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BIOCONVERSION OF WATER-HYACINTH TO NUTRITIONALLY ENRICHED ANIMAL FEED BY SOLID STATE FERMENTATION USING *Pleurotus sajor-caju*

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ABSTRACT

This study was undertaken to improve nutritional values and digestibility of water-hyacinth by solid-state fermentation with a white rot fungi, *Pleurotus sajor-caju*. At the end of 56 days fermentation of $CaCO_3$ treated water-hyacinth, significant ($p<0.05$) changes of crude protein, lipid, carbohydrate, ash, lignin, cellulose, hemicellulose, cellulose-lignin ratio and reducing sugar contents were detected. Crude protein, ash, cellulose-lignin ratio and reducing sugar contents were increased by 685, 47, 106 and 680%, respectively. In contrary, crude fiber, lipid, carbohydrate, lignin, cellulose and hemicelluloses contents were decreased by 36.8, 72, 19, 72.33, 37.5 and 4.57%, respectively. Ascorbic acid and carotenoid were increased by 42.9 and 122.8%, respectively. At 49 days of fermentation, the crude water-hyacinth extract showed very high CMCase, avicelase and amylase, moderate cellobiase and very poor pectinase and xylanase activities. *In-vitro* dry matter digestibility was also increased by 76%. The study concluded with the finding that *P. sajor-caju* has the potential for efficient degradation of water-hyacinth to convert the lignocellulosic wastes into nutritionally improved animal feed.

Keywords: Bioconversion, water-hyacinth, solid-state fermentation, *Pleurotus sajor-caju*, animal feed

INTRODUCTION

Lignocellulosic wastes (LCW), refer to plant biomass wastes, are composed of cellulose, hemicellulose, and lignin. They may be grouped into different categories such as wood residues (including sawdust and paper mill discards), grasses, waste paper, agricultural residues (including straw, stover, peelings, cobs, stalks, nutshells, non food seeds, bagasse), domestic wastes (lignocelluloses garbage and sewage), food industry residues and municipal solid wastes (**Qi *et al.*, 2005; Roig *et al.*, 2006; Rodríguez *et al.*, 2008**). Even though LCW are considered as the largest reservoir of potentially fermentable carbohydrates on earth (Mtui and Nakamura, 2005) these are mostly wasted in the form of pre-harvest and post-harvest agricultural losses and wastes of food processing industries. Due to their abundance and renewability, there has been a great deal of interest in utilizing LCW for the production of protein rich food, fuel and other value-added products (**Pandey *et al.*, 2000; Mukherjee *et al.*, 2004; Foyle *et al.*, 2007**). The barrier to the production of valuable materials from LCW is the structure of lignocelluloses which has evolved to resist degradation due to crosslinking between the polysaccharides (cellulose and hemicellulose) and the lignin via ester and ether linkages (**Yan and Shuya, 2006; Xiao *et al.*, 2007**). Cellulose, hemicelluloses and lignin form a structure called microfibril, which are then organized into macrofibrils and gives structural stability in the plant cell (**Rubin, 2008**). The main target of lignocelluloses degradation, therefore, is to amend or eliminate structural and compositional hurdles for hydrolysis and subsequent degradation processes in order to improve digestibility, rate of enzymatic hydrolysis and product yields (**Mosier *et al.*, 2005; Hendriks and Zeeman, 2009**). The degradation can be achieved by single or combined implementation of mechanical, physico-chemical or biological treatments.

