Journal of Scientific & Industrial Research
Vol. 72, Sept - Oct 2013, pp. 585-590

# Development of a microbial consortium for production of blend of enzymes for hydrolysis of agricultural wastes into sugars

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*Received 19 March 2013; revised 19 May 2013; accepted 29 May 2013*

This study presents development of a blend of enzymes capable of degrading lignocellulosic biomass from a locally isolated microbial consortium through solid state fermentation. Different agro wastes (wheat bran, rice husk and pine needles) were taken in all combinations as a substrate and at 1:1:1 ratio, optimum production of blend of enzymes was obtained with 0.673 U/ml, 0.214 U/ml and 0.032 U/ml activities respectively after 3 days of incubation at 37°C. This blend of enzyme hydrolysed pine waste and released 0.454 g/g of sugar at 37°C at 120 rpm for 48 h. Produced blend of enzymes was found very potent and can have great application in biofuel, phytochemical and other industries.

**Keywords**: Enzymes, Lignocellulosic waste, Microbial consortium, Pine waste, Sugar yield

## Introduction

Lignocellulose biomass is one of the major renewable sources of energy with annual 1 x $10^{10}$ million tonnes production[1]. Main components of lignocelluloses are cellulose (35-50%), hemicelluloses (25-30%) and lignin (25-30%)[2-5]. Most of the biomass is disposed of by burning[6]. Production of reducing sugars for fermentation purposes is still a costly process[7]. Biological pretreatment with enzymes is slower than chemical but does have better efficiency and also do not require large volume of chemicals[8]. For production of blend of enzymes, solid state fermentation (SSF) has been more advantageous than other approaches due to less water requirement and lesser chances of contamination[9-14]. Economical production of blend of laccase, cellulase and xylanase enzymes is an urgent need for bioethanol and biofuel industries throughout the world. In nature, microbes degrade lignocelluloses cooperatively[15]. A microbial consortium is more effective in biodegradation then single microbes[16]. Successive enrichment culture technique can be used for establishing microbial consortia[17]. A number of reports support co-cultivation of microbes with abilities to release lignocellulosic waste degrading enzymes[9]. Basidomycetes consortium capable of producing cellulase, xylanase and peroxidases has been employed for lignocellulosic waste degradation[18,19]. Haruta *et al* [20] prepared a consortia that degraded many cellulosic substances (filter paper, printing paper, cotton and rice straws) by 60% within 4 days. Microbial consortia prepared by Sarkar *et al* [21] was capable of producing enzyme with concomitant activity and used to degraded kitchen wastes (55-65%, by vol) with less time of degradation and less foul smell. Pine needles as a substrate is available throughout the year and is totally a forest waste responsible for forest fire and animal abortion[22,23]. With dead pine needles, *Pinus roxburghii* [22] occur wild in Himalayan ranges on a wide area.

This study presents development of a blend of enzymes capable of degrading lignocellulosic biomass from a locally isolated microbial consortium through solid state fermentation.

## Experimental Section

### Chemicals, Reagents and Cellulosic Material

All chemicals and reagents were of ACS grade and purchased from Himedia and S D Fine chemicals from local distributor at Allahabad, India. Whatman filter paper no. 1 was used to study stabilization of microbial consortia. Cellulosic material [pine needles (PN), wheat bran (WB), and rice husk (RH)] were used for substrate optimization. Only PN was used for degradation experiment. PN were grinded into powder form (2-5 mm) and soaked in 1% NaOH for 24 h, washed with distilled water until neutrality and dried at 60°C. Cellulosic

