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Biodegradation of agroindustrial wastes by *Pleurotus* spp for its use as ruminant feed

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Abbreviations: ADF: acid detergent fibre
cfg/g: colony forming units per gram
ITS: internal transcribed spacer region
NDF: neutral detergent fibre
PCR: polymerase chain reaction
RFLP: restriction fragment length polymorphism
SSF: solid substrate fermentation

The increasing expansion of agro-industrial activity has led to the accumulation of a large quantity of lignocellulosic residues all over the world. In particular, large quantities of rice straw (300.000 t) and citric bagasse (50.000 t) are annually produced in Uruguay. In this work we present the study of the bioconversion of these substrates with the edible mushroom *Pleurotus* spp so as to increase nutritional values and digestibility for its use as animal feed. The SSF process was optimized and the products after different periods of

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mushroom growth were evaluated. The microbial counts (cfu/g) for the inoculated substrates 44 days after incubation were 15 x 10^4, < 10 and < 10 for aerobic microorganisms, coliforms and *E. coli*, respectively. After 14 days of SSF the percentage of dry matter, ADF and NDF decreased, and the content of protein increased. These results show that vegetal cell-wall components were degraded during the period of mushroom incubation. PCR – RFLP analysis of the ITS region was used to characterize the *Pleurotus* species produced in Uruguay and discriminate between DNAs of *Pleurotus ostreatus* 814 and other fungi from the different substrates.

From the production, processing and consumption of agricultural products, there are a great variety of remainders, which create increasing problems of elimination. In Uruguay, the great majority of the industries that process agricultural products discard their remainders with no treatment, implying a huge aggression to the environment. For example, the citrus processing plants produce 50,000 tons per year of citric bagasse which represents 40-50% in weight of the fresh fruit. Its composition is relatively adequate for the feeding of ruminants, but present palatability problems and is contaminated with normal flora of the rinds, some of which are mycotoxin producers. With respect to rice straw it is obtained at the rate of 2000 kg per harvested hectare (310,000 ton of straw per year); and although it is used in the feeding of ruminants, it presents a very low protein content and low digestibility.

Other authors have shown that some fungi, particularly some species of *Pleurotus* are able to colonize different types of vegetable wastes, increasing their digestibility (Platt et al. 1984; Commanday and Macy, 1985; Rajarathnam and Bano, 1989; Villas-Boas et al. 2002; Zhang et al. 2002; Mukherjee and Nandi, 2004; Salmones et al. 2005). Previous studies have shown the feasibility of using these kind of wastes to produce animal feed (Calzada et al. 1987; Adamovic et al. 1998), and as substrate for mushroom production (Breene, 1990; Sermanni et al. 1994; Kakkar and Dañad, 1998; Yildiz et al. 2002).

In the present work, we study the biodegradation of these wastes by *P. ostreatus* 814 for its use as ruminant feed.

MATERIALS AND METHODS

Determination of the capacity to grow on the wastes under study

The strains used in this study (*P. ostreatus* 814, *P. ostreatus* 816, *P. cornucopiae and P. djmour*) were provided by Trinidad Mushrooms. They were grown in malt agar (1,25% malt extract, 1,5% agar, Oxoid) at 28ºC. The mycelium was then transferred to bottles with sterilized wheat grains and incubated again at 28ºC until colonization of the substrate was observed (approximately 7 days). These fermented grains were used as inocula (10% in weight) for the growth tests in trays at 28ºC on 500 g of substrate without sterilizing (citrus bagasse, rice straw and the mixture of both (1:9 w/w)).

From these experiments, *P. ostreatus* 814 was the most promising strain.

Evaluation of the characteristics of the fermentation products

1. Determination of the microbiological quality was performed using Petrifilm (3M). Total aerobes, total coliforms and *E. coli* were determined on the substrates without inoculation and after 44 days of fermentation with *P. ostreatus* 814.

