

## Effect of thermophilic fungus *Humicola fuscoatra* MTCC 1409 on paddy straw digestibility and biogas production



### Microbiology

**KEYWORDS :** Paddy straw;  
Pretreatment; *Humicola fuscoatra*;  
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### ABSTRACT

*The ability of *Humicola fuscoatra* MTCC 1409 to pretreat paddy straw for enhancing its digestibility and biogas production was investigated in this study. The potential of pretreatment of paddy straw was studied at regular intervals of 0, 5, 10, 15 and 20 days by determining the change in chemical composition of paddy straw like NDF, ADF, cellulose, hemicellulose, lignin and silica. Results indicated that the pretreatment of paddy straw with culture for 10 days was appropriate for increasing paddy straw digestibility and biogas production. The pretreatment significantly reduced the concentrations of NDF, ADF, cellulose and hemicellulose in the paddy straw by 16.9, 5.5, 56.1 and 42.9 % respectively. Reducing sugars and hydrolysis rate also increased significantly with pretreatment. These results showed that *Humicola fuscoatra* is an efficient cellulolytic fungus which is capable of increasing paddy straw digestibility and hence biogas production increased by 27.7% within 10 days pretreatment. The microscopic structural changes were examined by scanning electron microscopy (SEM) under reasonable conditions.*

### INTRODUCTION

In the recent years, there has been an increased interest in the development of technologies for exploiting renewable energy sources such as biomass (especially energy crops) for energy/power generation either directly or indirectly through various conversion routes (Kashyap et al. 2003). Anaerobic digestion is a biological process in which biodegradable biomass is decomposed in the absence of oxygen by the sequential action of hydrolytic, acetogenic and methanogenic bacteria to produce biogas. Biogas is a mixture of CH<sub>4</sub> (50-65%), CO<sub>2</sub> (30-45%), H<sub>2</sub> (0-3%), N<sub>2</sub> (1-5%), CO (0-0.3%), H<sub>2</sub>S (0.1-0.5%), O<sub>2</sub> and water vapors (traces) (Pauss et al. 1987).

Paddy straw consists of cellulose (35-40%), hemicellulose (20-24%), lignin (8-12%), ash (14-16%) and extractives (10-12%) which are associated with each other (Saha, 2003). Although, paddy straw has high cellulose content but the lignin complex and silica incrustation shields the microbial action for biogas production. Therefore, the paddy straw needs to be pretreated in order to enable cellulose to be more accessible to the microbial/enzymatic attack. Microbial pretreatment employs the use of micro-organisms especially fungi such as *Pleurotus ostreatus*, *P. chrysosporium* NRRL 6361, *Ceriporiopsis subvermispora* and *Cyathus stercoreus* (Gammal et al. 1998; Taniguchi et al. 2005). Thermophiles are a good source of novel catalysts that are of great industrial interest. The thermophiles have more stable enzymes as compared to mesophiles (Li et al. 2005). Thermophilic enzymes are also active at low temperature. Thermophiles developed more rapidly to higher peaks as compared to mesophiles and stability of obligate thermophiles increased with process temperature. Enzymes synthesized by thermophiles and hyper-thermophiles are known as thermozymes. These enzymes are typically thermostable or resistant to irreversible inactivation at high temperature. Thermozymes can be used in several industrial processes, in which they replace mesophilic enzymes or chemicals. The main advantages of performing process at higher temperature are reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved solubility of substrates. Due to these multifarious potentialities, they appear to be nature-borne biotechnologists. No doubt, reports are available for biological pretreatment of paddy straw using mesophilic fungi; however there is negligible work done on pretreatment using thermophilic fungi. Therefore, the present study was undertaken to optimize the conditions for thermophilic fungal pretreatment of paddy straw by *Humicola fuscoatra* MTCC 1409 and to study the implications of enhanced paddy straw digestibility on biogas production.

### MATERIALS AND METHODS

#### Procurement of the materials

Paddy straw was procured from the research field of Punjab Agricultural University, Ludhiana after harvesting of the crop. The paddy straw was chopped to 3-4 cm with a chopping machine and was stored in polythene bags at room temperature. Microbial culture of *H. fuscoatra* MTCC 1409, was procured from Institute of Microbial Technology, Chandigarh and was maintained on yeast peptone soluble starch (YPSS; yeast extract = 0.4%, soluble starch = 1.5%, K<sub>2</sub>HPO<sub>4</sub> = 0.1%, MgSO<sub>4</sub> = 0.1% & agar = 2.0%) agar slants. The culture was stored in refrigerator after sub-culturing at monthly intervals. Digested cattle dung slurry was procured from a working biogas plant of School of Energy Studies for Agriculture, PAU, Ludhiana.