Microbial conversion of lignocelloses to energy and nutritionally enriched ruminant feed is becoming popular day-by-day. Water-hyacinth, a very fast-growing ubiquitous aquatic herb which is mainly used as cheap animal feed in Bangladesh has a promising possibility to convert as nutritionally improved animal feed after proper delignification and solid-state fermentation. The agro-waste grows so abundantly in rivers and other navigable waters where it obstructs the passage of boats and ships, and it is also troublesome in irrigation ditches. Its abundant growth sometimes threatens fish and other water life in the rivers and lakes by depriving them of oxygen and causing significant changes in aquatic habitats. The bioconversion of water-hyacinth is thus has a dual advantage of handling the waste for cleaner environment and production of value added animal feeds. White rot fungi, capable of degrading lignin, cellulose and hemicelluloses, have already been reported for efficient bioconversion of many lignocellosic wastes (**Anwar *et al.*, 2015; Dashtban *et al.*, 2009; Shrivastava *et al.*, 2014**). Conversion of water-hyacinth to ruminant feed by several white rot fungi including *Pleurotus. sajor-caju* has also been reported (**Mukherjee *et al.*, 2004; Mukherjee and Nandi, 2004**). However, combination of chemical and biological treatment is expected to further improve the bioconversion. In the present study, $CaCO_3$-pretreated water-hyacinth was used for SSF by *P. sajor caju* to enhance delignification and *in-vitro* dry matter digestibility in addition to several nutritional parameters. We further checked the augmentation of antioxidantive properties and enzyme activities of crude water-hyacinth extracts during the SSF.

MATERIALS AND METHODS

Preparation of substrates

Water-hyacinth collected from different sources were first cleaned off all dirt and unwanted materials. Then they were sun dried and cut into tiny pieces between 2-3 cm. It was stored at 5^0C until used.

Pretreatment of substrates

500 g of untreated water-hyacinth was soaked with a calcium carbonate solution (2.67 g $CaCO_3$/L DH_2O). The substrates were left in soaking condition overnight. Then the lime solution was drained out by tap water. Treated substrates were then spread over aluminum foils and allowed to dry overnight at 60^0C.

Collection and storage of *P. sajur-caju*

Stock culture of *Pleurotus sajur-caju* was obtained as Potato dextrose agar (PDA) slant from Microbiology and Industrial Irradiation Division of Bangladesh Atomic Energy Commission. The culture was maintained on PDA medium at 4°C and sub-cultured every 15 days.

Solid-state fermentation

P. sajor-caju was sub cultured from stock PDA slant to PDA plate. After 14 days of incubation at 30^0C three pieces of mycelia growth (about 1 cm in diameter) were inoculated in 100 ml Erlenmeyer flask containing 50 ml PDA broth. The flask was incubated at 30^0C in shaking condition in an orbital shaker for 7 days and then the inoculums was transferred in pre-sterilized soaked substrates (into 1000 ml Erlenmeyer flask) containing 20 g substrates and 50 ml distilled water and incubated at 30^0C for 56 days.

Biochemical analyses

Water-hyacinth with different periods of fermentation were collected aseptically, oven dried at 60^0C and used for biochemical analysis. The substrate without $CaCO_3$ treatment and SSF was used as control and also dried overnight at 60^0C before biochemical analyses. Ash, fat, crude fiber and moisture contents were determined following the methods of **A.O.A.C (1980)**, while the crude protein contents (N×6.25) were determined using micro-kjeldahl method (**ISO 20483 2006**). The carbohydrate contents were determined by Dubois et al. (1956). Gravimetric determination of lignin, cellulose and hemicellulose of the substrates were estimated according to **Sun *et al.* (1996)** and **Adsul *et al.* (2005)**. The cellulose to lignin ratio was also determined. Reducing sugar contents in control and fermented substrates at their various stages of fermentation were determined by the dinitrosalicylic acid (DNS) method (**Miller, 1959**).

Determination of enzyme activity

The crude enzyme solution was obtained by soaking moldy substrate with considerable volume of 0.01 M acetate buffer (pH 5.5). The mixture was shaken for 2 h and centrifuged at 5000 rpm for 10.0 m to remove cells and residual substrate. The clarified extract representing crude enzyme was used for assaying endoglucanase (CMCase), exoglucanase (Avicilase), xylanase, (**Saddler *et al.*, 1987**) pectinase (**Shimizu and Kunoh, 2000**), cellobiase (**Lowe *et al.*, 1987**) and Amylase (**Pandey *et al.*, 2000**) activities. Enzyme assays were carried out in triplicate using three culture replicates at 25°C. The enzymatic activities are expressed as international units (IU), defined as the amount of enzyme required producing 1 µmol product/minute, and are reported as IU/g substrate used in the SSF as described by **Shrivastava *et al.* (2011)**.

