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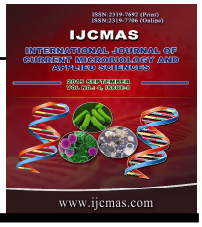


Original Research Article

Lignocellulose Biomass Degradation by microbial consortium Isolated from Harvested Rice Field

Shruti, Archit Sharma and Deepak Kumar Malik*

Department of Biotechnology, U.I.E.T, Kurukshetra University Kurukshetra, Haryana, India
**Corresponding author*

A B S T R A C T

Keywords

Lignocellulose, Rice straw, Gram staining, Lactophenol cotton, *Aspergillus*

Lignocellulosic containing plants are those types of biomass that include wood, agricultural residues and paper wastes. They are composite polymeric material containing primarily cellulose, hemicellulose and lignin. In the present work rice straw degrading microbial consortium was isolated. Nineteen microorganisms were isolated, among these microorganisms 14 were bacterial strains and four were fungal strains. The isolated bacterial strains were characterized using gram staining and biochemical tests. Four isolated fungal species were characterized by lactophenol cotton blue staining. Three of the isolated fungal strains were identified as *Aspergillus* and one of them was identified as *Gliocladium*.

Introduction

Lignocellulose is a renewable organic material and major structural component of all plants. Lignocellulose consists of three major components: cellulose, hemicellulose and lignin. In addition, small amounts of other materials such as ash, proteins and pectin found in lignocellulosic residues in different degrees based on the source (Sanchez *et al.,* 2009). Cellulose is major constituent of all plant material and most abundant organic molecule on the Earth. It is a linear biopolymer of an hydroglucopyranose-molecules, connected by β-1, 4-glycosidic bonds. The coupling of adjacent cellulose chains by hydrogen bonds, hydrophobic interactions and Van der Waal's forces leads to a parallel alignment of crystalline structures known as microfibril. Hemicelluloses, the second most abundant component of lignocellulosic biomass, are heterogeneous polymers of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids. Composition of hemicelluloses is very variable in nature and depends on the plant source (Saha, 2003). Lignin, the third main heterogeneous polymer in lignocellulosic residues, generally contains three aromatic alcohols including coniferyl alcohol, sinapyl and *p*-coumaryl. Lignin acts as a barrier for any solutions or enzymes by linking to both hemicelluloses and cellulose and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. Not surprisingly, lignin is the most recalcitrant component of lignocellulosic material to degrade (Sanchez *et al.,* 2009).

Lignocellulosic wastes are produced in large amounts by different industries including forestry, pulp and paper, agriculture, and food, in addition to different wastes from municipal solid waste (MSW), and animal wastes (Champagne, 2007; Wen *et al.,* 2004). These potentially valuable materials were treated as waste in many countries in the past, and still are today in some developing counties, which raises many environmental concerns (Palacios-Orueta *et al.,* 2005).

Significant efforts have been made to convert these lignocellulosic residues to valuable products such as biofuels, chemicals and animal feed (Howard *et al.,* 2003). Using sugars or corn as the main source for ethanol production caused a great deal of controversy due to its effect on food production and costs, which has made it difficult for ethanol to become cost competitive with fossil fuels. These concerns became a driving force in the generation of new biofuel research using lignocellulosic wastes produced by many different industries. In the present study our aim was to biologically degrade the lignocelluloses material under mesospheric conditions.

Materials and Methods

Materials and Pretreatment of rice straw and preparation of basal media

The chemicals and glassware used in this study were procured from Hi Media, Rankem and Central Drug House. The rice straws and soil sample were taken from harvested rice fields nearby Kurukshetra University. The rice straws were taken, cut into equal pieces of 3 cm and washed properly. The pretreatment of these straw pieces were done in 1% NaOH solution in distilled water for a period of 24 hours and after that these were rinsed properly with distilled water for at least thrice. After washing these pieces were dried properly in oven at 50 °C.

Isolation of lignocellulose (rice straw) degrading microbial consortium from wood rot and rice field soil sample

The isolation of microbial consortium was done by using enrichment technique (Martinus, 1901). The soil sample was taken from rice field near Kurukshetra University. The 1g soil was weighed and poured 100 ml basal media (5 gl^{-1} Peptone powder, 3g l^{-1} Yeast extract powder, pH 7) and 1g of pre-treated rice straw. The flasks were incubated for 15 days at 30 °C during first enrichment. The 10 ml culture broth from first enrichment was transferred into fresh rice straw media containing rice straw i.e. lignocelluloses as a sole carbon source. The second enrichment was carried out for 10 days followed by third and fourth enrichment with same conditions. The microbial consortium was isolated from enriched medium by spread plate method. All isolated bacterial and fungal consortium was purified by streak plate method using nutrient agar and potato dextrose medium respectively.

