

AMINO ACIDS PROFILE OF THE LIGNOCELLULOSIC FEED TREATED WITH CELLULASE-FREE LIGNOLYTIC MUTANTS OF *PLEUROTUS OSTREATUS*

Vijaya Lakshmi Chalamcherla,^a M. A. Singaracharya,^{a*} and Vijaya Lakshmi M.^b

Defining and quantifying amino acid requirements will become an important consideration in the next generation of feeding schemes for dairy cattle beyond the current emphasis on identification of limiting amino acids. In this context different amino acid profiles of untreated, urea treated, fungal treated, and urea plus fungal treated lignocellulosic feed by both *P. ostreatus* wild and its two cellulase-minus/ less lignolytic mutants were analyzed. Cellulase-free mutant strains were obtained after 20 minutes of exposure to UV light and 0.4 seconds to X-rays. A UV mutant of *P. ostreatus* (POM₁) exhibited better performance than the X-ray mutant (POM₂) in terms of production of less cellulolytic and more lignolytic enzymes. Urea treatment of straw enhanced the total amino acid content by less than a factor of two, while the fungal treatment improved it by 13-14 times. Fungal treatment of urea-treated straw improved the total amino acid content by a factor of 15, indicating the importance of urea in the straw. Further, the fungal treatment of urea-treated straw enhanced the quantity of amino acids such as glutamine, glycine, aspergine, etc. by 15-20 times. The quantity of limiting amino acids such as methionine, lysine, and histidine was also enhanced by 8 to 10 times through the fungal treatment. Maximum amounts of all the amino acids were found in urea plus fungal (POM₁) treated paddy straw than in only fungal treated and only urea treated paddy straws.

Keywords: Essential amino acids; Nutritive value; Biological treatment; *P. ostreatus*; Mutant strains; Lignolytic activity

Contact information: a: Department of Marine Biology, Vikrama Simhapuri University, Nellore, Andhra Pradesh INDIA. a*: Department of Microbiology, Kakatiya University, Warangal, Andhra Pradesh INDIA. b: Department of Microbiology, A.N.U., Guntur, Andhra Pradesh. INDIA.. * Corresponding author: E-Mail: vijayalch@gmail.com

INTRODUCTION

Ruminant production in mixed farming has lagged behind in productivity because of factors such as low genetic potential, poor health management, and imbalanced feeding (Singh et al. 1998). Nutrition remains the most critical constraint to increase animal productivity in the developing countries due to the practice of feeding crop residues and their bulkiness and poor nutritive value. The viable option in this context is the use of protein supplements of biological origin (Devendra 1997). Many researchers (Hinders 1998; Hall 2002; Sharma et al. 2006) have evaluated the nutritive value and biodigestibility of many novel feed formulations supplemented with edible mushrooms, oil cake, and leguminous tree leaves. However, current feeding schemes sub-divide essential amino acid (EAA) requirement into net requirements for the maintenance

functions, i.e. the first priority and for the growth, lactation and/or reproduction (NRC 2001). In that regard, there is limited, yet intriguing, information on what comprises the amino acid requirements for producing ruminants.

In recent years the significance of EAAs has been realized, not only in terms of their nutritional availability for anabolic use but also the fact that they are involved in a number of metabolic pathways that serve important functions in protein and energy metabolism, gluconeogenesis, lipid-fatty acid metabolism, and in mammary synthesis of milk protein and lactose (Gupta et al. 1991; El-kadi et al. 2006). Therefore, defining and quantifying these requirements will, in future, become important considerations in the next generation feeding schemes for dairy cattle beyond current emphasis on identification of limiting amino acid. In this context, a detailed study pertaining to the change in amino acid content was undertaken on the most abundantly available and widely used crop residue in India i.e., paddy straw treated with a selective delignifying white rot fungi *Pleurotus ostreatus* wild type (POW) and its two developed mutant forms– POM₁ (*P. ostreatus* UV mutant) and POM₂ (*P. ostreatus* X-ray mutant) in the presence or absence of urea.

