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Biodegradation of Saw in Plant Fertilizer

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Abstract

Many horticulture crops and cultivars of corn, soybean and cotton prefer applying saw dust as an additive in plant fertilizer, composting is an aerobic biological process. Composting wood wastes, reduces the waste volume, detoxifies the waste and transform it to humus which is a valuable amendment to soil and improves aeration of soil, but decomposition of saw dust takes years of time for cultivation. This is due to its slow biodegradation and this can be overcome by treating them with fungal enzymes as similar to fungal biological succession, partially degraded saw is a better substrate for decomposition. Main aim of this work is to produce raw information about biodegradation of saw which can be used in the studies on examining the amelioration of applying fungal enzyme treated saw as fertilizer to seek immediate benefit. On this aspect cellulose degrading fungi were isolated from natural sources, cultured on saw dust substrate, extracted crude enzymes were assayed on filter paper substrate and Fpase activity was measured by DNS reagent method. Aspergillus sp. and Trichoderma sp. depicted activity of 26.35and 25.8 FPU/ml respectively whereas their percentage of biodegradation depicted 1.88 and 1.84% respectively.

Keywords: Biodegradation, saw, cellulose, fertilizer, horticulture.

Introduction

Saw dust is a common waste generally emitted by timber factories or saw mills, plywood and wood scraps. It is used as animal feed, in early days it was widely used as fuel in cookers. They are also used in landfilling, and in the production of some aesthetic products. However it cannot be used as instant fertilizer as it takes long time for degradation, and due to its high content of carbon it is extremely hygroscopic nature, thus it absorbs more water from the soil and causing plants wilt. In addition, carbon to nitrogen ratio in saw dust is 400:1 this may cause nitrogen depletion in soil, high carbon content increases activity of soil microorganisms and increases the immobilization (depletion in nitrate ions caused by excessive use of minerals by microbes) of nitrate ions[1], this is disadvantageous to plants, due to these reasons saw is not solely added as fertilizer, usually it is mixed with plant litter and animal manure but unlike animal manure saw does not increase the salinity of soil[1]. Moreover, saw consist of about 30% lignin[2], and also bears anti-microbial substances such as tannins and resins as it is from wood, because of these properties it takes years of time for degradation. However, most of the horticulturists and farmers of soybean, cotton and corn using saw as an additive to compost fertilizer, here the amendments are chick manure, cow and horse dung and cotton gin trash, to absorb excess water in muddy land and in mulching to protect the soil from water loss and soil erosion, but when it is used as fertilizer farmers can cultivate only after about 4-5 years later[3].

Figure- 1
Decomposing saw particles in compost

Our main objective is to provide raw facts to the horticulturists to examine the amelioration of using fungal enzyme treated saw for composting (figure 1), where the decomposition process can occur rapidly, that facilitates the supply of inorganic ions to plants, detoxify the soil and improves the aeration since wood particles are bigger in size. In the kingdom of Fungi, most of the individuals are saprophytic and are efficient in degradation of major polymers such as cellulose and lignin. Fungi or their byproducts are used in recycling cellulosic materials such as paper mills, Purified fungal cellulolytic enzymes are used for commercial food processing such as production of coffee, it performs hydrolysis of cellulose during drying of beans, they are also widely used in textile industry and in laundry detergents such as modern washing powders, and fermentation of biomass into biofuels, even in medicine for example fungal cellulase is used as a treatment for phytobezoars (a form of cellulose bezoar found in the human stomach)[4]. In this work cellulolytic fungi collected from various natural sources were screened and

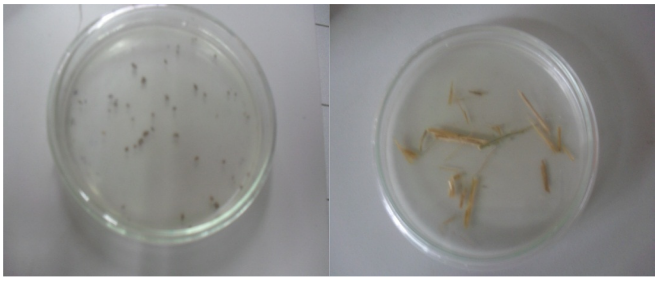
cultured on saw, degrading activity of extracted enzymes was measured using filter paper assay and DNS reagent method comparison was made on empirical data. Furthermore, optimum temperature and pH for each extracted fungal enzyme also determined for future applications.