Quantification of antioxidants

Amount of ascorbic acid was quantified by spectrophotometric method after extraction with 3% HPO_3 as described in the **Methods of Vitamin Assay (1966)**. Total carotenoid was extracted in 80% acetone and absorption was taken at 663, 645 and 480 nm. Finally the amount of carotenoid was calculated using the following formula as described by **Hiscox and Isrealstam (1979)**.

Total carotenoid (mg /g) $= A_{480} + (0.114 \times A_{663}) - (0.638 \times A_{645}) \times V / 1000 \times W$

***In-vitro* dry matter digestibility (IVDMD)**

Dry matter digestibility was assessed following the methods of Tilley and Terry (1963) and Minson and McLeod (1972) and expressed as loss of dry matter. Ruminal fluid was obtained from a lactating goat after 4 h feeding on a mixed ration consisting of 75% grass forage and 25% grain mixture (20% ground corn, 4% soybean meal, 1% vitamin and mineral mix).

Statistical Analysis

Data from different biochemical analyses of non-fermented and fermented samples at different periods were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Analyses were performed using statistical applications and differences and were considered significant at an alpha level of 0.05.The statistical program used was Stat-View^R 5.0 (Mind Vision Software, Abaccus, Concepts, Inc. Berkeley, CA, USA).

RESULTS AND DISCUSSION

Changes in the proximal composition during SSF

The proximal composition of water-hyacinth was changed significantly after solid-state fermentation ($p<0.05$) compared to non-fermented one (Table 1). The crude fiber content decreased 36.86% after 56 days fermentation. This indicates secretion of cellulose/hemicellulose-degrading enzymes by the fungus during fermentation (**Lateef *et al.*, 2008**). The protein content of fermented water-hyacinth was increased by 685.34% refereeing enormous increase of the fermenting fungal growth on water-hyacinth (Figure 1). The finding was in accordance with several previous reports (**Murata *et al.*, 1967; Hammond and Wood, 1985; Matsuo, 1997; Iluyemi et al., 2006; Moore *et al.*, 2007**). Besides fungal growth, secretion of certain extracellular enzymes also contributed to the increase of protein (**Kadiri, 1999**). Earlier studies of fungal growth on cassava byproducts, wheat straw, coffee husk, corn bran, and rice bran have also reported similar increase in protein content (**Leifa and Soccol *et al.*, 2001; Iyayi and Aderolu, 2004., Das and Mukherjee, 2007**). The ash content was also found to increase with fermentation time and a total of 47.35% increased at the end. Since the ash content determination is a measure of mineral levels in the substrates, it can be inferred that SSF contributed to the elevation of mineral levels in the fermented products. Similar improvement of ash content, following fermentation of lignocelluloses has been reported by **Sanni and Ogbonna (1991)**, **Bressani (1993)**, and **O'Toole (1999)**. In contrary, **Fadahunsi *et al.* (2010)** and **Akinyele *et al.* (2011)** reported decrease of ash content due to SSF of agricultural wastes. Generally, fermentation led to reduction in the crude fat content. Here, the reduction was 72.65 % after 56 days SSF. In a similar study, the