EXPERIMENTAL PROCEDURES

Development of Cellulase-free Lignolytic Mutants

Mutant strains of *Pleurotus ostreatus* were obtained with the objective of enhancing their lignolytic activity. For the development of mutants, actively growing *P. ostreatus* was inoculated in to the central part of the sterile malt agar plates and incubated for 3 days at 37°C. After sufficient growth, the plates were exposed to UV. rays and X-rays. In the UV irradiation, the plates were exposed for 10, 20, 30, 40, 50, and 60 minutes at an intensity of 83 μ Wcm⁻². During X-ray irradiation, the plates were exposed for 0.4, 0.8, 1.2, 1.6, 2.0, and 2.4 seconds at 40 mA and 70 KV. Unexposed plates served as control (Vijaya et al. 2006). After the exposure all the plates including control were further incubated at 37°C for 4 days, and approximately 2mm² of the fungal mycelium from the spreading edge of each plate was transferred in to separate sterile malt broth (50mL) flasks and then incubated at 37°C for 7 days. After incubation the culture filtrates prepared were used for the estimation of cellulolytic and lignolytic enzymes. Cellulolytic enzyme C_x – exoglucanase activity was measured by the viscometric method suggested by (Reese et al. 1950). Another cellulolytic enzyme, C₁ – endoglucanase, was estimated by the DNS method suggested by Miller (1959). Among lignolytic enzymes, lignin peroxidase was measured by the method suggested by Crawford and Crawford (1976) and laccase by Kirk and Farrell (1987). All the experiments were conducted in triplicate.

Estimation of Amino Acid Contents:

For the estimation of amino acid content, paddy straw was chopped into pieces that were 2 cms long, soaked for overnight in water, and taken in 50 grams quantity into each conical flask and sterilized. Different sets of conical flasks (500 ml capacity) containing paddy straw alone, paddy straw + 2% urea, paddy straw + fungal spore suspension, and paddy straw + urea (2%) + fungal spore suspension were prepared in

triplicate. For the preparation of spore suspension wild as well as mutant mycelia was grown on sterile solid substratum (paddy straw) and the resultant fruit bodies were used for spore collection. Spore suspension was prepared in 10 ml of sterile distilled water as 1×10^6 CFU/ml and added to each conical flask and incubated at room temperature (average room temperature 30°C during the day and 25°C during the night) for 20 days with periodical shaking. The sterilized paddy straw was used to avoid the contamination by other fungi during incubation. After the incubation with *P. ostreatus*, samples were harvested by grinding the contents in distilled water (1:4) and filtered through Whatman No.1 filter paper. The filtrate was centrifuged at $10,000 \times g$ for 20 minutes, and the supernatant was dialyzed and used for the estimation of amino acids (Vijaya and Singaracharya 2005). Individual amino acid analysis was carried out by using the Amino Acid Analyzer (Model- AAA 400 apparatus Ingos Praha (Van Vuuren et al. 1992).

The data obtained were analysed by ANOVA and tests of significance were carried out using Duncan's multiple range tests. Standard deviation, % deviation over control, and F values were calculated and interpreted accordingly. Percent deviation over control indicates the percent increase / decrease in the amino acid content as a result of the treatment.

RESULTS AND DISCUSSION

Development of Cellulase-free Lignolytic Mutants of *P. ostreatus*

The primary objective of developing the mutant strain is to enhance the lignolytic activity while suppressing the cellulolytic activity of *P. ostreatus*. This high lignolytic and low cellulolytic mutants are desirable in bio-delignification practices, where selective delignification is preferred to prevent the dry matter loss during treatment. Keeping this objective in mind, when the data were analyzed, it was apparent that exposure of *P. ostreatus* to UV light for 20 minutes enhanced the lignolytic activity by 71% (Lac) to 81% (Lip), while suppressing the cellulolytic activity by 50% in the case of Cx (from 40 REA to 20 REA) and 14% in the case of C₁ (from 42 mg/ml to 36 mg/ml) (Table. 1). Similarly, the exposure of the culture to X-rays for a period of 0.8 seconds enhanced the activity of lignolytic enzyme Lip by 11% (from 36 U to 40 U) and Lac by 28% (from 32 U to 39 U) while suppressing the cellulolytic activity by 50% in the case of Cx (from 66 REA to 33 REA) and 23% in the case of C₁ (from 34 mg/ml to 26 mg/ml). The exposure of cultures to U.V. light for more than 20 minutes and to X-rays for more than 0.8 seconds did not enhance the lignolytic enzyme activity. Therefore, the mutant strains obtained after 20 minutes of exposure to UV light and 0.8 seconds exposure to X-rays are considered as desirable cultures or cellulase-free mutant strains of *P. ostreatus*. Based on the results (Table. 1) cultures of *P. ostreatus* exposed to UV rays for 20 minutes and to X-rays for 0.8 seconds were designated as cellulase-free mutants and denoted as POM₁ and POM₂ respectively. These strains were deposited in K.U. Microbial Culture Collection Centre, Warangal, Andhra Pradesh, India.