Material and Methods

Source of fungi and initial culturing: Samples were collected from sawdust, straw dust and sprinkled soil (garden, beach, Mud). Decaying wood particles and decaying leaf collected from the surroundings. Samples were collected into sterile containers and stored separately. Potato dextrose agar medium was used to grow the initial cultures, where samples were cultured by streak plate method and sprinkle method.

Methods of inoculation: Streak plate method: In order to isolate the fungi this method is best in practice. Initial streak is made with the sample then all other streaks are continuum of the previous strike using separate sterile tooth peck for each strike.

Sprinkled method: This is more suitable for the soil samples and sawdust. Particles were sprinkled on the medium. Well-spaced sprinkled particles would result in separation of colonies. Finally, petri-dishes were sealed with Para film, labeled and inverted dishes were incubated in a dark place. Visible colonies were observed after 4-7days (figure 2).



Sawdust sprinkled **Straw sprinkled**

Figure-2
Inoculation of samples for the first time on PDA medium by sprinkle method

Media used for sub culturing: Selective water agar medium: To separate the fungi causing cellulose digestion, a medium consisting cellulose as a sole carbon source was prepared. For cellulose Whatman no. 1 filter paper made of 100% cellulose was used. Water agar medium was prepared by dissolving 4g of agar in 250ml of distilled water. Autoclaved pieces of filter paper (1cm×1cm) were used for inoculation.

After pouring the water agar medium on petri dishes, paper strips were carefully placed on top of the agar bed by a sterile forceps. After the inoculation, sealed dishes kept for 4-7 days of incubation. To avoid bacterial growth, antibiotic was added to the medium. This made the medium more selective to fungi.

Table-1
Antibiotics and their concentrations that used in the culture

Antibiotic	Ampicillin	Tetracycline
In a ml of stock solution	50mg/ml	100mg/ml
Final concentration in a ml of water Agar medium	50µg/ml	100µg/ml

Table 1 depicts the amount of antibiotics that were added for the preparation of selective water agar medium. Sub culturing continued until the pure culture was available.

Culturing fungi in liquid medium containing saw dust and preparation of crude enzymes: In order to extract the secreted cellulolytic enzymes by each fungal colony, it is important to culture them in a liquid medium. Cellulolytic basal medium (CBM) was chosen for this purpose. Cellulolytic basal medium (g /250ml in distilled water) was prepared[5].

Diammonium tartrate ($C_4H_{I2}N_20_6$)	1.5
Potassium dihydrogen phosphate (KH_2P0_4)	0.25
Yeast extract	0.02
$MgS0_4$. $7H_20$	0.15
$CaCl_2.2H_20$	0.0002

CBM medium was autoclaved and 10ml aliquots were transferred to sterile 20ml bottles.

Saw particles of same amount were submerged into the CBM medium aseptically inside the laminar airflow. Samples which were previously obtained from pure culture were inoculated by streaking on the submerged oven dried saw dust aseptically by sterile tooth peck. Always a control bottle was kept without inoculation.

Caps of the bottles were loosely fitted to allow the adequate air exchange. All the bottles were incubated for 4 days at room temperature (25^0C). After the incubation, observations were made, and the liquid medium, which contains the crude fungal enzymes, was collected.

Each bottle contains the cellulolytic enzymes that were secreted as extra cellular enzyme by each fungus colony. At the time of isolation, which is after a period of incubation it is better to vortex in slow speed in order to ensure the distribution of enzymes all over the liquid medium. About 1ml of aliquot was taken by sterile micro pipet and placed into the 1.5ml sterile centrifuge tubes. Centrifugation was done at 12,000 r.p.m for 15 minutes. The resulted supernatant consists of proteins that are mostly the fungal crude enzymes. Centrifugation is important for the separation of fungal spores since spores could not be allowed in the later filter paper assay.