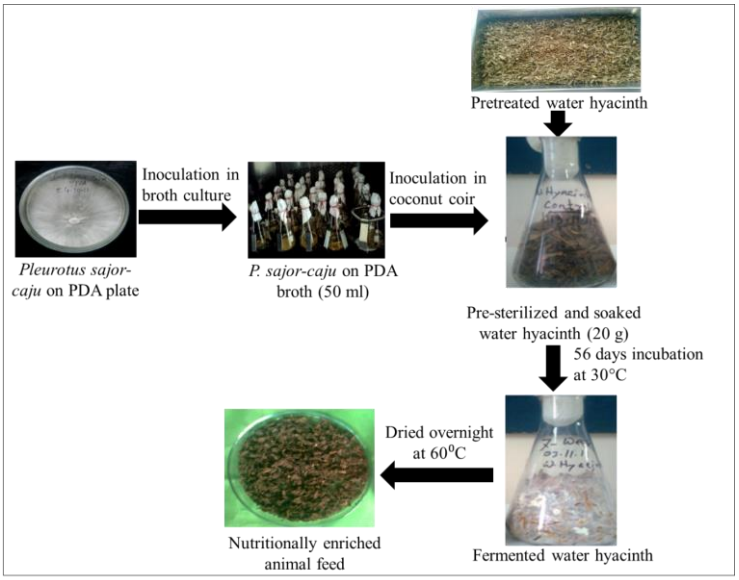

Figure 1 Biological conversion of water-hyacinth to nutritionally enriched animal feed. *P. sajor-caju* was sub cultured from PDA plate to 50 ml PDA broth and incubated at 30^0C in shaking condition for 7 days. The inoculum was then transferred in pre-sterilized soaked substrates containing 20 g substrates and 50 ml distilled water and incubated at 30^0C for 56 days. Final product was achieved after drying at 60^0C for overnight.

Fat content of okara was reduced from 15 to 9% by fermentation with *N. intermedia* (**Matsuo, 1997**). Previous studies have shown reduction in the lipid content of different substrates fermented with different microorganisms. During SSF, lipolytic strains assimilate lipid from substrates for biomass production and cellular activities leading to a general reduction of the overall lipid content (**Das and Weeks, 1979; Ejiofor and Okafor, 1987; Sanni and Ogbonna, 1991; Iluyemi *et al.*, 2006; Lateef *et al.*, 2008**). The carbohydrate content of water-hyacinth was also decreased significantly because of the SSF. Carbohydrates are used through different biochemical processes by microorganisms to produce simple sugars during bioconversion of lignocelluloses (**Howard et al., 2003; Akinyele *et al.*, 2011**).

Table 1 Proximate composition (% of dry substrate) of water-hyacinth at various period of solid-state fermentation with *P. sajor- caju*

Period of Incubation	Crude fiber	Protein	Ash	Lipid	Carbohydrates
control	4.07±0.16^{g}	2.32±0.19^{a}	9.80±0.28^{a}	1.06±0.16^{g}	76.80±0.60^{h}
7	3.89±0.04^{g}	5.68±0.25^{b}	10.16±0.23^{b}	0.905±0.02^{f}	75.17±0.36^{g}
14	3.70±0.04^{f}	8.89±0.33^{c}	10.68±0.02^{c}	0.867±0.01^{f}	71.52±0.18^{f}
21	3.57±0.06^{ef}	9.98±0.27^{d}	11.22±0.13^{d}	0.716±0.01^{e}	70.81±0.12^{f}
28	3.39±0.02^{de}	11.72±0.38^{e}	11.75±0.09^{e}	0.646±0.01^{de}	69.42±0.92^{e}
35	3.24±0.06^{d}	12.92±0.26^{f}	12.57±0.04^{f}	0.526±0.02^{cd}	68.3±0.34^{d}
42	3.04±0.08^{c}	14.42±0.27^{g}	12.67±0.02^{f}	0.444±0.02^{bc}	67.2±0.27^{c}
49	2.78±0.06^{b}	16.09±0.49^{h}	13.86±0.05^{g}	0.353±0.01^{ab}	64.45±0.13^{b}
56	2.57±0.06^{a}	18.22±0.93^{i}	14.44±0.08^{h}	0.290±0.01^{a}	62.01±0.74^{a}

Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different superscripts are significantly different at $p< 0.05$.

The reducing sugar content of water-hyacinth was increased dramatically and correlated directly with increase of biomass and decrease of carbohydrates during 56 days fermentation period (Table 2). The reducing sugar content of fresh water-hyacinth was found to increase up to 49 days of fermentation indicating enzymatic degradation of cellulose, hemicelluloses and pectin fractions of the substrate (**Sherief *et al.*, 2010**). However, the decreased free sugar content after 49 days fermentation can be explained by decreased rate of the degradation as compared to the rate of free sugar metabolism by *P. sajor caju*. This submission corroborates the findings of **Sanni and Ogbonna (1991)** where they reported a sharp decrease of enzymatic activity at 24h of fermentation during the production of 'Owoh" from cotton seed.

Table 2 Lignin, cellulose, hemicelluloses, C/L and reducing sugar contents (% of dry substrate) of water-hyacinth at different period of SSF by *P. sajor caju*.