Table 1. Effect of U.V. Rays and X-Rays on the activity of Lignocellulolytic Enzymes of *P. ostreatus*

S.No	Effect of U V rays					Effect of X - rays				
	Time of Exposure (minutes)	C _x	C ₁	Lip	Lac	Time of Exposure (seconds)	C _x	C ₁	Lip	Lac
1	0	40 ±0.02	42 ±0.01	32 ±0.04	35 ±0.08	0	66 ±0.03	34 ±0.08	36 ±0.03	32 ±0.06
2	10	20 ±0.03	36 ±0.08	58 ±0.10	60 ±0.05	0.4	66 ±0.05	26 ±0.12	35 ±0.01	34 ±0.09
3	20	28 ±0.06	44 ±0.06	46 ±0.06	55 ±0.09	0.8	33 ±0.06	26 ±0.06	40 ±0.11	39 ±0.03
4	30	33 ±0.03	40 ±0.11	40 ±0.08	50 ±0.12	1.2	40 ±0.08	32 ±0.06	34 ±0.06	35 ±0.02
5	40	28 ±0.08	46 ±0.07	36 ±0.06	48 ±0.11	1.6	33 ±0.05	32 ±0.05	34 ±0.04	32 ±0.03
6	50	25 ±0.04	44 ±0.05	35 ±0.09	40 ±0.05	2.0	45 ±0.07	34 ±0.03	32 ±0.08	32 ±0.09
7	60	23 ±0.08	46 ±0.09	30 ±0.04	30 ±0.04	2.4	40 ±0.04	38 ±0.01	20 ±0.09	30 ±0.02

C_x – REA (Relative Enzyme Activity); C₁ – mg/ml of glucose liberated in 6 hours;
 LiP – Lignin Peroxidase – 0.1 increase in OD equal to one unit of enzyme;
 Lac - Laccase – 0.1 increase in OD equal to one unit of enzyme.

Amino Acids Profile of Treated Straw

After the incubation of samples for 20 days, different amino acid profiles of untreated, urea-treated, fungal-treated and urea-plus-fungal-treated paddy straw by both *P. ostreatus* wild and mutant varieties were analyzed and results are presented in Table 2.

The total amino acid content of the fresh or untreated paddy straw was 37.8 μ mol / g, and it was 67.35 μ mol / g in the case of urea treated straw, recording an increase of 78% of total amino acid content due to chemical treatment of paddy straw with 2% urea for 20 days. On the other hand, the total amino acid content of fungal treated straw was improved from 37.8 μ mol / g in the untreated straw to 555.14 μ mol / g in the case of wild type, 581.24 μ mol / g in the case of UV mutant (POM₁), and 565.4 μ mol / g in the case of X-ray mutant (POM₂), indicating an increase in the total amino acid content by 13 – 14 folds due to biological treatment of paddy straw using *P. ostreatus* for 20 days. In the case of urea-plus-fungal-treated straw, the improvement in total amino acid content was as high as 615.37 μ mol / g in the case of wild type and it reached its maximum of 646.68 μ mol / g with POM₁ followed by POM₂ (632.59 μ mol / g), indicating a rise of total amino acid content by a factor of 15 times compared to untreated straw. The total amino acid content of fungal treated straw was 555.14 μ mol / g, and the urea plus fungal treated straw was 615.37 μ mol / g, indicating a rise of 10% in the total amino acid content of the straw treated with wild strain of *P. ostreatus* for 20 days.

Table. 2. Amino Acids Content (μ mol/g) of Untreated, Urea-Treated and Fungal Treated Paddy Straw after 20 Days of Incubation