Characterization of isolated bacterial and fungal strains

The identification of fungal strains was done using lactophenol cotton blue staining. In order to identify the fungal colonies, colony colour, shape, border and spores (if the spores are available) were recorded. Microscopic examination was done. The fungi were classified up to the genus level by their morphological features. Characters used in classification were compared by considering mycelial characters such as presence of septa, whether mycelium was

branched or not, on mature colonies the presence of reproductive structures such as sporangia, conidia and their morphology. The biochemical identification of bacterial strains was done by using Hi Assorted biochemical tests kits on the basis of changes in colour of various coloured medium.

Analysis of lignocellulose degradation

The individual bacterial and fungal strains were also tested for degradation of lignocellulose to check their individual effect on degradation of rice straw in the rice straw medium at 37 °C. The analysis of lignocelluloses degradation was checked by using whole consortium. The consortium was cultured in basal medium containing 1% (w/v) lignocellulose material (rice straw) for 5 days at 30 °C under static conditions. The uninoculated medium was act as a control. The rice straws were degraded into fibers, the solid filtrate was suspended in 100 ml acetic acid/nitric acid reagent and heated at 100 ºC for 30 min to remove the biological cells. The acetic acid/nitric acid suspension was filtered again. The remaining cellulosic material was washed three times by using distilled water. After washing and filtration the filtered solid was dried. The weight loss of lignocellulosic materials was calculated by subtracting the weight of the residual substrates from the total weight of the lignocellulosic materials before degradation. The degradation ratio was calculated as followed equation: Where M_t is total weight of the cellulosic materials before degradation and M_r is the weight of the residual substrates after degradation.

$$\text{Degradation ratio (\%)} = (M_t - M_r / M_t) \times 100$$

Optimization of environmental conditions for the degradation of lignocellulose

To obtain high percentage of lignocellulose degradation, following environmental factors was optimized i.e. pH and temperature. In order to investigate the influence of pH on the degradation of lignocellulosic material medium of different pH (5, 6, 7 and 8) were incubated with 3% inoculum at 30 °C. The contents of the flasks were harvested after 96 hr and assayed for the amount of degradation. The effect of temperature was investigated by inoculating the production medium (pH 7.0) with 3% inoculum at two temperatures (30° C, 37º C) with static conditions. The contents of the flasks were harvested after 96 h and assayed for the percentage degradation of lignocellulose.

Optimization of nutritional conditions for the degradation of lignocellulose

To obtain high percentage degradation of lignocellulose, two nutritional factors was optimized i.e. carbon and nitrogen source. The effect of carbon source on degradation of lignocellulosic material was studied by adding 1% D-fructose and dextrose in the lignocellulosic media (pH 7.0). The medium were inoculated with 3% inoculums at 30 °C for 96 h with static conditions. The degradation efficiency was monitored thereafter. The effect of nitrogen source on of degradation of lignocellulosic material was studied by adding 1% ammonium sulphate and urea in the lignocellulosic media (pH 7.0). The flasks with medium were inoculated with 3% inoculums at 30 °C for 96 h with static conditions. The degradation efficiency was monitored thereafter.

Results and Discussion

Isolation of lignocellulose degrading microbial consortium

A total of 14 morphologically different bacterial strains coded as (SLB1, SLB2,

SLB3, SLB6, SLB7, SLB8, SLB9, SLB11, SLB13, SLB14, SLB15, SLB16, SLB17 & SLB18) and four fungal strains coded as (SLF1, SLF3, SLF5 & SLF6) were isolated by using enrichment technique. Among all the bacterial strains, only one strain SLB15 was showing fluoroscence. The qualitative microbial degradation of rice straw was checked in rice straw medium, there was no degradation in uninoculated media. The degradation was found in inoculated media after 5 days as shown in figure 1. The rice straw degradation indicated that microbial consortium was using the rice straw as a sole source of carbon and energy.