Filter paper Assay: International Union of Pure and Applied Chemists recommended filter paper assay (FPA) as the standard measure of cellulase activity. Enzymatic reactions often occur in the presence of buffer, which helps to keep the

reaction environment stable. This is obtained by maintaining the ionic balance and the pH unchanged. 2ml 0.05M of Trisodium citrate dihydrate ($C_6H_5Na_3O7•_2H_2O$) buffer was used with the crude enzymes and filter paper strips (0.5cm×0.1cm) were used as substrate. 0.1ml of crude enzyme of each fungus was added with 0.15 ml of Trisodium citrate dihydrate solution, while the pH was maintained at 4.8. Always a blank was maintained in one tube without adding any fungal enzymes. Instead, it was replaced by same volume of sterilized distilled water. Then the Whatman no. 1 filter paper strip (0.5cm×0.1cm) was added as the substrate. Each tube was then incubated in 50^0C shaking incubator running at 100 r.p.m[6].

Measuring the activity of cellulolytic enzymes: The activity of extracted fungal enzymes can be measured quantitatively. Here the concentrations of reducing sugars (products of enzyme activity) were measured using DNS reagent test since the optical absorbance can be more accurately measured numerically using spectrophotometer at 540nm.

Dinitrosalicylic acid method: Dinitrosalicylic acid reagent was prepared by adding 1g 3, 5-dinitrosalicylic acid in 50ml of distilled water. 200mg crystalline phenol (optional) and 30g of Sodium potassium tartrate were added to the solution, which turns the solution into yellow colour. To this, 20ml of 2N NaOH was added. This turns the colour of the solution into transparent orange yellow. Finally, the stock was made into 200ml by adding distilled water. Stock was stored at 4°C in refrigerator, to prevent deterioration[7].

After the incubation, filter paper strips were carefully removed from the tubes using a glass rod. Then 0.5ml of DNS reagent was pipetted into each tube. This terminated all enzymatic reactions occurred in the tube. Then the lids of tubes were tightly closed, and placed in a water bath at 95-100^0C for 10 minutes. After this, tubes were immediately transferred into an ice cold bath and kept for few minutes. 1ml of distilled water was pipetted into each tube before measuring the optical absorbance. Colour change in each tube was measured by using UV spectrophotometer at 540nm wavelength. Finally, the optical absorbance readings were compared and plotted with the standard glucose curve to find the glucose (product) concentrations[7].

From each glucose and buffer mixture, 0.1ml of solution was added to 0.15ml of Trisodium citrate dihydrate buffer solution. Then each centrifuge tube was transferred into a water bath where tubes were incubated at 50^0C temperature for an hour, same as the conditions given for the enzyme filter paper assay. After the incubation, 0.5ml of DNS reagent was pipetted into each tube and the lids of all tubes were tightly closed. Then the temperature in the water bath was raised to 95-100^0C and kept for 10 minutes. Finally, the tubes were immediately transferred into an ice cold bath for few minutes and 1ml of distilled water was pipetted to each tube before measuring the absorbance of optical absorbance, and the samples were examined for the colour change.

Colour change in each tube including the control blank was measured by using UV spectrophotometer at 540nm wavelength. Finally, the optical absorbance readings were plotted against the concentration of glucose.

As given in the table 2 optical absorbance differ according to the concentration of glucose, this is ranging from 1.00mg/0.5ml to 3.35mg/0.5ml resulted in optical absorbance ranging from 0.228 to 0.766 respectively.

Table-2
Glucose concentration vs. Optical absorption at 540nm

Glucose concentration	Optical absorption
3.35mg/0.5ml	0.766
2.50mg/0.5ml	0.580
1.65mg/0.5ml	0.378
1.00mg/0.5ml	0.228

This standard curve (figure 3) was used to find the unknown concentrations of reducing sugars in all samples, dilutions used were translated into enzyme concentrations. Concentration of enzyme which would have released exactly 2.0 mg/ 0.5ml of glucose by means of a plot of glucose liberated against the logarithm of enzyme concentration was estimated.

Filter paper unit was calculated according to IUPAC-FPU. As given below:

$$FPU = \frac{0.37}{\text{Enzyme concentration to release 2mg glucose}} \text{units} \bullet \text{ml-1}$$

This quantitatively shows the activity of cellulolytic enzymes.

Percentage of degradation of saw dust by each enzyme is calculated by using given formula.

Degradation (%) = {Glucose (mg/0.5 ml) / Substrate (mg/0.5 ml)} x 100

Since saw dust consists of 50% cellulose[2], substrate concentration in 0.5ml can be derived as 25mg (cellulose). By applying the product (glucose) concentration retrieved from the standard glucose curve the percentage of degradation was calculated.