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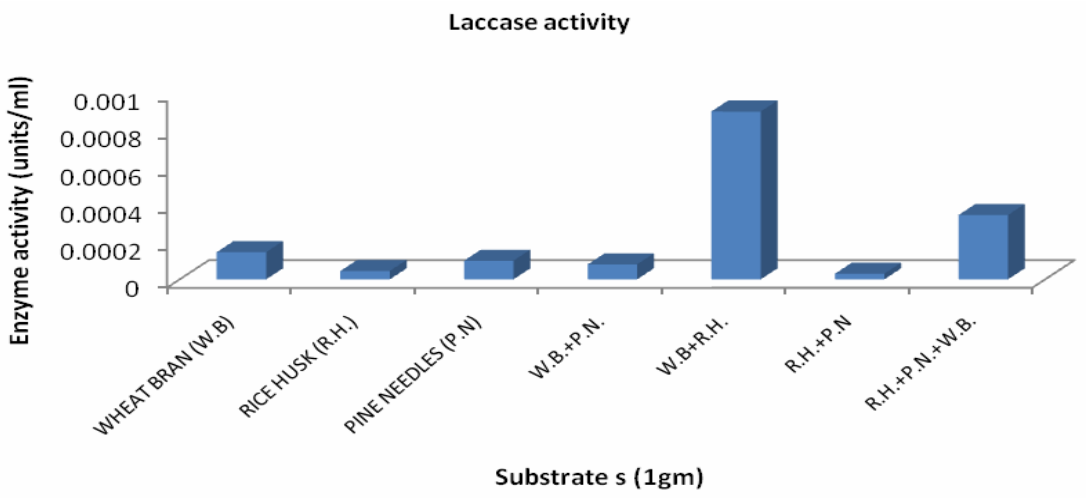


Fig. 1ð Laccase enzyme production in terms of enzyme activity with various lignocellulosic substrates

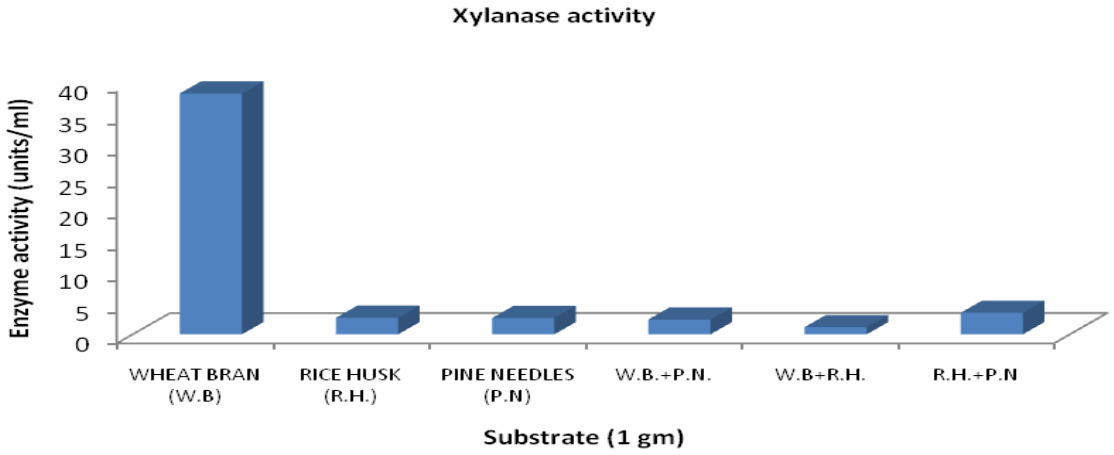


Fig. 2ð Xylanase enzyme production in terms of enzyme activity with various lignocellulosic substrates

material was autoclaved at 121°C for 15 min, prior to use.

**Screening Media Composition**

Peptone cellulose solution (PCS) media (pH 7) contained: NaCl, 0.5; peptone, 0.5; yeast extract, 0.1; $CaCO_3$, 0.2; and filter paper, 0.5%. Laccase producing microbes screening media[24] (pH 6) contained: $ZnSO_4$, 0.001; Peptone, 3.0; glucose, 10.0; $KH_2PO_4$, 0.6; $MnSO_4$, 0.5; $K_2HPO_4$, 0.0005; $FeSO_4$, 0.05; $MgSO_4$, 0.5; and agar, 20 g/l, besides guaiacol, 0.2%. Xylanase producing microbes screening media[25] contained: Birch wood xylan, 1.0; peptone, 5.0; yeast extract, 5.0; $K_2HPO_4$, 0.2; agar, 20.0 g/l. Cellulase producing microbes screening media[26] (pH 9-10) contained: $K_2HPO_4$, 1.0; NaCl, 5.0; $MgSO_4$, 0.2; KCl, 0.5; yeast extract, 5; peptone, 5.0; and agar, 20 g/l, besides CMC, 1%.