#### Biological pretreatment of paddy straw

For the preparation of inoculum of *Humicola fuscoatra* MTCC 1409, wheat grains were washed and boiled for 20-30 minutes. The excess water was drained off. The grains were then mixed with 2% gypsum (CaSO<sub>4</sub>) and 4% CaCO<sub>3</sub> and dispensed into empty glucose bottles (250 g/bottle). The bottles were plugged and autoclaved for 90 minutes. After cooling, the bottles were inoculated with 5mm bits of 7-8 days old culture of *H. fuscoatra* MTCC 1409 and incubated at 50±2°C. The mycelium impregnated grains were used to inoculate paddy straw. Chopped paddy straw was soaked in water overnight. The excess water was drained off, so as to have approximately 65-70% moisture content. It was then mixed with inoculum at 10% w/w ratio (i.e. 25 g inoculum /250 g PS). After proper mixing, paddy straw was filled in polythene bags and incubated at 50±2°C for different times i.e. 0, 5, 10, 15 and 20 days, respectively. After the completion of required incubation, each set of treated paddy straw was removed and used to determine the change in chemical composition, dry matter loss, reducing sugars and hydrolysis rate of paddy straw.

#### Chemical analysis

Dry matter (DM) loss was determined by difference between dry weight of sample before and after pretreatment and described as percentage of initial weight of sample. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the standard method of AOAC (2000). Hemicellulose content was estimated as the difference between NDF and ADF while cellulose, lignin and silica were estimated by acid detergent lignin (ADL) as per standard procedure (AOAC, 2000).

#### Estimation of reducing sugars and hydrolysis rate

The total amount of reducing sugars was determined by 3, 5-dinitrosalicylic acid colorimetry (DNS method) (Ghose, 1987).

**Sample preparation:** Total sugars were extracted from the sample as follows:

Sample (0.1g) was taken in a beaker and 20 ml 80% alcohol was added in it. The beaker containing sample was kept on the boiling water bath for 20 min. The samples were covered with a lid in order to avoid evaporation. After 20 min, the alcohol containing sugars was extracted. These steps were repeated 4-5 times with 80% and 70% alcohol, alternatively. All the aliquots of alcohol were collected and then evaporated by keeping the beaker containing alcohol extract on water bath at 80-100°C leaving behind the extracted sugars. The final volume was made 10 ml with distilled water.

#### Assay procedure

Duplicate test tubes containing 3ml sample and 3ml DNS reagent were heated for 15 minutes in a boiling water bath. One ml Rochelle salt (sodium potassium tartarate) solution was added to each tube and the tubes were allowed to cool to room temperature. O.D. was measured at 575nm using UV-VIS spectrophotometer. A control was run simultaneously using 3ml distilled water. The glucose was used as a standard for the calibration of DNS reaction. The hydrolysis rate was calculated as follows:

$$\text{Hydrolysis rate (\%)} = \frac{(\text{Reducing sugars} \times 0.9)}{(\text{Cellulose} + \text{Hemicellulose})} \times 100$$

#### Biogas production

Biogas production experiments were carried out in glass biogas digesters of 2 litre capacity following single phase digestion and biogas produced was measured by water displacement method for a period of 35 days. The 250 g pretreated paddy straw was mixed with 250 ml of the digested cattle dung slurry and 100 g cattle dung and fed to the biogas digesters. These digesters were incubated at 40±2°C. A control was also run where untreated paddy straw was used instead of treated paddy straw. The experiment was conducted in triplicate.

#### Scanning electron microscopy

The change in surface structures of pretreated paddy straw was observed by scanning electron microscopy (SEM). SEM of untreated (control) and pretreated paddy straw was done in Nano and Electron microscopy laboratory (NEML) at PAU, Ludhiana. For SEM, the paddy straw was dried in oven at 60°C for 24h. Dried samples were treated with 10% ethanol w/v, dried in air for 24hr and grind to powdered form. The samples were sticked carbon glue for inspection and were observed using scanning electron microscope at 10.8 mm and 2.5K magnification.

#### STATISTICAL ANALYSIS

The Standard error (SE at 5% level) and least significance difference (LSD at 5% level) were calculated for triplicate data.

