African Journal of Biotechnology Vol. 10(29), pp. 5648-5653, 22 June, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2353
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Bioconversion of rape straw into a nutritionally enriched substrate by *Ganoderma lucidum* and yeast

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Accepted 24 March, 2011

This work aims to select biological treatments and conditions for the bioconversion of rape straw by the mixed-strain fermentation of *Ganoderma lucidum* and yeasts (*Saccharomyces cerevisiae, Candida tropicalis and Candida utilis*), into an enriched substrate with increased crude protein and digestibility. Orthogonal experiment showed that the optimal experimental condition for the crude protein enrichment was: 10% (v/w) *C. utilis* inoculum was added to the rape straw medium after 7 days of *G. lucidum* growth; the crude protein content of the substrate was 16.23%; the yield rate were increased by 75.70 and 225.90%, respectively when compared to the *G. lucidum* individual-fermentation and without fermentation substrate. The results in this study also indicated that: the co-culture of 2 fungi (*G. lucidum* + *C. utilis*) was better than individual (*G. lucidum*) culture on the degradation of cellulose and lignin of rape straw substrate and the secretion of ligninolytic enzyme system including laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP).

Key words: Bioconversion, rape straw, nutritionally enrichment, *Ganoderma lucidum*, yeast.

INTRODUCTION

The increasing expansion of agro-industrial activity has led to the accumulation of a large quantity of lignocellulosic residues all over the world (Villas-Bôas et al., 2002b), particular, large quantities of rape straw are annually produced in China (Wang, 2007). With the improvement of the living standards of farmers, coal, liquefied petroleum gas, electricity, gas and other new energy have been popularized in the rural areas. The traditional cooking values, as an alternative to steam explosion of rape straw, are gradually being lost, and the results are mostly fields burning or natural degradation. The former would be a direct result of serious air pollution, while the latter will be doing nothing to release a large number of greenhouse gases and water pollutants (Petersson et al., 2007; Zhu et al., 2005).

The potential for microbiologically modifying or enriching agricultural wastes as stock-feed has been investigated extensively in the past (Gélinas and Barrette, 2007; Ghaly et al., 2005; Stabnikova et al., 2005; Tengerdy and Szakacs, 2003; Bisaria et al., 1997; Nigam and Singh, 1996). Microbial conversion, especially fungal bio-conversion of wastes seems to be a practical and promising alternative for increasing their nutritional value, transforming them into animal feed and thus producing a value-added product (Agosin et al., 2006; Villas-Bôas et al., 2003; Villas-Bôas et al., 2002a), fungal bioconversion of agricultural by-products is an environmentally friendly biotechnological process (Mukherjee and Nandi, 2004; Zhang et al., 2002; Huettermann et al., 2000; Karunanandaa et al., 1995). From an animal nutrition point of view, rape straw is not a suitable feed as it is deficient in digestible protein (Song et al., 2009). Growth of yeast on the agriculture residue increases protein and vitamin contents (Villas-Bôas et al., 2002b). However, the low level of fermentable sugars limits protein enrichment of rape straw by yeasts, and a major portion of the rape straw comprises lignocelluloses. Therefore, in the present investigation, an attempt has been made to use a co-culture system (with a white rot fungi as the lignocelluloses organism and yeast as the fermentative organism) to increase the protein content and digestibility

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Abbreviations: Lac, Laccase; **MnP,** manganese peroxidase; **LiP,** lignin peroxidase; **ABTS,** 2,2-azino-bis (3-ethylbenzthiazoline)-6-sulfonate; **SSF,** solid state fermentation.

Table 1. Levels of 3^3 full factorial experimental design.

Level	Factor		
	Kinds of yeast (A)	**Inoculation time of yeast (B)**	**Inoculation volume of yeast (v/w) (C)** (%)
1	*S. cerevisiae*	At the same time with *G. lucidum*	5
2	*C. tropicalis*	After 7 days of *G. lucidum* growth	10
3	*C. utilis*	After 14 days of *G. lucidum* growth	15

of the rape straw substrate.