Period of Incubation (days)	Lignin	Hemicelluloses	Celluloses	Cellulose and Lignin ratio (C/L)	Reducing sugar
control	15.25±0.85^{e}	18.15±1.15^{e}	23.75±1.52^{e}	1.56±0.22^{a}	0.30±0.02^{a}
7	12.77±0.80^{d}	17.32±0.93^{de}	20.32±0.92^{d}	1.60±0.176^{ab}	0.68±0.03^{b}
14	10.23±0.63^{c}	16.89±0.80$_{cde}$	19.58±1.76^{cd}	1.91±0.057^{bc}	0.81±0.03^{c}
21	9.84±0.70^{c}	16.78±1.03^{cde}	18.02±0.49^{bcd}	1.84±0.051^{abc}	1.03±0.02^{d}
28	9.70±0.42^{c}	15.67±0.95^{abcd}	17.67±0.95^{bc}	1.82±0.078^{abc}	1.07±0.03^{d}
35	9.19±0.68^{c}	16.03±0.50^{bcde}	17.06±0.56^{ab}	1.87±0.014^{bc}	1.51±0.01^{e}
42	7.67±0.47^{b}	14.86±0.76^{abc}	16.03±0.51^{ab}	2.08±0.078^{cd}	1.89±0.08^{f}
49	6.75±0.35^{b}	13.95±0.88^{ab}	15.02±0.49^{a}	2.22±0.078^{d}	2.88±0.11^{h}
56	4.22±0.16^{a}	13.75±1.06^{a}	14.83±0.70^{a}	3.52±0.042^{e}	2.34±0.04^{g}

Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different superscripts are significantly different at $p< 0.05$.

Degradation of lignin, cellulose and hemicelluloses

The chemical pretreatment of water-hyacinth with $CaCO_3$ prior to SSF enhanced the delignification and resulted in a decrease of lignin content from 15.25% of total dry weight to 14% (8.2% loss). While comparing the contents of lignin, hemicelluloses and cellulose during SSF, a significant degrease ($p< 0.05$) of all these contents were observed. However, cellulose and lignin ratio (C/L ratio) of fermented agro-industrial wastes was significantly increased ($p< 0.05$) compared with their unfermented samples. The percentage of lignin content was decreased by 72.33 % (Table 2) for SSF indicating the ability of *P. sajor-caju* to bulk of ligninases production such as laccases and peroxidases (**Leonowicz *et al.*, 1999; Baldrian *et al.*, 2005; Hoegger *et al.*, 2007**) while fermenting water-hyacinth. The finding was in accordance with the previous reports of **Lechner and Papinutti (2006)** and **Sherief *et al.* (2010)** where lignolytic activities of fermenting microorganisms were found during biodegradation of rice straw, saw dust, wheat straw, coffee pulp and banana leaves. The percentage of cellulose was found to reach 14.83% of the total dry weight at the end of 56 days fermentation (Table 2) after a reduction of 37.5% from the initial cellulose content that indicates the increased production of cellulases. Cellulose degradation is a usual phenomenon during SSF of lignocelluloses as reported by **Bisaria *et al.* (1997)**, **Sherief *et al.* (2010)** and **Jahromi *et al.* (2011)**. Unlike cellulose, hemicellulose degradation was found lower and at the end the reduction was 24.24% compared to non-fermented one. The decrease in the values of hemicellulose could be indicative of the degradation of the cell wall component of the substrates produced by the extracellular enzymes (xylanase, xylosidase, arabinase and pectinase) of the fungi used.

Cellulolytic enzyme activities

Edible mushrooms (*P sajor-caju* and *P. pulmunarium*) are able to convert a wide variety of lignocellulose materials due to the secretion of extracellular enzymes (**Buswell *et al.*, 1996 and Rajarathman *et al.*, 1998**). Increase of free sugar and decrease of cellulose and hemicellulos (Table 2) during SSF indicated the presence of degradation cellulolytic enzyme activities of *P. sajor-caju* while growing on water-hyacinth. Therefore, crude enzymatic activities of *P. sajor-caju* were measured at the period of 49 days SSF as maximum reducing sugar was found at this point. CMCase, avicelase and cellobiase activities were 1.23, 0.92 and 0.31 IU/g respectively (Figure 2). These activities directly correlate with the degradation of cellulose. A very low enzymatic activity of xylanase was expected as the hemicelluloses degradation was lower compared to cellulose degradation. However, the fungus also showed low pectinase activity and moderate amylase activity. Very low xylanase activity was also reported by **Kumar *et al.*, (1997)** during SSF of Sago hampus, a starchy lignocellolosic by-product prepared from sago palm.

