RESEARCH ARTICLE

Enhancement of nutritive value of tea leaf waste by solid-state fermentation with *Lentinus sajor–caju*

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ABSTRACT
Nutritional value of tea leaf waste was improved significantly ($p<0.05$) by solid-state fermentation for 8 weeks with a white rot fungus, *Lentinus sajor-caju*. The proximate analysis revealed that crude protein, ash, cellulose-lignin ratio and reducing sugar contents were increased by 2001.53, 117.62, 31.38, and 619.10%, respectively. In contrary, crude fiber, lipid, carbohydrate, lignin, cellulose and hemicelluloses contents were decreased by 40.70, 71.87, 47.65, 35.63, 15.26, and 61.03%, respectively. Ascorbic acid and carotenoids were also increased by 129.17 and 398.79%, respectively. At 7 weeks of fermentation, the crude tea leaf waste extract showed very high endoglucanase, exoglucanase, cellobiase and amylase activity, moderate pectinase and poor xylanase activity. Furthermore, In vitro dry matter digestibility was increased by 50.35% at the end of fermentation. Therefore, it was concluded that *L. sajor-caju* efficiently degraded tea leaf waste and improved its nutritive value.

Key words: Solid-state fermentation, *L. sajor-caju*, tea leaf waste, protein, lignocellulose

Introduction

Lignocellulosic wastes (LCW) are composed of cellulose, hemicellulose, and lignin. These abundant and largest renewable fermentable carbohydrates have a great potential for the production of protein rich food, feed, fuel and other industrially important products (Pandey et al., 2000; Mukherjee et al., 2004; Mtui & Nakamura, 2005; Foyle et al., 2007). However, the main impediment to produce valuable materials from LCW through biodegradation 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 & Shuya, 2006; Xiao et al., 2007; Rubin, 2008). The degradation can be achieved by single or combined implementation of mechanical, physico-chemical or biological treatments (Mosier et al., 2005; Hendriks & Zeeman, 2009).

Microbial conversion of lignocellulooses to energy and nutritionally enriched ruminant feed is becoming popular day-by-day. White rot fungi, capable of degrading lignin, cellulose and hemicelluloses, have already been reported for efficient bioconversion of many lignocelluloosic wastes. Although tea is the most popular beverage in Bangladesh, like other countries, leaf waste of this popular drink has almost no use and just throughout with other kitchen waste. Until now, there are only a few reports evaluating the nutritive value of tea leaf waste and even fewer reports

attempting augmentation the value by bioconversion. However, combination of chemical and biological treatment is expected to improve the nutritional quality of this useless-lignocellulosic waste. In the present study, CaCO3-pretreated tea leaf waste was used for Solid-state fermentation (SSF) by *L. sajor-caju* to enhance delignification and in vitro dry matter digestibility in addition to several nutritional parameters. We further checked the rise of antioxidantive properties and enzyme activities of crude tea leaf waste extracts because of SSF.

Materials and Methods

Pretreatment of substrates

Cellulosic materials collected from different sources were first cleaned off all dirt and unwanted materials. Then they were sun dried, cut into tiny pieces between 2─3 cm and stored at 5^0C until used. 500 g of untreated tea leaf waste was soaked with a calcium carbonate solution (0.27% CaCO3 in distilled H2O). 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 L. 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 slant medium at 4^oC and sub-cultured every 15 days.

Solid-state fermentation

L. sajor-caju was sub cultured from stock PDA slant to PDA plate. After 7 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 PDB. The flask was incubated at 30^0C at 150 rpm for 7 days and then the inoculum was transferred in pre-sterilized 30 g substrates (into 1000 ml Erlenmeyerflask) and incubated at 30^0C for 8 weeks.