#### RESULTS AND DISCUSSION

The results of various experiments conducted are discussed under following sub heads:

##### Lignocellulose content

The effect of *H. fuscoatra* on lignocellulose content of paddy straw is shown in Table 1. Pretreatment have significant effect on acid detergent fibre (ADF), neutral detergent fibre (NDF), cellulose and hemicellulose contents of paddy straw ( $P \leq 0.05$ ) but not on lignin and silica. The results also suggested that, 10 days pretreatment is most appropriate to achieve significant reduction of holocellulose by *H. fuscoatra*. Duration of pretreatment has great importance in biological pretreatment of biomass, as most of the white rot fungi have slow growth rate and need a long incubation period (Kirk & Moore, 1972). Long incubation period is of disadvantage under field scale conditions because of limited space and also it is not economically feasible. On the other hand, long time incubation will increase the dry matter loss of pretreated sample. Results of this study show that *H. fuscoatra* have faster growth, thus 10 days pretreatment is sufficient for reduction in holocellulose content in the biomass. Based on the data of 10 days pretreatment, 37.6% of cellulose was degraded and only 18.7% of hemicellulose was degraded within same duration of pretreatment (Table 1). The 2 fold high degradation rate of cellulose than the hemicellulose is reflected

by significantly higher degree of cellulolytic enzyme activity.

Similar results were also observed by Zafar et al. (1980), who reported reduction in cellulose content after treatment of rice straw by *Pleurotus sajor caju*. Jafari et al. (2007) too reported decrease in hemicellulose, acid detergent fibre and neutral detergent fibre after pretreatment of rice straw with *Pleurotus spp*.

##### Dry matter (DM) loss, Reducing sugars and Hydrolysis rate

The effect of *H. fuscoatra* on DM loss, reducing sugars and hydrolysis rate are shown in Table 2. As discussed previously, based on lignocellulose degradability data, 10 days pretreatment was sufficient for pretreatment of paddy straw using *H. fuscoatra*. At this incubation period, the DM loss was 13.47%, which is lower than that reported using white rot fungi (Jung et al. 1992; Jalc et al. 1998). One of the problems of biological pretreatment using white rot fungi is high dry matter loss, as a result of long incubation period. Jung et al. (1992) studied the effect of five white rot fungi (basidiomycetes) for quality improvement of oat straw. Although, 30 days of fermentation using *Phanerochaete chrysosporium* had shown enhancement in *in vitro* digestibility, but 42.3% of dry matter lost due to required long incubation time. Jalc et al. (1998) reported 43.0% DM loss after pretreatment of wheat straw by *Daedalea guercina* (white rot fungus).

Pretreatment using *H. fuscoatra* had significant ( $P \leq 0.05$ ) effect on reducing sugar content of paddy straw (Table 2). Enhancement of reducing sugar over a period of time was correlated with reduction of holocellulose content as described earlier. Zadrazil (1984) reported that cellulose and hemicellulose are converted to soluble sugars during the incubation. Enhancing the concentration of reducing sugars is an additional advantage besides the reduction of holocellulose in improving the quality of biomass through biological pretreatment because microorganisms can easily utilize reducing sugars.

The hydrolysis rate increased significantly ( $P \leq 0.05$ ) with increase in pretreatment time (Table 2). The hydrolysis rate was 9.7%, 20.9%, 35.8%, 41.4% and 42.4% for 0, 5, 10, 15 and 20 days pretreatment, respectively. The result clearly showed that the increase of pretreatment period improved the removal rate of cellulose and hemicellulose and thereby enhanced the biodegradability of paddy straw as represented by the increase of hydrolysis rate. The reason is simple; disruption of barrier caused by hemicelluloses made cellulose more accessible to the microorganism and more easily converted into fermentable sugars.

##### Biogas production:

Results from Table 3 indicate that biogas production increased in 5 and 10 days pretreated paddy straw. A maximum of 27.7% biogas production was observed in 10 days pretreated straw. Petersson et al. (2007) too reported enhanced biogas production with pretreatment. However, further increase in pretreatment time led to a decrease of biogas production as 20 days pretreated straw reflected 25.6% decrease in biogas production as compared to control. The increase in biogas production is due to the increase in digestibility of paddy straw by increase of reducing sugars and breakage of bonds between cellulose and hemicellulose (Fox & Noike, 2004). Further reduction in biogas production can be correlated with the decrease in substrate, preferably cellulose, which is preferable source for methanogens.