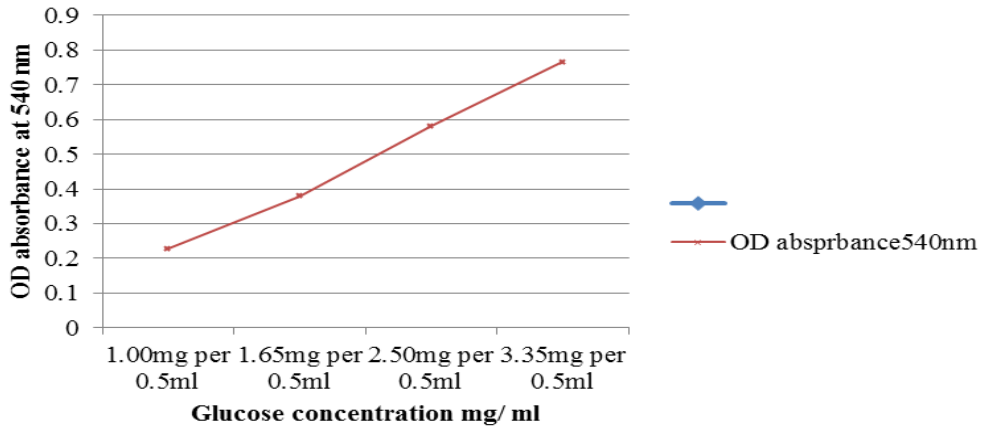


Figure-3
Glucose concentrations vs. Optical absorbance (540nm): standard glucose plot

Identification of fungi: In order to identify the fungal colonies, colony colour, shape, border, and spots (if the spores are available) were recorded as given in table 4.2. Microscopic visuals were observed under high power (40×10) objective. Spores and the mycelia were observed so clearly (figure 4), and the data were recorded and used in classification. Fungi were classified up to the genus level by their morphological features. Classification was based on microscopic observation of mycelia as well as reproductive structures such as spores and fruiting bodies, if available. 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, types of spore they generate, whether spores are septate or not and position of rhizoids on the mycelium etc.

Figure-4
Fungal samples, Optimization of pH and Temperature for fungal enzyme activity

Enzyme activity related to variation in temperature and pH were measured separately. Filter paper assays of each fungal crude enzyme were kept in water baths at temperatures of 37^0, 50^0 and 60^0C. After an hour of incubation DNS reagent test was done.

Similarly, pH of the each buffer solution was changed to 3, 6, 8, and 13 by adding either dill NaOH or dill HCl. Then crude enzyme of each fungus was added and filter paper strips were placed. Assay was incubated at 50^0C for an hour and products were measured using DNS reagent test. Concentrations of reducing sugars were obtained from the standard glucose curve and, finally activity of enzymes was calculated as FPU/ml.

Results and Discussion

Fungal colony on saw dust broth: After 4 days of incubation at room temperature (25^0C) partially degraded saw particles in CBM (cellulose basal medium), (figure 5).

Figure-5
Fungal colony on saw dust; immersed in CBM medium after 4 days of degradation at 25^0C.

Degradation of saw by enzymes of fungi: According to the results (figure 6) *Aspergillus* sp. showed the highest degradation of 1.88%, whereas *Trichoderma* sp. was 1.84%. Rate of degradation was lower than that of when using cellulolose Whatman no. 1 filter paper as substrate. This is due to low concentration of cellulose here (about 50%) and saw consist of 30% of lignin[1], furthermore wood usually have anti-microbial properties such as resins and tannins in miner quantities these properties may slow down the degradation process.

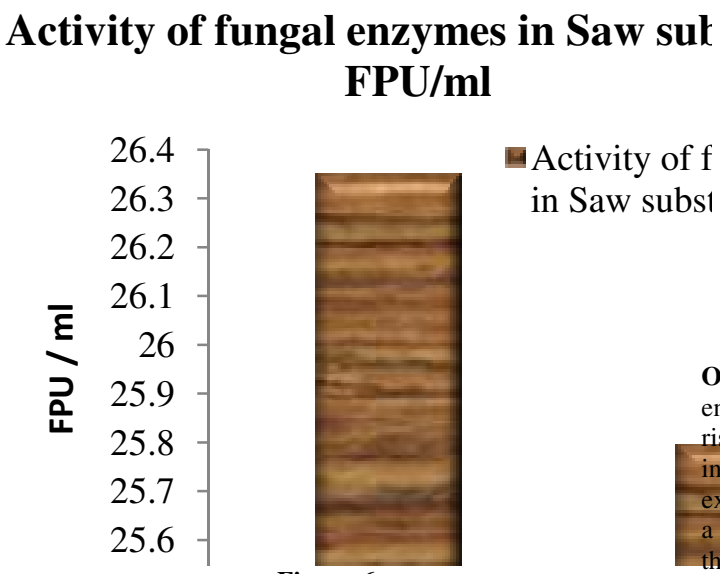


Figure-6
Degradation % of saw dust by each fungal enzymes.