Analytical methods

Biochemical analyses: Tea leaf waste 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 content was determined by the method of 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). In brief, a considerable weight of air-dried moldy substrate was fragmented into small pieces and suspended in 200 ml 1.0% (w/v) aqueous solution of NaOH. The mixture was autoclaved at 121°C for 1.0 h in 500 ml Erlenmeyerflask. The residues were collected and extremely washed by tap water until neutral pH; then dried at 80°C for 48 h and weighted. The loss of weight corresponded to lignin content. For determination of cellulose and hemicellulose, a considerable weight of air dried delignified substrate (after lignin determination) was milled and screened to about 0.1 cm and suspended in 100 mL sulfuric acid 1.0% (v/v). The mixture was then autoclaved at 121°C for 1.0 h in 250 ml Erlenmeyer flask. The residues were collected and washed extensively with tap water until neutral pH, dried at 80°C for 48 h and then weighted. The difference between started weight and residual weight corresponded to hemicellulose fractions; while, residual weight after acid hydrolysis corresponded to cellulose content.

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).

The crude enzyme solution was obtained by soaking 1 g moldy substrate with 10 ml 0.01 M acetate buffer (pH 5.5). The mixture was shacked for 2.0 h and centrifuged at 5000 rpm for 10.0 min to remove cells and residual substrate. The clarified extract representing crude enzyme was used for assaying endoglucanase (CMCase, EC 3.2.1.4), exoglucanase (avicilase, EC 3.2.1.91), xylanase (EC 3.2.1.8), (Saddler et al., 1987), pectinase (EC 3.2.1.15) (Shimizu & Kunoh, 2000), cellobiase (EC 3.2.1.21) (Lowe et al., 1987) and amylase (EC 3.2.1.1) (Pandey et al., 2000) activities. Enzyme assays were carried out in triplicate. The enzymatic activities were expressed as international units (IU), defined as the amount of enzyme required for production of 1 µmol product/min, and were 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 Vitamins Assay (1966). Total carotenoids were extracted in 80% acetone and absorption was taken at 663, 645 and 480 nm. Finally the amount of carotenoids was calculated using the following formula as described by Hiscox & Isrealstam (1979).

Total carotenoid mg /g of sample = A480 + (0.114 x A663) – (0.638 x A645) x V/1000 x W

A = Absorption at given wavelength, V = Total volume of sample in extraction medium (ml) and W = Weight of the sample (g)

In-vitro dry matter digestibility (IVDMD): Dry matter digestibility was assessed following the methods of Tilley & Terry (1963) and Minson & 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 viewR 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 tea leaf waste was changed significantly after solid-state fermentation ($p<0.05$) compared to non-fermented one (Table 1). The crude fiber content decreased 40.70% after 8 weeks fermentation indicating secretion of cellulose/hemicellulose-degrading enzymes by the fungus during fermentation (Lateef et al. 2008). The protein content of fermented tea leaf waste was increased by 2001.53% refereeing enormous increase of the fermenting fungal growth on the lignocellulosic waste. The finding supports similar improvement for lignocellulosic wastes reported elsewhere (Murata & Miyamoto, 1967; Bender, 1970; Odunfa, 1983; Hammond & Wood, 1985; Matsuo, 1997; Leifa et al., 2001; Iyayi & Aderolu, 2004; Iluyemi et al., 2006; Moore et al., 2007; Das & Mukherjee, 2007; Akinyele et al., 2011). Besides fungal growth, secretion of certain extracellular enzymes also contributed to the increase of protein (Kadiri, 1999). The ash content was also increased 117.62% at the end of fermentation. 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. The improvement of ash content due to enhanced decomposition of total organic carbons to carbon dioxide by SSF was in accordance with the report of Sanni & Ogbonna (1991), Bressani (1993), O'Toole (1999). In contrary, Fadahunsi & Sanni (2010) and Akinyele et al., (2011) reported a decrease of ash content due to SSF of agricultural wastes. Generally, fermentation led to the reduction in the crude fat content. Here, the reduction was 71.88% after 8 weeks SSF. In a similar study, the fat content of okara was reduced from 15 to 9% by fermentation with *Neurospora intermedia* (Matsuo, 1997). Earlier studies showed adecrease in the fat content of different lignocellulosic substrates fermented with different microorganisms. During SSF, lipolytic strains assimilate lipids from substrates for biomass production and cellular activities leading to a general reduction of the overall lipid content (Lateef et al., 2008; Iluyemi et al., 2006; Sanni & Ogbonna, 1991; Das & Weeks, 1979; Ejiofor et al., 1987). The carbohydrate content of tea leaf waste was also decreased (47.66%) 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, 2011).