##### SEM of untreated and treated paddy straw

Because a large fraction of holo-cellulose content was removed by pretreatment, there were some physical changes in the straw. For this reason, SEM pictures of pretreated and untreated paddy straw were produced. Fig.1 (a & b) showed the longitudinal section of paddy straw before and after biological pretreatment i.e. a) untreated paddy straw and b) paddy straw treated with *Humicola fuscoatra* MTCC 1409. The distinct changes in surface structure are visible in the basic tissue of paddy straw. The untreated paddy straw exhibited a rigid and highly compact structure whereas pretreated sample showed opening of the

holo-cellulose fibrils due to creation of pores of different sizes. Micro-fibrils were separated from initial connected structure and are fully exposed, thus increasing the external surface area and porosity of paddy straw. Similar results were also reported by Zhang and Cai (2008), who observed changes in histological structures of rice straw after 2% NaOH treatment. Yu et al. (2009) reported that both morphological and structural characteristics are changed due to organic polar substances and inorganic silica partly dissolved which leaves higher surface area with more pores on acid treated plant residues.

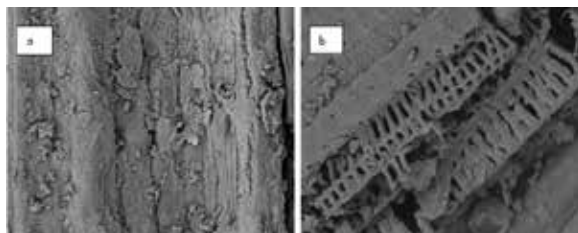
### CONCLUSION

So from the above studies, it is concluded that pretreatment of paddy straw by thermophilic fungus, *H. fuscoatra* MTCC 1409 for a period of 10 days is advantageous for increasing paddy straw digestibility, which ultimately leads to increase in biogas production by 27.7%.

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**Fig. 1: Scanning electron micrographs (SEM) of paddy straw before and after biological pretreatment, (a) longitudinal section of paddy straw before treatment, (b) longitudinal section of paddy straw after treatment with *Humicola fuscoatra* MTCC 1409**



**Table 1: Effect of incubation time on lignocellulose composition of paddy straw (% of dry matter)**

Pretreatment time (days)	NDF	ADF	Hemicellulose	Cellulose	Lignin	Silica
0	83.6 ± 0.95 <sup>a</sup>	58.0 ± 0.82 <sup>a</sup>	25.6 ± 0.85 <sup>a</sup>	37.8 ± 0.49 <sup>a</sup>	9.8 ± 0.66	9.2 ± 0.52
5	78.8 ± 1.22 <sup>b</sup>	57.4 ± 1.16 <sup>a</sup>	21.4 ± 0.79 <sup>b</sup>	30.8 ± 0.62 <sup>b</sup>	10.2 ± 0.41	9.6 ± 0.39
10	75.3 ± 1.14 <sup>c</sup>	55.4 ± 1.08 <sup>b</sup>	19.9 ± 0.68 <sup>bc</sup>	23.6 ± 0.84 <sup>c</sup>	10.7 ± 0.48	9.8 ± 0.44
15	71.9 ± 0.85 <sup>d</sup>	53.8 ± 0.91 <sup>c</sup>	18.1 ± 0.83 <sup>cd</sup>	18.6 ± 0.39 <sup>de</sup>	11.2 ± 0.61	10.1 ± 0.50
20	69.4 ± 1.06 <sup>e</sup>	54.8 ± 1.01 <sup>b</sup>	14.6 ± 0.78 <sup>e</sup>	16.6 ± 0.75 <sup>e</sup>	11.5 ± 0.64	10.4 ± 0.29
LSD	2.1	0.5	1.6	1.8	NS	NS

LSD: least significance difference at 5% level ( $P \leq 0.05$ ); NS: non significant; a, b, c, d and e: indicating means within row differed significantly,  $\pm$  values indicate per cent standard error for triplicate data, 0 day: control or untreated paddy straw

**Table 2: Effect of incubation time on dry matter loss, reducing sugar content and hydrolysis rate of paddy straw**

Pretreatment time (days)	Dry matter loss (%)	Reducing sugars (mg/g DM)	Hydrolysis rate (%)
0	3.9 ± 0.11 <sup>e</sup>	6.8 ± 0.95 <sup>e</sup>	9.7 ± 0.56 <sup>d</sup>

5	7.9 ± 0.15 <sup>d</sup>	12.1 ± 1.22 <sup>d</sup>	20.9 ± 0.66 <sup>c</sup>
10	11.5 ± 0.11 <sup>c</sup>	17.4 ± 1.14 <sup>a</sup>	35.8 ± 0.63 <sup>b</sup>
15	18.4 ± 0.16 <sup>b</sup>	16.8 ± 0.85 <sup>ab</sup>	41.4 ± 0.87 <sup>a</sup>
20	22.6 ± 0.14 <sup>a</sup>	14.7 ± 1.06 <sup>c</sup>	42.4 ± 0.59 <sup>a</sup>
LSD	2.1	0.5	0.8