Activity of fungal enzyme from saw dust broth (by filter paper assay)**:** Enzyme activity is determined by the binding ability of their enzymes to the available substrate. According to the graph (figure 7) *Aspergillus sp.* and *Trichoderma sp.* respectively showed high activity of 26.35and 25.8 FPU/ml.

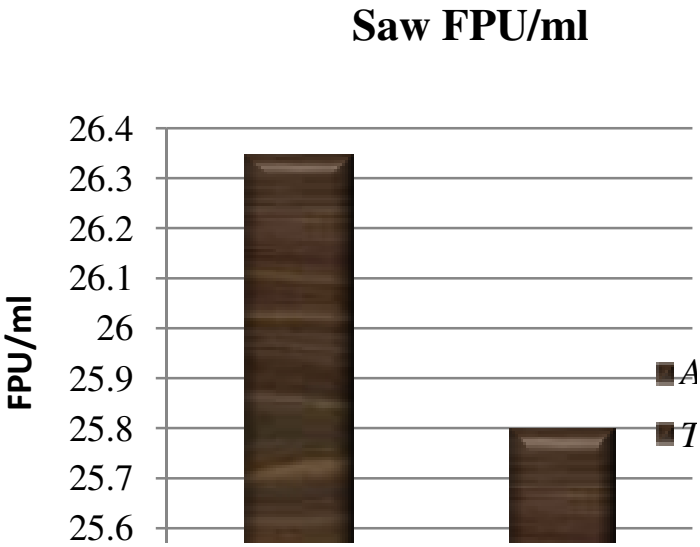


Figure-7
Activity of fungal enzymes from saw dust substrate in cellulose basal medium

Optimization of Temperature (FPU/ml): The rate of an enzyme catalyzed reaction increases as the temperature has risen. Variations in reaction temperature by 1 or 2 degrees may introduce changes of 10 to 20% in the results. In this experiment, enzymatic reaction of given fungi (figure 8) showed a peak at 50^0C by reaching a peak of 32.5 FPU/ml. This shows that the temperature for the cellulolytic enzymes of three given fungi was 50^0C. However, if further high temperatures are tested it is possible to find the point they get denature. Normally animal enzymes get denatured even at 40^0C. Nevertheless, for fungi it is higher. It is also possible to observe the tolerance as well as the preference of high temperature (since 50^0C as optimum) by fungal enzymes. According to the graph, the fungus *Trichoderma* sp. shows an increase in the rate of reaction until 50^0C followed by decline afterwards.

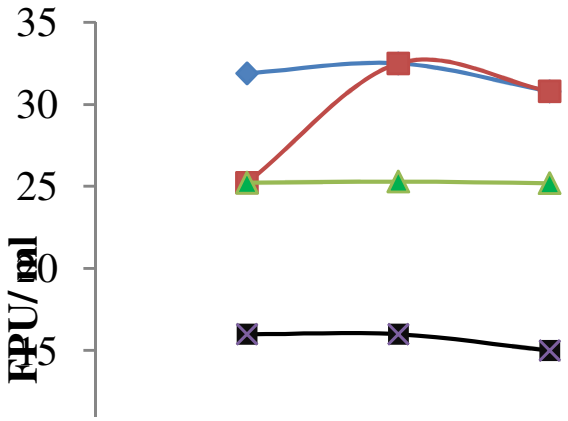


Figure-8
Temperature vs. activity of fungal enzymes (FPU/ml)