The reducing sugar content of tea leaf waste was increased significantly and correlated directly with the increase of biomass and decrease of carbohydrates during 8 weeks fermentation period (Table 2). The reducing sugar content of fresh tea leaf waste was found to increase up to 7 weeks of fermentation indicating enzymatic degradation of cellulose, hemicellulose and pectin fractions of the substrate (Sherief et al., 2010). However, the decreased free sugar content after 7 weeks fermentation can be explained by decreased rate of the degradation as compared to the rate of free sugar metabolism by *L. sajor caju*. These findings were supported by the findings of Sanni & Ogbonna (1991) where they reported a sharp decrease of enzymatic activity at 24 h of fermentation during the production of "Owoh" from cotton seed.

Degradation of lignin, cellulose and hemicelluloses

The chemical pretreatment of tea leaf waste with $CaCO_3$ prior to SSF enhanced the delignification and resulted in a decrease of lignin content from 27.90% of total dry weight to 24.27% (13.01% loss). While comparing the content of lignin, hemicellulose and cellulose during SSF, a significant decrease ($p< 0.05$) of all these compounds was observed. However, cellulose to lignin ratio (C/L ratio) of fermented agro-industrial wastes was significantly increased ($p<0.05$) compared with their non-fermented samples. The percentage of lignin content was decreased by 35.62% (Table 2) for SSF indicating the ability of *L. 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 tea leaf waste. The finding was in accordance with the previous reports of Vega et al. (2005), Lechner & 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

Table 1. *Proximal composition (% of dry substrate) of tea leaf waste at various periods of solid state fermentation with L. sajor- caju. (Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different alphabets are significantly different at p< 0.05).*

Cultivation period	Crude fibers	Protein	Ash	Lipids	Carbohydrates
Week-0	5.11±0.04h	1.96±0.12a	5.9±0.14a	0.985±0.09g	80.01±0.23i
Week-1	4.71±0.03g	6.52±1.19b	7.13±0.18b	0.820±0.01f	78.19±0.80h
Week-2	4.32±0.05f	13.49±1.02c	8.25±0.35c	0.594±0.01e	71.01±0.74g
Week-3	4.20±0.04ef	19.11±0.38d	8.43±0.04c	0.470±0.01d	65.46±0.14f
Week-4	4.05±0.07de	22.08±1.48e	8.65±0.06c	0.427±0.02cd	62.54±0.24e
Week-5	3.84±0.08cd	26.84±0.55f	9.30±0.02d	0.396±0.01cd	57.27±0.23d
Week-6	3.66±0.06c	30.17±0.40g	10.33±0.47e	0.367±0.01bc	52.95±0.67c
Week-7	3.36±0.08b	35.54±0.68h	11.60±0.28f	0.361±0.01ab	46.55±1.32b
Week-8	3.03±0.03a	41.19±3.08i	12.84±0.23g	0.277±0.01a	41.88±0.43a

Table 2. *Lignin, cellulose, hemicelluloses, C/L and reducing sugar contents (% of dry substrate) of tea leaf waste at different periods of SSF by L. sajor caju. (Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different alphabets are significantly different at p< 0.05).*

Cultivation time	Lignin	Hemicelluloses	Celluloses	Cellulose to Lignin ratio (C/L)	Reducing sugars
Week-0	27.9±0.42e	10.78±0.78e	38.0±0.56d	1.37±0.007a	0.89±0.03a
Week-1	22.08±0.68d	9.75±0.78e	34.80±0.56c	1.58±0.021b	1.16±0.02ab
Week-2	20.95±0.21cd	7.80±0.85d	34.05±0.35bc	1.63±0.007bc	1.28±0.02ab
Week-3	20.87±0.37cd	7.65±0.92d	34.10±0.42bc	1.64±0.007c	1.42±0.04ab
Week-4	20.75±0.78cd	7.0±0.28cd	34.20±0.84bc	1.65±0.021cd	1.60±0.0.02b
Week-5	19.67±0.93cd	6.85±0.35bcd	33.15±0.49abc	1.69±0.057de	1.74±0.06b
Week-6	18.90±0.42ab	5.75±0.21abc	33.15±0.15abc	1.76±0.007fg	4.99±0.68c
Week-7	18.81±0.57ab	5.3±0.42ab	32.65±0.91ab	1.72±0.014ef	7.21±0.47e
Week-8	17.96±0.50a	4.2±0.57a	32.20±0.85a	1.80±0.007g	6.40±0.15d