Morphological and biochemical characteristics of isolated microbial consortium

All 14 isolated bacterial strains were characterized by gram staining. It was observed that majority of the bacterial strains were gram negative (SLB1, SLB2, SLB3, SLB6, SLB8, SLB9, SLB11, SLB13, SLB16, SLB17) and only a few of them were gram positive (SLB7, SLB14, SLB15, SLB18). The biochemical tests of the gram negative bacteria were performed by using HiAssorted KB002 kits. The results of biochemical testof all gram negative bacteria shown in table 1. The morphological characteristics of four isolated fungal strains were carried out in our laboratory. The morphological characteristics of these fungal strains are shown in figure 2. After close examination of isolated fungal strians, it was observed that strain SLF 1 had condia stained blue and had structure of hyphae and conidiophores as *Gliocladium,* similarly it was observed that strains SLF3, SLF5 and SLF6 had hyphae and conidia structures as that of *Aspergillus.*

Analysis of lignocellulose degradation

There was no degradation observed when individual bacterial or fungal isolates were inoculated in rice straw medium and for a week. When the whole microbial consortium was used for degradation of lignocellulose only then the rice straws were converted into fibrous structures. The degradation of rice straw in the medium was assayed with the help of acetic acid/nitric acid test. The M_t i.e. total mass of the rice straw before degradation was 2 g and M_r i.e. the residual mass after the acetic/nitric acid test was found to be 0.59 g after 5 day of incubation at 30 °C, pH 7.0. The percentage degradation of lignocellulose was observed as 74.6 % as compared to the control.

Effect of environmental conditions on lignocellulose degradation by the microbial consortium

The pH and temperature of the medium had significant effect on degradation activity by microbial consortium as shown in table 2. The degradation was least in medium having pH 5.0, the total mass (M_t) was 1 g with residual mass (M_r) 0.37 which was least and percentage degradation assesed was 63 %. The lignocellulose degradation increases with increasing pH. At pH 8.0 in which total mass (M_t) was 1 g with residual mass (M_r) 0.15 g and degradation was found to be 85 %. The lignocellulose degradation increased 13.94 % at pH 8.0 as compared to pH 7.0. The degradation was performed at three different tempratures to find out which one gives the best degradation results. The optimum temprature for degradation was 37 °C, at which 79 % degradation of lignocellulose observed. At 30 °C, 70 % degradation of lignocellulose was observed. At 50 °C there was no degradation observed. The lignocellulose degradation was increased 5.8 % at 37 °C as compared to at 30 °C. This indicated that pH has an significant effect on lignocellulose microbial degradation.

Effect of nutritional sources on degradation of lignocellulose by microbial consortium

It was found that degradation activity decreases in the presence of both the carbon sources i.e. D-fructose &dextrose. In case of both D-fructose & dextrose degradation was found to be 40 %. In the absence of both carbon sources was added in the medium the percentage of lignocellulose degradation was 74.9 % after 5 days of incubation. So after the addition of additional carbon source (D-fructose & dextrose- 1 %), the % of degradation decreased upto 46.3 % as show in table 2. It was found that degradation activity decreases in the presence of both the nitrogen sources i.e. ammonium sulphate and urea. The percentage degradation in case of 1 % urea was found to be 70 % which indicated a decrease of 6.1 % degradation as compared to the medium with out any additional urea as a nitrogen source. The percentage degradation in case of 1 % ammonium sulphate was found to be 67 % which indicated a decrease of 10.1 % degradation of lignocellulose as compared to the medium with no additional ammonium sulphate as a nitrogen source.

Table.1 The biochemical test of isolated bacterial consortium

Biochemical tests	Bacterial strains						
	SLB1	SLB2	SLB3	SLB9	SLB13	SLB16	SLB17
Citrate utilization	+	-	-	-	+	+	-
Lysine utilization	+	+	+	+	+	+	+
Ornithine utilization	+	+	+	+	+	+	+
Urease	-	-	-	-	+	-	-
Phenylalanine deamination	-	-	-	-	-	-	+
Nitrate reduction	-	+	-	-	+	+	-
H_2s production	-	-	-	-	-	-	-
Glucose	-	-	-	-	+	-	-
Adonitol	+	-	-	-	+	-	-
Lactose	-	-	-	-	-	-	-
Arabinose	+	-	-	-	+	-	-
Sorbitol	-	-	-	-	-	-	-