LSD: least significance difference at 5% level ( $P \leq 0.05$ ); a, b, c, d and e: indicating means within row differed significantly,

$\pm$  values indicate per cent standard error for triplicate data, 0 day: control or untreated paddy straw

**Table 3: Biogas production from *H. fuscoatra* pretreated paddy straw**

Biogas	Pretreatment time (days)					LSD
	0	5	10	15	20	
l/250g PS	42.6±2.21 <sup>c</sup>	47.4±1.18 <sup>b</sup>	58.9±1.06 <sup>a</sup>	41.0±2.07 <sup>c</sup>	31.8±1.03 <sup>d</sup>	1.3
l/kg PS	170.4±12.6 <sup>c</sup>	189.6±10.4 <sup>b</sup>	235.6±11.9 <sup>a</sup>	164.0±12.7 <sup>c</sup>	127.2±11.8 <sup>d</sup>	

LSD: least significance difference at 5% level ( $P \leq 0.05$ ); a, b, c and d: indicating means within row differed significantly,

$\pm$  values indicate per cent standard error for triplicate data, 0 day: control or untreated paddy straw

## REFERENCE

- AOAC (2000) Association of Official Analytical Chemists, Official Methods of Analysis. 17th Edition. Maryland, USA. | Fox, M. and Noike, T. (2004) Wet oxidation pretreatment for increase in anaerobic biodegradability of newspaper waste. *Bioresource Technology* 91, 273-81. | Gammal, A.E.E., Kamel, Z., Adeb, Z., Helmy, S.M. (1998) Biodegradation of lignocellulosic substances and production of sugars and lignin degradation intermediates by four selected microbial strains. *Polymer Degradation and Stability*. 61, 535-42. | Ghose, T.K. (1987) Measurement of cellulase activities. *Pure and Applied Chemistry*. 59, 257-68. | Jalc, D., Nerud, E., Siroka, P. (1998) The effectiveness of biological treatment of wheat straw by white rot fungus, *Folia Microbiology* 43, 687-89. | Jafari, M.A., Nikkhab, A., Sadeghi, A.A., Chamani, M. (2007) Effect of *Pleurotus* spp. on chemical composition and in vitro digestibility of rice straw. *Pakistan Journal of Biological Sciences* 10, 2460-64. | Jung, H.G., Valdez, F.R., Abad, A.R., Blanchette, R.A., Hatfield, R.D. (1992) Effect of white rot basidiomycetes on chemical composition and in vitro digestibility of oat straw and alfalfa stems, *Journal of Animal Science* 70, 1928-35. | Kashyap, D.R., Dadhich, K.S., Sharma, S.K. (2003) Biomethanation under psychrophilic conditions: a review, *Bioresource Technology* 87, 147-53. | Kirk, T.K. and Moore, W.E. (1972) Removing lignin from wood with white rot fungi and digestibility of resulting wood. *Wood Fiber* 4, 72-79. | Li, W.F., Zhou, X.X., Lu, P. (2005) Structural features of thermozymes. *Biotechnology* 23, 271-81. | Paus, A., Naveau, H., Nyns, E.J. (1987) Biogas Production. In: Hall, D.O. and Overend, R.P. (eds.) *Biomass*, A Wiley-Interscience Publication, Great Britain, pp. 273-91. | Petersson, A., Thomsen, M.H., Nielsen, H., Thomsen, A. (2007) Potential bioethanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. *Biomass Bioenergy* 31, 812-19. | Saha, B.C. (2003) Hemicellulose bioconversion. *Journal of Industrial Microbiology and Biotechnology*. 30, 279-91. | Taniguchi, M., Suzuki, H., Watanabe, D., Sakai, K., Hoshino, K., Tanaka, T. (2005) Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw. *Journal of Bioscience Bioengineering*. 100, 637-43. | Zadrazil, F. (1984) In: F Sundstol & E Owen (Ed) *Straw and other fibrous by-products*, Elsevier, Amsterdam. pp-276. | Zafar, S.I., Kausar, T.K., Shah, F.H. (1980) Biodegradation of cellulose component of rice straw by *Pleurotus sajor caju*. *Folia Microbiology* 26, 394-97. | Zhang, Q. and Cai, W. (2008) Enzymatic hydrolysis of alkali pretreated rice straw by *Trichoderma reesei* ZM4-F3, *Biomass Bioenergy* 32, 1130-35. | Yu, C.T., Chen, W.H., Men, L.C., Huang, W.S. (2009) Microscopic structure features changes of rice straw treated by boiled acid solution. *Industrial Crops Production* 29, 308-15.