MATERIALS AND METHODS

Substrate

Rape straw was collected in the suburb of Wuhu region, the residues were dried at 50°C, and the straw was cut in to pieces of 0.5 to 1.0 cm.

Fungi

Candida tropicalis AS 2.587, *Candida utilis* AS2.281 and *Saccharomyces cerevisiae* CGMCC 2.118 were purchased from Chinese Academy of Agricultural Sciences and maintained on malt extract agar slants, *Ganoderma lucidum* GF-1 was found and isolated in the south of Anhui province of People's Republic of China and maintained on potato dextrose agar (PDA) at 4°C with periodic transfer.

The inocula of yeasts were prepared by growing yeasts on a rotary shaker at 130 rpm and 28°C in 500 ml flasks containing 100 ml of malt extract medium (2%malt extract, 2% glucose, pH 5.5); and the inocula of *G. lucidum* was prepared by growing mushrooms on a rotary shaker at 130 rpm and 25°C in 500-ml flasks containing 200 ml of following synthetic medium (per litre): 30 g glucose, 5 g peptone, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.5 g KH_2PO_4, 100 µg vitamine B_1 and the medium was adjusted to pH 6.0 to 6.2 with 2 M NaOH. After 18 h of three yeasts and 7 days of *G. lucidum*, cultivation was carried out.

Protein enrichment of rape straw in co-culture

The following factors were considered: kinds of yeast, inoculation time of yeast and inoculation volume of yeast (v/w) in the protein enrichment of rape straw substrate, and they were analyzed by 3^3 factorial experimental design (completely randomized design, three variables in three levels), as shown in Table 1.

The solid-state fermentation (SSF) of rape straw has been carried out at 27°C in plastic bags containing 200 g of lignocellulosic substrate (moistened with distilled water to 65%): 95.9% rape straw, 2% wheat bran, 2% bean dreg, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.05% KH_2PO_4. The initial pH of the medium was adjusted to 6.0 to 6.2 prior to sterilization by adding 2 M NaOH. Solid state fermentation (SSF) medium were sterilized at 121°C for 1.5 h. The substrate was cooled to room temperature and then inoculated with 10% (v/w) of *G. lucidum* inoculum, then the yeasts were also inoculated, the time and volume of inoculation of *S. cerevisiae, C. tropicalis and C. utilis* are shown Table 1. According to the orthogonal experiment design, 9 combinations were executed.

Validation of solid-state fermentation efficiency

After determining the best culture conditions, a second solid-state fermentation with the optimize culture condition was performed using rape straw substrate. The optimize culture condition were as following: 10% (v/w) *C. utilis* inoculum was added to the rape straw growth medium after 7 days of *G. lucidum* growth. At the same time, single-strain fermentation with *G. lucidum* was also performed. Kinetic parameters were evaluated during fermentation of rape straw.

Crude protein analyses

After 50 days of fermentation, the growth medium were dried at 50°C and milled with a grinder. Crude protein was determined using the Kjeldahl method (Clesceri et al., 1992).

Cellulose, lignin and enzyme activity assessment

Every 10 days, cellulose was determined using a semimicro method described by Updegraff (1969), and the amount of lignin present in rape straw substrate was determined using a modified Klason method (Hatfield et al., 1994). The production of extracellular ligninolytic enzymes were also investigated, 3 g culture medium samples were extracted with 30 ml distilled water for 4 h at room temperature, filtered using 4 layer gauze, and the filtrate were centrifuged at 4000 g for 10 min and the supernatants were used for the enzyme assays. Laccase (Lac) activity were determined via the oxidation of 2,2-azino-bis (3-ethylbenzthiazoline)-6-sulfonate (ABTS) (Wolfenden and Willson, 1982), manganese peroxidase (MnP) activity was determined by the method of Wariishi et al. (1992), and lignin peroxidase (LiP) was assayed according to Arora and Gill (2001).