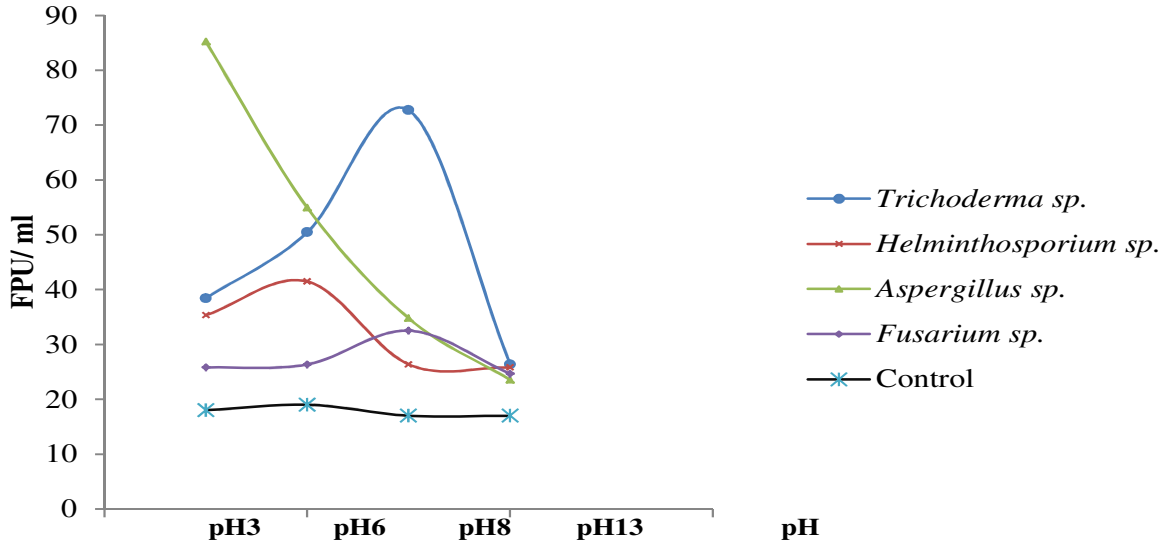


Figure-9
Effect of pH on the activity of cellulolytic enzymes from fungi

Optimization of pH (FPU/ml): Since enzymes are proteins, they are very sensitive to changes in pH. Each enzyme has its own optimum range for pH, where it is most active, and the result is determined by the effect of pH on a combination of factors such as binding of the enzyme to substrate, catalytic activity of the enzyme, ionization of the substrate, and the variation of protein structure. The initial rates for many enzymatic reactions exhibit bell-shaped curves. The most favourable pH value (optimum pH) may vary among enzymes of different fungi. In this experiment (figure 9) the optimum pH for fungus *Trichoderma sp.* and *Fusarium* sp. was closer to neutral, and for *Helminthosporium sp.* it is 3. It means *Helminthosporium sp.* prefers slightly acidic medium. For *Aspergillus sp.* the curve was peaking at very low pH that showed the preference of *Aspergillus sp.* towards acidic environment. To increase the nitrate content it is also possible to allow the saw particles to react with nitric acid and subsequently mixed with compost, similar work was done to paper pulp by a US scientist[8], however, this reaction is allowed to occur after the enzymatic degradation and it will not affect the pH during the enzyme activity.

Conclusion

Based on empirical calculations *Aspergillus* sp. and *Tricchoderma* sp. depicts high cellulolytic activity of 26.35 and 25.8 FPU/ml respectively on raw saw, in addition their ability of biodegradation depicted 1.88 and 1.84% respectively. This showed the minimal eligibility of these two fungal enzymes to be used in pretreatment of saw. Aapplication of extracted cellulolytic fungal enzymes to speed up the biodegradation process of saw, which is to be used as fertilizer, plants required mineral ions which is resulted after the decomposition of microbes on complex organic substances, fungal enzymes partially digests cellulose and lignin substances as in animal manure fertilizer, this makes the substrate an easy platform for decomposers, enzyme treatment is to be given under optimum temperature and pH after a partial degradation of complex components such as cellulose and lignin. Thereafter, saw is applicable with other traditional organic fertilizers, this will reduce the preparation time for horticulturists using saw.

Further scope of the study: Facts obtained on this work paves a way on the production of quality fertilizer using saw, this may also lead to waste reduction from saw mills, reduce transportation cost, reduce dumping effort in landfills, and reduce toxicity of soil. Moreover, production of biofuel (ethanol) by using enzyme treated saw in fermentation could be an additional advantage, cultivation of mushroom also depends on saw as substrate and there is a room to study about the applications of degraded saw pulp in various other useful ways.

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