2. Analysis of the chemical composition of the fermentation product consisted in the analysis of dry weight (AOAC, 1990), proteins (AOAC, 1990) and neutral and acid detergent fiber (AOAC, 1996) of the different substrates without inoculating and on different days of fermentation with *P. ostreatus* 814. The results were analyzed using t-Student test with $P < 0.001$.

The significance of the differences were estimated by using the Mann-Whitney U test (Mann and Whitney, 1947), with the limit of significance set at $P < 0,05$. Statistical analyses were performed on SPSS 9.0 Windows.

Molecular characterization

DNA extraction. Genomic DNA was obtained from pure cultures of fungi belonging to the mushroom producers "Trinidad Mushrooms" (*Pleurotus ostreatus* 814, *Pleurotus*

Table 1. Determination of the microbiological quality. The values reported are the mean of two measurements.

Microbial count (cfu/g)			
	Total aerobic count	**Total coliforms**	***E. coli***
Citric bagasse day 0	< 100	< 10	< 10
Fermentation product day 44	15 x 10^4	< 10	< 10

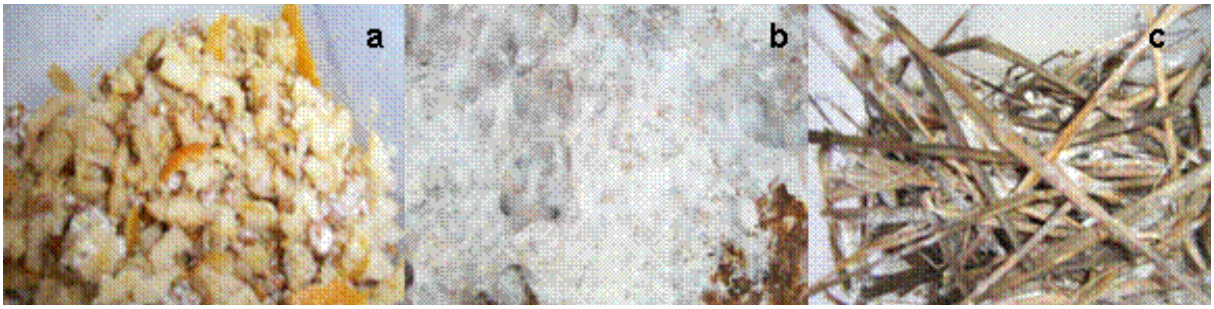

Figure 1. Mycelial colonization by *P. ostreatus* 814 on citrus bagasse and rice straw.
(a) 21 days of fermentation on citrus bagasse.
(b) 44 days of fermentation on citrus bagasse.
(c) 21 days of fermentation on rice straw.

ostreatus 816, *Pleurotus cornucopiae* y *Pleurotus djmour*) or from the fermentation product after inoculation of wheat seeds or citrus bagasse. Fresh mycelia or the corresponding fermentation product was grounded under liquid nitrogen in a sterile mortar, to obtain a fine powder. The extraction technique performed followed (Jasalavich et al. 2000), and is based in the classical extraction with cetyltrimethylammonium bromide (CTAB) in the presence of β-mercaptoethanol, followed by organic extractions and isopropanol precipitation of the DNA. DNA was quantified by spectrophotometrical measurement at 260 and 280 nm and its integrity was evaluated in 0,8% agarose gels, using ethidium bromide (EtBr). DNA bands were visualized by the fluorescence of the intercalated EtBr under UV light.