Statistical analysis

All the experiments were performed in triplicate, and the data presented are the averages of triplicate measurements with a standard error less than 10%.

RESULTS AND DISCUSSION

Protein enrichment of rape straw substrate after mixed-strain fermentation by *G. lucidum* and three yeasts, respectively (orthogonal experiment)

Experiments were conducted by using orthogonal experiment design. The kind of yeast, the inoculation time of yeasts and the inoculation volume of yeasts were used to compare their effects on the increased crude protein of rape straw substrate after SSF. The results obtained for the 3^3 factorial design can be seen in Table 2. Orthogonal experiment results showed that, the principal–secondary

Table 2. Treatment combinations and experimental results of 3^3 full factorial orthogonal experimental design.

Combination number	Factor			Crude protein content of the fermented substrate (%)
	A	B	C	
1	1	1	1	9.67±0.58
2	1	2	2	13.55±1.03
3	1	3	3	10.34±0.98
4	2	1	2	10.96±0.85
5	2	2	3	11.49±1.02
6	2	3	1	9.59±0.68
7	3	1	3	11.65±0.99
8	3	2	1	14.73±1.23
9	3	3	2	15.84±1.37
K_1	33.56	32.28	33.99	
K_2	32.04	39.77	40.35	
K_3	42.22	35.77	33.48	
Range	3.39	2.49	2.29	
Optimization level	A_3	B_2	C_2	

Table 3. Protein enrichment of rape straw substrate after single-strain fermentation of *G. lucidum* and mixed-strain fermentation of *G. lucidum* and *C. utilis*.

Protein enrichment	Treatment		
	Without fermentation	Fermentation by *G. lucidum*	Fermentation by *G. lucidum* and *C. utilis*
Crude protein content (%)	4.98±0.25	8.75±0.42	16.23±1.37
Increased rate of crude protein (%)	0	75.70	225.90

sequence of factors was: the kind of yeast, the inoculation time of yeast and the inoculation volume of yeast; and the optimal condition was $A_3B_2C_2$: 10% (v/w) *C. utilis* inoculum was added to the rape straw substrate growth medium after 7 days of *G. lucidum* growth.

The co-culture of *G. lucidum* and C. *utilis* proved to be the best combination. In the co-cultures, the white rot fungi, *G. lucidum* hydrolyses the cellulose or hemi-cellulose component of the rape straw by secreting extracellular enzymes (cellulases and xylanases) and the yeast then uses the sugar released. The higher yield of protein from the *G. lucidum* and C. *utilis* combination probably results from the enzymatic hydrolysis of the lignocellulosic component of the rape straw by the *G. lucidum* releasing hexoses and pentoses which C. *utilis* can efficiently metabolise. *S. cerevisiae,* however, uses only hexoses (Bhalla and Joshi, 1994) and consequently is less efficient than the C. *utilis*. In addition, C. *utilis* itself has high content of protein.

Validation of solid-state fermentation efficiency under the optimal condition

Experiments were conducted according to the optimal condition: 10% (v/w) *C. utilis* inoculum was added to the rape straw growth medium after 7 days of *G. lucidum* growth. At the same time, single-strain fermentation with *G. lucidum* was also performed. Kinetic parameters were evaluated during fermentation of rape straw.

Protein enrichment and the degradation of cellulose and lignin of rape straw substrate after single-strain and mixed-strain fermentation

After 50 days of fermentation, the content of crude protein was evaluated, and cellulose and lignin were also evaluated every 10 days. The effects of single-strain and mixed-strain fermentations on the yields of crude protein

Table 4. The content (%) of cellulose of rape straw substrate during single-strain fermentation of *G. lucidum* and mixed-strain fermentation of *G. lucidum* and *C. utilis*.