Amino acid	Paddy straw							
	Untreated	Urea treated (2%)	Fungal treated			Urea + Fungal treated		
			S+PO W	S+PO M ₁	S+PO M ₂	S+U+PO W	S+U+PO M ₁	S+U+PO M ₂
Lys	0.82 ± 0.05	1.06 ± 0.02	8.24 ± 0.37	9.24 ± 0.37	8.75 ± 0.04	9.05 ± 0.16	11.00 ± 0.06	10.70 ± 0.05
His	0.26 ± 0.05	0.17 ± 0.02	2.09 ± 0.07	3.00 ± 0.05	2.75 ± 0.15	3.56 ± 0.05	4.05 ± 0.41	3.75 ± 0.17
Arg	0.29 ± 0.07	0.50 ± 0.08	3.12 ± 0.17	4.10 ± 0.01	3.80 ± 0.02	3.98 ± 0.13	4.98 ± 0.15	4.25 ± 0.50
Asp	7.22 ± 0.30	3.30 ± 0.56	90.97 ± 0.26	95.90 ± 0.03	93.10 ± 0.07	94.90 ± 0.11	98.50 ± 0.10	96.50 ± 0.24
Thr	2.34 ± 0.43	9.50 ± 0.02	47.42 ± 0.13	49.40 ± 0.13	48.20 ± 0.12	53.51 ± 0.30	59.00 ± 0.22	56.75 ± 0.05
Ser	3.39 ± 0.14	6.00 ± 1.01	41.30 ± 0.02	44.30 ± 0.05	42.00 ± 0.10	47.00 ± 0.02	50.55 ± 0.08	48.90 ± 0.03
Glu	7.39 ± 0.05	19.50 ± 0.01	171.00 ± 0.03	174.00 ± 0.09	173.00 ± 0.01	190.50 ± 0.07	195.00 ± 0.06	193.00 ± 0.03
Pro	2.80 ± 0.01	2.20 ± 0.11	16.95 ± 0.23	18.00 ± 0.32	17.90 ± 0.06	18.90 ± 0.04	20.95 ± 0.16	19.75 ± 0.45
Gly	7.49 ± 0.80	15.75 ± 0.16	86.51 ± 0.05	89.61 ± 0.32	88.15 ± 0.19	100.05 ± 0.28	102.85 ± 0.31	101.95 ± 0.17
Ala	2.68 ± 0.20	4.92 ± 0.16	34.75 ± 0.02	36.80 ± 0.06	35.70 ± 0.16	38.75 ± 0.24	40.05 ± 0.26	39.60 ± 0.07
Val	0.84 ± 0.05	0.90 ± 0.27	11.22 ± 0.04	12.44 ± 0.07	12.00 ± 0.19	12.02 ± 0.14	13.00 ± 0.11	12.75 ± 0.01
Met	0.68 ± 0.02	1.50 ± 0.05	23.91 ± 0.32	25.00 ± 0.27	24.55 ± 0.21	24.90 ± 0.04	26.00 ± 0.01	25.09 ± 0.02
Leu	1.07 ± 0.76	1.55 ± 0.03	14.94 ± 0.18	15.50 ± 0.13	12.30 ± 0.09	15.05 ± 0.01	16.00 ± 0.16	15.55 ± 0.05
Tyr	0.53 ± 0.17	0.50 ± 0.04	2.72 ± 0.13	3.95 ± 0.02	3.20 ± 0.03	3.20 ± 0.21	4.75 ± 0.11	4.05 ± 0.08
Total	37.8 PDC*	67.35 (+ 78)	555.14 (+1369)	581.24 (+1438)	565.4 (+1396)	615.37 (+1528)	646.68 (+1611)	632.59 (+1574)

POM₂ : *P. ostreatus* X-ray mutant

S : Straw

U : Urea (2%)

POW : *P. ostreatus* wild typePOM₁ : *P. ostreatus* UV. mutant

All values are Statistically Significant at P<0.001

PDC* : Percent deviation over control

All values are Mean ± SD of three individual observations

Similarly the rise in the total amino acid contents of the straw treated with POM1 and POM2 and urea was about 11% higher than the fungal treated straw without urea. The improvement of 11% in the total amino acid content of the straw contributed by the mutant strains and 10 % improvement contributed by the wild strains indicates that the

addition of urea to the paddy straw at a 2% level before fungal treatment would improve the total amino acid content of the straw by at least 10% irrespective of the type of strain used. The presence of urea in the straw would supply the nitrogen content required for the growth of the organism. Thus, it could be noted from the analysis that the combined treatment using chemical as well as biological methods would improve the nutritive value of paddy straw in terms of total amino acid content by 15 times.

These results were analyzed statistically (Two factor ANOVA), and it was observed that the treatment of paddy straw with urea was insignificant ($P > 0.05$) (Table 3). This clearly demonstrates that the treatment of paddy straw with urea alone cannot improve the amino acid content of the straw. Further, the statistical analysis showed that the variations between amino acid contents of the straw treated with wild, UV, and X-ray mutants of *P. ostreatus* were significant ($P < 0.001$), indicating that the fungal treatment of straw had significant impact on the amino acid content of the straw. The ANOVA results for amino acid contents of urea treated paddy straw incubated with wild, UV and X-ray mutants of *P. ostreatus* recorded much more significant variations with regard to total amino acid content ($P < 0.001$). The statistical analysis emphasizes that the biological treatment of paddy straw with mutant strains improved the amino acid content significantly compared to wild strains of *P. ostreatus*.