**Isolation and Screening of Microbes**

For construction of a microbial community, soil samples were collected from lignocellulosic waste decomposing sites in an around MNNIT campus. Soil samples were mixed together, crushed and final mixture (1 g) was taken in distilled water (10 ml) and serial dilution was performed. Water (200 µl) from each test tube was taken and then spread over the plates with respective screening media. To screen xylanase producing microbes, plates spread with different dilution factors were incubated for 5 days at 30°C. Xylanase producing microbes were detected based on the clear zone of hydrolysis after flooding plates with 0.1% aqueous congo red dye followed with repeated washing with 1 M NaCl solution[25]. To screen laccase producing microbes, plates were spread with 200 µl of various dilutions separately and were identified on the basis of brown/reddish brown

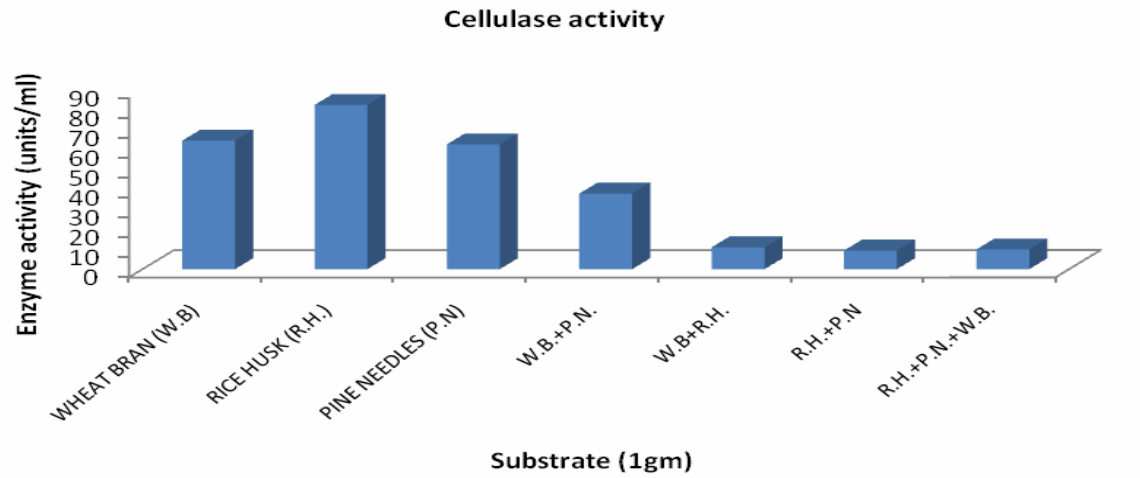


Fig. 3ð Cellulase enzyme production in terms of enzyme activity with various lignocellulosic substrates

colour due to oxidation of guaiacol in presence of peroxidase[24]. To screen microbes with cellulase activity, plates enriched with cellulose were spread with various dilutions separately for 48 h at 28°C, and flooded with iodine solution for 3-5 min[26]. Clear zone of hydrolysis around fungal or bacterial strains was indicator of cellulose producing microbes. Microbes isolated were inoculated into 250 ml flasks containing autoclaved PCS medium (100 ml) supplemented with PN (1.0 g), and with a filter paper strip (0.3 g) as an indicator for cellulase activity[27].

**Consortium Stabilization and Activity**

For stabilization of consortia, cultures were incubated at 40°C under static conditions. Once strip of filter paper was completely degraded and PN softened, culture (1 ml) was transferred into fresh enrichment medium with filter paper. Resulting cultures were combined together in fresh PCS medium and sub cultured for several times with culture transfers every 3 days. This culture was stored at 4°C in PCS medium. After consortia stabilization, consortia was cultured in PCS medium containing 0.5% (w/v) cellulosic materials (filter paper and PN) for 72 h at 40°C as per reported[28] procedure. By providing the same conditions, uninoculated medium was used as control. Inoculated flask containing Whatman paper strip for degradation was observed by placing it in incubator. Incubation was followed by filtration and solid filtered residue was then mixed with 100 ml acetic acid/nitric acid reagent[28] and heated for 30 min at 100°C to remove microbial cells. Acetic acid/nitric acid treated suspension was filtered and residue was washed three times with distilled water (100 ml) each time. Filtered solids were dried at 80°C and determined gravimetrically[3, 28]. To calculate loss in lignocellulosic material, subtract residual weight from total lignocellulosic weight. Degradation ratio[3] was calculated as: Degradation ratio (%) = ($M_t$ ï $M_r$ / $M_t$) x 100, where $M_t$ is total weight of cellulosic materials before degradation and Mr is weight of residual substrates after degradation. All experiments were performed in triplicate and average values are reported.