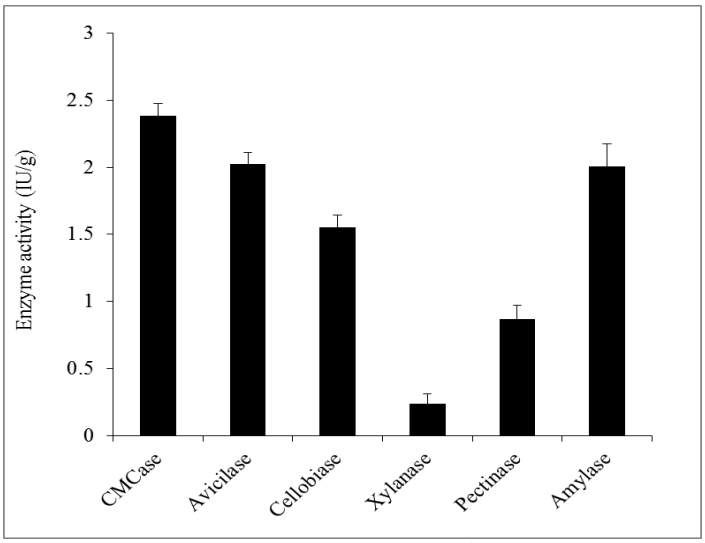

Figure 2 Cellulolytic enzymatic activities (IU/g) of *P. sajor-caju* at 49 days SSF with water-hyacinth. Values are mean±SD of three independent experiments.

Improvement of antioxidative nature and *in-vitro* digestibility

We also checked the change of dry matter, anti-oxidative properties and *in-vitro* dry matter digestibility (IVDMD) of water-hyacinth as a consequence of SSF (Table 3). Increase of dry matter by 9.3% was because of increased biomass as a mycelial growth of the fungus. Ascorbic acid was improved by 42%. Growth of *P. sajor-caju* also contributed to improving significant level of total carotenoid by 122.8%. More importantly, the IVDMD was changed in significant level. Improved IVDMD of water hyacinth after solid-state fermentation has also been reported by **Mukherjee *et al.* (2004)**, however, in our study the improvement was higher as we used a chemical pretreatment which increased the delignification. This result was supported by the findings that digestibility is usually inversely related to the lignin concentration (**Kamra and Zadrazil,**

1985). **Karunanandaa *et al.* (1995)** also reported higher digestibility of paddy straw because of faster delignification than other lignocellulosic wastes by mutant strains of *P. forida* in SSF. As ruminal microbes do not secrete any ligninolytic enzyme (**Zadrazil *et al.*, 1995)**, the chemical pretreatment aided in lignin reduction which facilitated the degradation of structural carbohydrates of water hyacinth by solid state fermentation. Thus, the SSF used in this study helped to accumulate higher amount of soluble sugars through bioconversion which will be easily digestible by ruminants.

Table 3 Amounts of total dry wt, ascorbic acid, total carotenoid and *in-vitro* digestibility in water hyacinth before and after SSF

Type of sample	Total dry wt (g)	Ascorbic acid (mg/g)	Total Carotinoid (mg/g)	*In-vitro* digestibility (% of dry substrate)
control	20.00±0.57[a]	0.0573±0.006[a]	0.105±0.01[a]	20.25 ±0.45[a]
Fermented substrates	21.86±0.83[b]	0.0819±0.018[a]	0.234±0.026[b]	35.65±0.75[b]

Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different superscripts are significantly different at $p< 0.05$.

CONCLUSION

The present study revealed that solid state fermentation of pre-treated water-hyacinth not only improved nutritive values such as protein and available polysaccharide fractions as energy source for ruminants but also made it more digestible due to higher delignification. In addition, the fermented product was also rich in some anti-oxidative agents. Therefore the bioconverted product can be used as nutritionally improved animal feed after an *in-vivo* feeding trial and toxicity tests.

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