Treatment	Time (day)					
	0	10	20	30	40	50
Single-strain fermentation	40.11±2.31	39.80±2.45	36.05±2.69	31.38±2.38	29.24±2.31	27.79±2.05
Mixed-strain fermentation	40.11±2.31	39.77±2.14	35.12±2.69	28.89±2.21	26.30±2.11	24.12±1.98

Table 5. The content (%) of lignin of rape straw substrate during single-strain fermentation of *G. lucidum* and mixed-strain fermentation of *G. lucidum* and *C. utilis*.

Treatment	Time (day)					
	0	10	20	30	40	50
Single-strain fermentation	19.82±1.24	17.31±1.58	16.25±1.36	14.13±1.24	13.23±0.98	12.77±0.96
Mixed-strain fermentation	19.82±1.24	18.27±1.10	17.13±1.27	12.97±1.06	11.18±1.06	9.41±0.87

of rape straw substrate were analyzed (Table 3). The results showed that the yield of crude protein of fermentation substrate by single-strain fermentation of *G. lucidum* was 8.75%, and that from mixed-strain fermentation was 16.23%, when compared to that without fermentation substrate, the yield rate was increased by 75.70% and 225.90%, respectively.

The variance of the content of cellulose and lignin of rape straw substrate during single-strain and mixed-strain fermentations is shown in Tables 4 and 5. Mixed-strain fermentation showed good cellulose and lignin decomposition and relatively low decomposition rates were determined for single-strain fermentation.

Taken together, our results indicated that rape straw can be bioconversed into an enriched substrate with increased crude protein and digestibility by solid-state fermentation. Therefore, mixed-strain fermentation in solid state could be a good way to make rape straw a useful resource.

The production of Lac, MnP and LiP in the rape straw substrate under single-strain and mixed-strain fermentations

White rot fungus *G. lucidum* was introduced as a kind of fungi that can degrade the lignin. Figure 1 shows the activities of ligninolytic enzyme system- Lac, MnP and LiP in the rape straw substrate during single-strain and mixed-strain fermentations.

G. lucidum reached maximal Lac, MnP and LiP activity on day 30 of cultivation. It is worth noting that, during the mixed-strain fermentation, higher levels of Lac, MnP and LiP activity were revealed, especially MnP. This finding is in accordance with other reports indicating that *C. utilis* also has MnP productivity (Villas-Bôas et al., 2002a). Lignin is the main factor that limits the utilization of the crops straw, so the efficiency of cellulose and hemicellulose saccharification depends heavily not only on the cellulolytic and hemicellulolytic enzyme activities but also on the efficiency of the ligninolytic enzyme system- Lac, MnP and LiP.

Conclusions

The highest content of crude protein was obtained with the following SSF treatment: 10% (v/w) *C. utilis* inoculum was added to the rape straw growth medium after 7 days of *G. lucidum* growth and production of crude protein increased by 225.90% with respect to that without fermentation substrate. Due to the fact that treatment with a mixture of two species of *G. lucidum* and *C. utilis* was obviously characterized by its higher contents of crude proteins, stronger degradation rates of lignin and cellulose and higher levels of Lac, MnP and LiP activity than the single-strain of *G. lucidum* treatment, it was concluded that there was a synergistic effect on crude protein production, lignin and cellulose degradation and the secretion of ligninolytic enzyme system for the treatment with a combination of *G. lucidum* + *C. utilis*. These results showed that the protein production and the strong degradation of lignin and cellulose after solid state fermentation (SSF) of the rape straw by the white rot fungus *G. lucidum* and yeast *C. utilis* enables it to be used as a feed supplement in diets for ruminant, and represents a useful destination for the rape straw and other agro-industrial residues.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support from the Provincial Key Laboratory of Biotic Environment and Ecological Safety in Anhui, the Key Project of Natural Fund Of Anhui Province Education Department (kj2008A135),