**Microbes and Production Media**

Microorganisms utilized for production of enzymes were grown in single as well as in consortia. Laccase producer (LSD), xylanase producer (XSD) and cellulase producer (CSD) were grown separately and in together. Each microbeôs enzyme production was compared with each other singularly as well in consortia. Substrates were utilized in all possible combinations but overall weight was kept at 1 g.

**Solid State Fermentation (SSF)**

SSF was carried out using Erlenmeyer flasks (250 ml each) Substrates (1 g) were added to each flask and moistened with mineral basal salt ($NaNO_3$, 2.5; $KH_2PO_4$, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 g/l) in 1:1 ratio to substrate. Then flasks were autoclaved at 121°C at 15 psi for 15 min. After sterilization, flasks were inoculated with specific microbes with all possible combinations singularly as well as in consortia. Incubate inoculated media for 3 days, at 37°C.

**Enzymes Extractions and Assays**

Enzymes were extracted by mixing fermented substrates with distilled sodium acetate buffer at pH 4.8 for cellulase, pH 6 for laccase and pH 5-6 for xylanase.

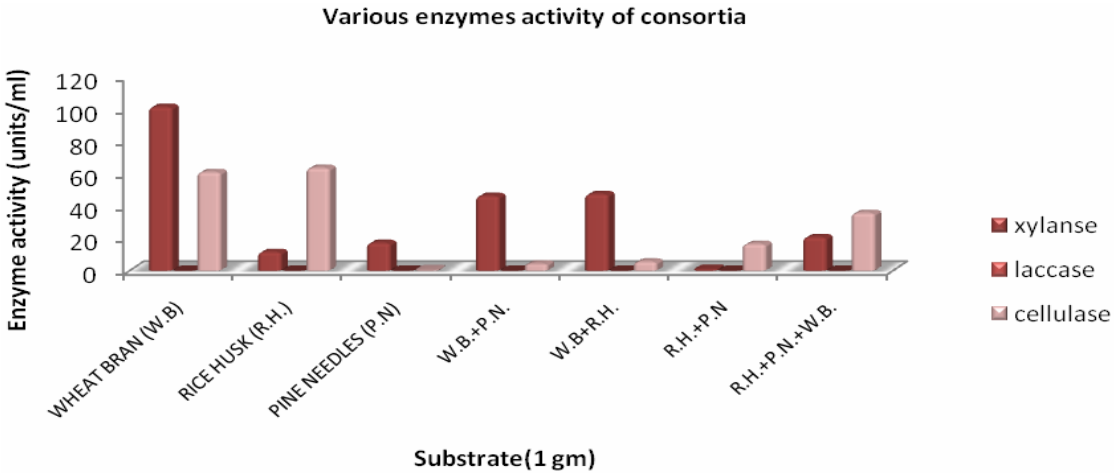


Fig. 4ð Enzyme production in terms of enzyme activity with various lignocellulosic substrate

Mixture was left for 2 h at room temperature. Solid residue was separated from enzymatic solution by filtering through Whatman no. 1 filter paper. Enzymes isolated were crude and stored at 4°C. To analyse production of enzymes by microbes, enzymatic assays were performed for each culture. Cellulase and xylanase activity were calculated by DNS reducing sugar method[29] and laccase activity as repored[30]. Enzyme activity was calculated as the amount of enzyme catalysing production of one µm of coloured product per ml per min.