PCR amplification. The ITS region was amplified by PCR from DNA isolated from pure cultures of each of the fungi under study and from the corresponding fermentation product. Primers ITS1-F (CTT GGT CAT TTA GAG GAA GTA A) which is specific for the higher fungi, and ITS4 (TCC TCC GCT TAT TGA TAT GC) the universal primer, were used together to amplify the ITS region from higher fungi. The primer pair ITS1-F and ITS4-B (CAG GAG ACT TGT ACA CGG TCC AG), which is specific for basidiomycetes, were used to specifically amplify the ITS region from only basidiomycetes. Amplification were performed in 50 µl reactions of PCR buffer (Invitrogen), 5 mM concentrations of each deoxyribonucleotide triphosphate, 10 pmol concentrations of each appropriate primers, 10 ng of total DNA isolated from a pure culture or from the fermentation product, and 1U of *Taq* polymerase (Invitrogen). PCR reactions consisted of denaturation at 94ºC for 1 min 25 s, 35 cycles of amplification, and a final extension at 72ºC for 10 min; each cycle of amplification consisted of denaturation at 95ºC for 35 s, annealing for 55 s (at 55ºC for reactions with ITS1-F and ITS4 and at 60ºC

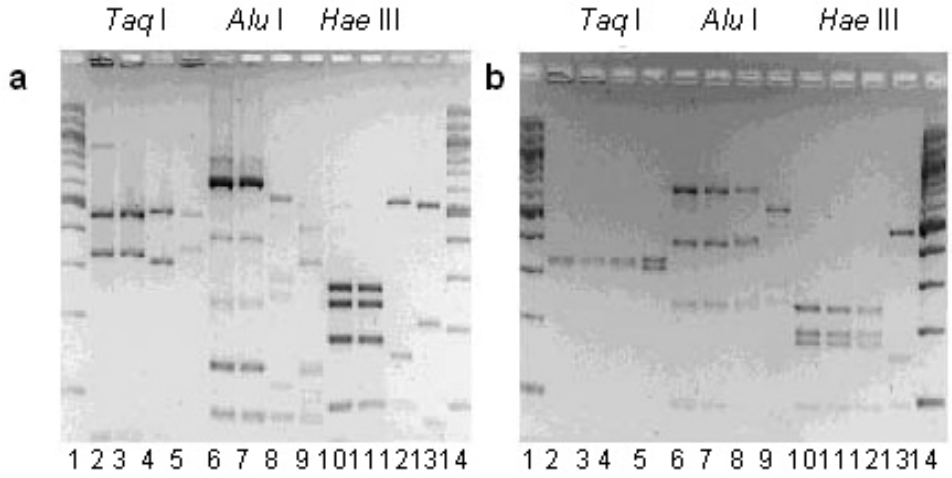

Figure 2. Restriction profiles obtained on restriction with *Taq* I, *Alu* I y *Hae* III.
(a) Amplification with specific primers. 2, 6 and 10: *P. ostreatus* 814 ; 3, 7 and 11: *P. ostreatus* 816, 4, 8 and 12: *P. cornucopiae*; 5, 9 and 13: *P. djmour*. 1 and 14: Molecular weight marker (Smart ladder, Eurogentec).
(b) Amplification with general primers. 2, 6 and 10: *P. ostreatus* 814 ; 3, 7 and 11: *P. ostreatus* 814 (SSF on citric bagasse); 4, 8 and 12: *P. ostreatus* 816; 5, 9 and 13: *P. cornucopiae*. 1 and 14: Molecular weight marker (Smart ladder, Eurogentec).

Table 2. Chemical analysis of the substrates under study at 0, 7 and 14 days of fermentation. The values reported are the mean of two measurements.

Substrate: citrus bagasse				
Ferm. day	**Dry weight (w/w)**	**Protein (w/w)**	**ADF (w/w)**	**NDF (w/w)**
0	19.8	0.6	4.5	4.7
7	16.8	0.8	3.5	4.0
14	16.7	0.8	3.1	3.6
Substrate: rice straw				
Ferm. day	**Dry weight (w/w)**	**Protein (w/w)**	**ADF (w/w)**	**NDF (w/w)**
0	27.3	0.7	17.5	18.5
7	22.3	0.7	12.2	14.0
14	18.3	0.9	10.2	11.2
Substrate: rice straw + citrus bagasse 1:9 w/w				
Ferm. day	**Dry weight (w/w)**	**Protein (w/w)**	**ADF (w/w)**	**NDF (w/w)**
0	20.0	0.5	6.12	6.3
7	14.8	0.7	4.8	5.3
14	14.4	0.6	4.3	4.8

for reactions with ITS1-F and ITS4-B), and extension at 72ºC for 1 min. PCR products were separated by electrophoresis in 0,8% (wt/vol) agarose gels in 1 x TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) with EtBr at 100 ng/ml in the geland running buffer; DNA bands were visualized by the fluorescence of the intercalated EtBr under UV light and photographed. Molecular weight markers were run together with the samples.