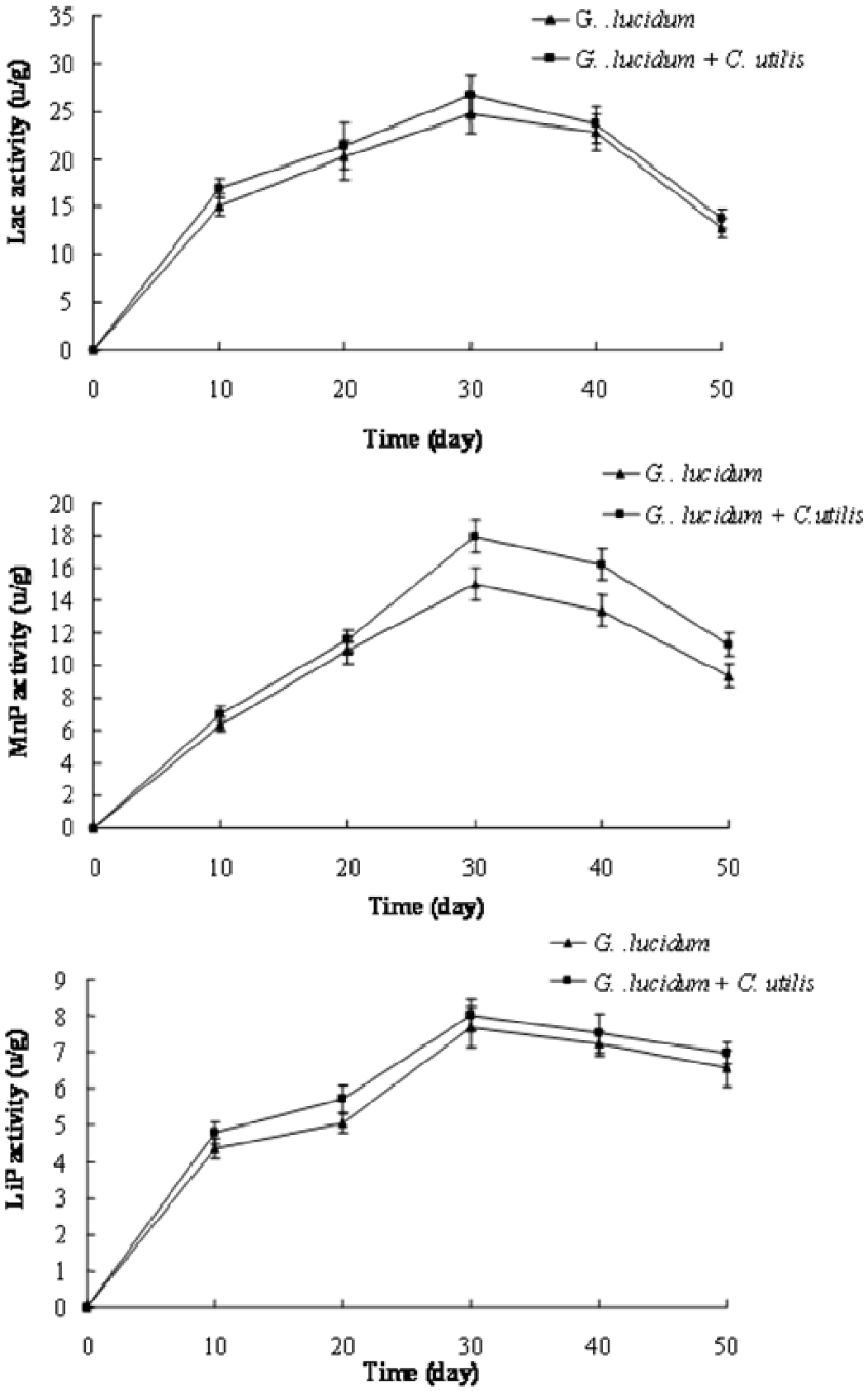

Figure 1. The activities of Lac, MnP and LiP in the rape straw substrate during single-strain and mixed-strain fermentations

the Doctoral Scientific Research Fund of Anhui Normal University and the Key Project of Science and Technology of Wuhu in 2009 (Agriculture 11-1), China.

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