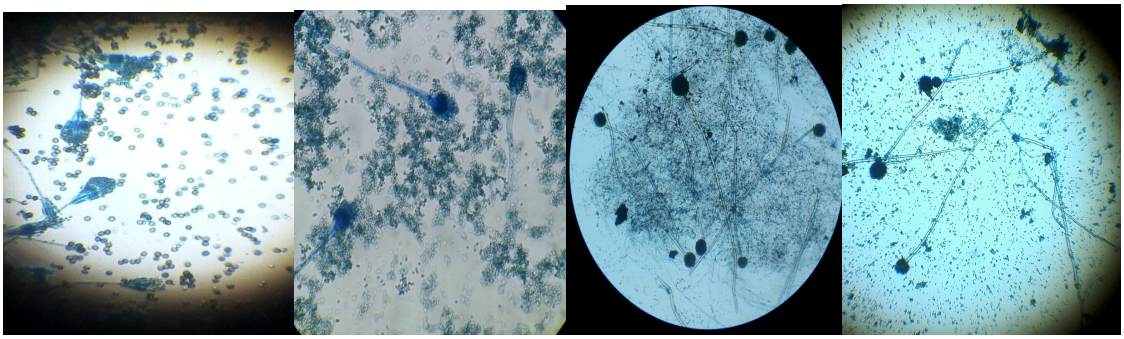
Table.2 The effect of pH, temperature, carbon and nitrogen sources on the degradation of lignocelluloses (rice straw)

Parameter	M_t	M_r	% degradation
Control	1.0	1.0	0
5.0 pH	1.0	0.37	63
6.0 pH	1.0	0.28	72
7.0 pH	1.0	0.25	75
8.0 pH	1.0	0.15	85
30 º C	1.0	0.3	70
37 º C	1.0	0.21	79
50 º C	1.0	1.0	0
D-fructose (1%)	1.0	0.6	40
Dextrose (1%)	1.0	0.6	40
Ammonium sulphate (1%)	1.0	0.33	67
Urea (1%)	1.0	0.3	70

Fig.1 The degradation of rice straw by microbial consortium after 5 days of incubation, (a) uninoculated (b) inoculated

(a) (b)

Fig.2 The lactophenol cotton blue staining of the isolated fungal strains



(a) SLF1-*Gliocladium* (b) SLF3-*Aspergillus* (c) SLF6-*Aspergillus* (d) SLF5-*Aspergillus*

In the present study lignocellulose degrading microbial consortium was isolated by using enrichment technique from soil sample from rice field near Kurukshetra University. Among the all bacterial strains only one bacterial strain SLB15 was showing flourosence. Identification of fungal strains was done by lactophenol cotton blue staining, among the four isolated fungal strains SLF3, SLF 6 & SLB5 were *Aspergillus* and SLF1 was *Gliocladium.* Khan *et al.* (2007) reported straw degradation was observed by *Trichoderma*, in this cellulase production was carried out by solid state bioconversion (SSB) method using rice straw, a lignocellulosic material and agricultural waste, as the substrate of three *Trichoderma* spp. and *Phanerochaete chrysosporium*in lab-scale experiments. Belal (2013) also isolated a rice straw - cellulose utilizing mold was from rotted rice straw residues. The efficient rice straw degrading microorganism was identified as *Trichoderma reesei.* Among all isolated fourteen bacterial strains 11 were gram negative (SLB1, SLB2, SLB3, SLB6, SLB8, SLB9, SLB11, SLB13, SLB16, SLB17) and only a few of them were gram positive (SLB7, SLB14, SLB15, SLB18). The gram negative strains were assessed biochemically for utilization of various substrates. Amitha and Reddy (2013) performed high-solids incubations were performed to enrich for microbial communities and enzymes that decompose rice straw under mesophilic 35 °C. The linocellulose degradation was assessed at pH 5, 6, 7 and 8, the highest degradation was found at pH 8.0. The percentage degradation was 85 % at pH 8.0 and 63 % at pH 5.0. Zhao (2014) also performed rice straw degradation and optimized the degradation of the cellulosic

content of the lignocellulose and the optimum pH range was found to be 5.9 to 8.5 which is quiet a stretch as compared to the single optimum pH observed in this current study. The lignocellulose degradation was not found at 50 °C. The maximum degradation (79 %) was found at 37 ºC and decreased upto 70% at 30 ºC. This was in contrast with the results obtained with Zhao (2014) in which optimum temperature for degradation was obtained as 60 ºC. In the present study, when additional carbon or nitrogen sources were added in to the medium then there was decrease in percentage degradation of lignocellulose. When carbon source D-fructose and dextrose were added at 1 % concentration in to the medium it observed that the degradation of lignocellulose observed up to 40 %. In a similar study Rashad *et al.* (2010) monitored microbiological and physicochemical parameters of lignocellulosic biomass degradation for 12 weeks during composting of rice straw, soybean residue and enriched with rock phosphate.

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