing 70 ml of Tween 80 (1.0%) and then stirred in a rotary shaker (150 rpm, 30 min, 4 °C). The suspension resulting from the extraction was centrifuged at 18,900 g for 20 min at 4 °C. The supernatant was stored at 4 °C and used as the enzyme extract in SFS and SHF.

2.5. Analytical methods

Filter paper activity (FPA) was assayed according to standard IUPAC procedures and expressed in international units (IU) [11]. Ethanol production was estimated by the dichromate colourimetric method (590 nm), which is based on the complete oxidation of ethanol by dichromate in the presence of sulphuric acid to form acetic acid. The reducing sugar was measured by the DNS method for reducing sugars [12].

3. Results and discussion

3.1. Media selection for solid-state fermentation and ethanol fermentation

The solid culture media were composed of the inexpensive and widely available agroindustrial byproducts RW, BW and RB. All *A. niger* strains produced enzyme complexes in the tested media; however, the enzyme complex activity depended on the media and strains. The strain *A. niger* ATTC 16404 had better activity in RW, BW and RB media and produced enzyme complexes with higher activity (40 U/g) when grown on RW than when grown on BW and RB (Fig. 1). Based on these results, *A. niger* ATTC 16404 was selected for further experiments.

The ethanol production by *S. cerevisiae* Y904 in SFS in three different media is given in Table 3. A significant increase in the final ethanol content was observed for the SRW medium relatively to that for other media; the ethanol content was 4.2 times that produced in the SW medium. However, the reducing sugar consumption indicated the existence of a nutrient limitation or inhibitory metabolites in the medium. These results are in good agreement with the results for similar tests with other substrates. Wilkins et al. [13] noted that fruit byproduct hydrolysis by commercial enzymes in SFS was not greatly affected by varying the enzyme activity above 0.02 U/g. Stenberg et al. [14] observed an