The improvement in the total amino acid content of the paddy straw is the result of an increase in the quantity of different amino acids. However, the increase in the quantity is not uniform for all the amino acids. A maximum amount of glutamine ($195 \mu\text{mol/g}$) was noticed in urea + POM₁ treated paddy straw. This represents a 10-fold increase in its content over control (urea treated straw) and a 22-fold increase compared to untreated straw. The concentration of other essential amino acids like tyrosine, histidine, lysine, threonine, methionine, valine was also enhanced by a factor of 7 to 13 over the controls. Maximum amounts of all these amino acids were found in urea + fungal (POM₁) treated paddy straw, in comparison to only fungal-treated and to only urea-treated paddy straws. Similar to the present observation, the amino acid content of wheat and paddy straws were found to increase by a factor of 2 after the fungal treatment as compared to the urea treatment (Singh et al. 1990; Taniguchi et al. 2005). Jalc et al. (1996) also reported an enhanced quantity of the amino acids profile in the mutant form of *Pleurotus ostreatus* over its wild form over 30 days of incubation at 28°C. On the other hand, Gupta et al. (1991) estimated the changes during the bioconversions by two fungi and noted that mycelial colonization while increasing the protein content of the substrates did not affect the balance of amino acids, which remained basically unmodified except for a prevalent increment in the relative contents of tyrosine, arginine, and lysine to a lesser degree. Singh et al. (1990) analysed the amino acid profiles of the untreated, urea-treated, and *Coprinus fumetarius* treated straws of wheat and rice and observed that there was a substantial increase in the amino acids on fungal treatment of both the straws. The present study indicates that the total amino acid content of paddy straw could be improved by as much as 15 times by the addition of urea and subsequent treatment with *Pleurotus ostreatus* for 20 days.

Ford and Kirwan (1948) used soft X-rays for the first time for the production of mutants in *Chaetomium globosum* and compared the so obtained mutant with those obtained by U.V rays. They reported that the performance of X-ray mutant was better

than the UV mutant. Similar to our present aim of enhancing the ability of white-rot fungi for increased production of lignolytic enzymes and suppressing the cellulase secretions for higher digestibility of lignocellulosic feeds, many researchers (Myers and Eberhart 1996; Toyama and Ogawa 1974; Johnsrud and Erikson 1985; Babasaki and Ohmasa 1991; Garzillo et al. 1992; Messner et al. 2003) have concentrated on this basic theme. The mutants having enhanced lignolytic ability induced by gamma ray irradiation were isolated from *P. ostreatus* PO₁ and characterized (Lee et al. 2000). Among four white-rot fungi tested (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Ceriporiopsis subvermispora* and *Pleurotus ostreatus*), *P. ostreatus* selectively degraded the lignin fraction (41%) of paddy straw more than the holocellulose component, supporting the selection of *P. ostreatus* for the development of cellulase-free mutants in order to further improve its ability (Taniguchi et al. 2005).

Higher concentrations of preformed amino acids in the livestock diets may allow more efficient microbial synthesis. The biological value of plant proteins is less than that of animal / microbial proteins because they contain less methionine, lysine, tryptophan, threonine, valine, and arginine (Thomas et al. 1980). The amino acid content of plant proteins can be enhanced by breeding, by adding animal proteins or amino acids to the fodder, or by biological treatment with fungi. It has been shown that individual amino acids such as methionine, lysine, and histidine can become a limiting factor for milk production or growth in cattle (Kim et al. 2000). The present study indicated that the amount of these important amino acids could be increased by biological treatment with *P. ostreatus* and thus the feeding of the fungal treated paddy straw would enhance the productivity of dairy cattle. The most important sources of amino acids are the microbial crude proteins (CP_M) synthesized during the process of its treatment with fungi. Korhonen et al. (2002) and Garg et al. (2007) recorded the role of different nitrogen sources, which influence the amino acid profiles of the CP_M. Dundar et al. (2008, 2009) during their study on 'Effect of different lignocellulosic wastes for cultivation of *P. ostreatus* (Jacq.) on mushroom yield, chemical composition & nutritional value' demonstrated that *P. ostreatus* is especially rich in lysine and leucine, which are lacking in most staple cereal foods. In the present study urea treated straw incubated with *Pleurotus ostreatus* (wild) and its mutant forms improved the amino acid profile of the straw. Hence, the treatment of straw with mutant forms for efficient feed formulations is strongly recommended.

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