Restriction digestion of PCR products. PCR reaction products were digested directly without further purification with restriction endonucleases to obtain RFLPs; each sample was digested with *Alu*I (Biolabs), *Hae*III (Biolabs) and *Taq*I (Biolabs) in single-enzyme digests. 10 µl of amplified PCR reaction was mixed with the appropriate restriction reaction buffer and 10 U of the appropriate enzyme and then incubated for 6 hrs at 37ºC for the *Alu*I or *Hae*III digests or at 65ºC for the *Taq*I digests. Restriction fragments were separated by electrophoresis in 3% (wt/vol) agarose in 1 x TAE (40 mM Tris acetate, 1 mM sodium EDTA) with EtBr at 100 ng/ml in the gel and running buffer. DNA bands were visualized by fluorescence under UV light and photographed.

RESULTS AND DISCUSSION

Determination of the capacity to grow on the wastes

At 7 days of fermentation fungal growth is already appreciated on all the substrates under study, with all the strains assayed. A greater colonization is achieved at day 21 with *P. ostreatus* 814 (Figure 1).

Determination of the microbiological quality

Although an increase in the total aerobic count is seen for citrus bagasse with respect to day 0, no appreciable increase in total coliforms or *E. coli* is appreciated (Table 1).

Analysis of the chemical composition

After fermentation, a decrease in the dry weight, increase in the levels of proteins and a decrease in the values of neutral detergent fiber (hemicellulose, cellulose and lignin) and acid detergent fiber (lignin and cellulose) were detected (Table 2). These last determinations could be indicative of the degradation of the cell wall components of the substrates produced by the extracellular enzymes of *P.*

ostreatus. Previous authors concluded that lignification of structural polysaccharides not only inhibited ruminal microbial digestion of polysaccharides by forming 3-D matrix, but also that the presence of highly lignified tissues formed a physical barrier preventing the accessibility of the otherwise highly digestible tissues to the action of hydrolytic enzyme of the rumen microorganisms (Karunanandaa et al. 1995), and have shown that increased digestibility was associated with the degradation of structural carbohydrates (Mukherjee and Nandi, 2004).

No nitrogen source was added, as previous work showed that the *in vitro* digestibility of the fermented substrate was decreased as compared with fermentation substrate not amended with nitrogen.

Molecular characterization of the strains under study

When using primers ITS1F and ITS4B, specific for basidiomycetes, an interspecies polymorphism is observed in the band profile obtained, whilst there is no difference observed within a same species (Figure 2a). When making the amplification with primers ITS1F and ITS4, general for higher fungi, the same restriction profile for fungi of the same species (*P. ostreatus* 814 and 816) is obtained. This same profile is obtained when DNA is extracted from the substrates under study colonized by *P. ostreatus* 814. It is important to emphasize that when using these general primers no additional bands, that could correspond to other fungi, were obtained (Figure 2b).

These results show not only that strain in use in the mushroom producing industry can be identified to species level using molecular techniques, but also that it is plausible to carry out a molecular characterization of the fermentation products (Villas-Boas et al. 2002).

CONCLUDING REMARKS

From the results obtained, we can conclude that *P. ostreatus* 814 is an appropriate strain to use as inocula of the agroindustrial wastes under study. Higher protein levels, better conservation of the substrate and an increase in *in vitro* digestibility is observed. A scale up process, using rice straw bales in the open, is under way.

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