Table 3. *Amounts of total dry weight, ascorbic acid and total carotinoids of fresh and fermented tea leaf waste before and after SSF. (Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different alphabets are significantly different at p< 0.05).*

Type of sample	Total dry weight (g)	Ascorbic acid (mg/g)	Total Carotinoids (mg/g)	In-vitro digestibility (% of dry substrate)
Control	30.00±0.97a	0.077±0.02a	0.166±0.014a	34.77±0.47 a
Fermented substrates	32.43±0.86b	0.176±0.035b	0.828±0.046b	52.28±0.36b

found to reach 32.20% of the total dry weight at the end of fermentation (Table 2) after a reduction of 15.26% from the initial cellulose content referring increased production of cellulases. Cellulose degradation is a usual phenomenon during SSF of lignocelluloses as reported by Bisaria et al. (1997), Vega et al. (2005), Sherief et al. (2010) and Jahromi et al. (2011). Hemicellulose degradation was found higher than that of cellulose and at the end the reduction was 61.04% compared to non-fermented one. The higher hemicelluloses degradation could be the indicative of the higher 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

Because of secretion of extracellular enzymes, edible mushrooms i.e., *L. sajor-caju* and *P. pulmunarium* are able to convert a wide variety of lignocellulose materials (Rajarathnam, 1981; Buswell et al., 1993). The increase of free sugar (Table 2) and decrease of cellulose and hemicellulos (Table 2) during SSF indicated the presence of degradation cellulolytic enzyme activities of *L. sajor-caju* while growing on tea leaf waste. Therefore, crude enzymatic activities of *P. sajor-caju* were measured at the period of 7 weeks SSF since the highest concentration of reducing sugars was found at this point. CMCase, avicelase and cellobiase activities were 2.38, 2.02 and 1.55 IU/g respectively. These activities directly correlate with the degradation of cellulose. The fungus also showed high α-amylase activity and low xylanase and moderate pectinase activity. Very low xylanase activity was also reported by Kumaran et al. (1997) during SSF of Sago hampus, a starchy lignocellulosic by-product prepared from sago palm.

Improvement of antioxidative nature and in-vitro digestibility

Total dry matter, anti-oxidative properties and in-vitro dry matter digestibility of tea leaf waste were also changed after

SSF (Table 3). Increase of dry matter by 8.1% was because of increased biomass of the fungal mycelial growth. Ascorbic acid production was improved by 129.16%. The growth of *L. sajor-caju* also contributed to improving significantly the levels of total carotenoid by 398.79%. Possession of high antioxidant activity of some common edible mushrooms has already been reported earlier (Cheung et al. 2003; Cheung & Cheung 2005). Most importantly, a very high increase of IVDMD (50.35%) was also observed. Delignification of tea leaf waste before fermentation through chemical pretreatment led this improved IVDMD. This result was supported by the findings that digestibility is usually inversely related to the lignin concentration (Kamra & Zadrazil 1985). Karunanandaa et al. (1995) and Garcha 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, 1995) the reduced lignin of the fermented material facilitated more efficient degradation of structural carbohydrates of hyacinth, thus, increased the digestibility. Therefore, the SSF used in this study helped to accumulate a higher amount of soluble sugars through bioconversion which will be easily digestible by ruminants.

CONCLUSION

Solid state fermentation of pre-treated tea leaf waste not only improved nutritive values such as protein and available polysaccharide fractions as an energy source for ruminants but also made it more digestible due to higher delignification. Furthermore, the fermented product was also rich in some anti-oxidative agents such as ascorbic acid and total carotenoids. However, the bioconverted product should be checked by in-vivo feeding trail and toxicity tests before using as nutritionally improved animal feed.

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