## Results and Discussion

Based on enzymatic activity, out of 50 microbial strains isolated, 15 strains were selected. Microbes that showed maximum clear zone were taken and named LSD, XSD and CSD. Microbial consortia are more efficient and stable to withstand changing environment conditions than single uniform populations, because of being able to communicñate and differentiate[16]. A consortium was prepared by mixing these microbes. To optimise growth of microbial consortia, different sets of temperature were taken and microbes were allowed to grow. Maximum activity was observed at 37°C for cellulase and xylanse. Degradation of filter paper along PN was used an indicator for stability of consortia and potency of the same for degrading complex agriculture wastes. Two flasks as control and test were used for studying degradation of lignocellulosic wastes by consortia. Within 24 h, paper strip started degrading and in 3.5 days, it was completely degraded by stabilised consortia. In this study, final transfer culture has: xylanase activity, 0.210; cellulase activity, 0.655; and laccase activity, 0.032 U/ml. All microbes from the end culture maintained their growth as was confirmed by streaking them on agar plates.

This consortium was then analysed for its enzyme producing ability. Production of microbes was carried out with different agricultural waste substrates. Production of enzymes was indirectly made associated with the amount of sugar released. Maximum enzyme production for cellulase with 62.5 µmole of sugar released from rice husk, 65.22 µmoles/ml with xylanase in wheat bran while 0.85U/ml activity for laccase in wheat bran. These kind of various agricultural residues generated biomass can be utilized for the production of laccase, cellulase and xylanase enzymes, which find great use in biofuel industries, environmental and other pharma sectors. Wongwatanapaiboon *et al*[31] used *T. ressei* TISTR 3081 for production of cellulolytic enzymes, which was carried on Mandels medium and xylolytic enzymes in media containing birch wood xylan as substrate at 30°C for 8 days; enzymes produced found use in ethanol production from grasses. Microbial consortia made up of *Ralstonia* sp., *Clostridium* sp., uncultured *Firmicutes, Propionibacterium acnes,* uncultured *betaproteobacterium*, and *Pantoea* sp. showed 51% degradation of rice corn stover powder and 81% of filter paper in 8 days within anoxic conditions at 40°C[28]. Okeke & Lu[33] characterized a consortium capable of degrading Bermuda grass. A consortia, developed from high temperature sugarcane bagasse compost mainly containing *Clostridium* sp., *Thermoanaerobacterium* sp., and *Shodocyclaceae*, showed high stability and degradation of rice straw (75%), corn stover (70%) and bagasse (60%) within 7 days at 50°C[16]. Guevara & Zambrano[32] consortia degraded pretreated sugarcane leaves were up to 90%. In present study, final transfer culture showed enzymatic activities of cellulase (0.673 U/ml), xylanase (0.214 U/ml) and laccase

(0.032 U/ml), whereas release of sugar was 0.454 g/g with PN as substrate. Microbes from this culture when transferred on the plates showed same type of colonies as were present on the first plate, from where they were isolated, thereby proving stability of microbes in the consortia, hence shows stabilize co-existences.

**Application of Enzymatic Cocktail Produced**

Pine forest wastes were pretreated for hydrolysing, with enzymes blend produced by consortia and sugar released was found to be 0.250 g/g, at 50°C at 100 rpm, 1 g/15 ml (w/v) PN and phosphate buffer saline, in 48 h of incubation in submerged fermentation. Similar studies are reported with Bermuda grass[32] and sugarcane leaves[33] for hydrolysis of lignocellulosic waste. Enzymes based pretreatment of grasses showed reducing sugar release of 500-600 mg/g[31].

## Conclusions

This paper dealt with isolation of microbes able to form consortia and can produce blend of lignocellulosic degrading enzymes. At optimum temperature (37°C) for production of enzymes, highest activities for consortia were observed for cellulase (28.6 U/ml) and xylanase (17.68 U/ml). Microbes were allowed to grown for three days in Czepkdox media with PN at 37°C at 100 rpm and filter paper as an indicator for degrading activities. Process was repeated for many a times and an enzymatic activity for final stable culture has: xylanase, 0.210; cellulase, 0.655; and laccase, 0.032 U/ml; and release of reducing sugar, 0.250 g/g. These experiment data of lab scale, if carried out at industrial scale, can be helpful to biofuel, pharma and environmental industries.

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