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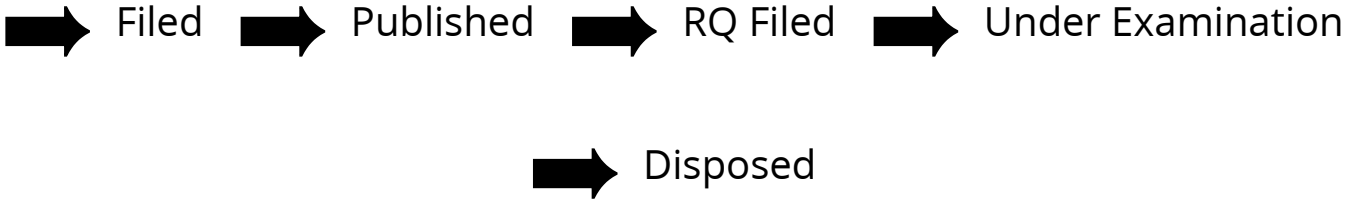


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**FORM 2**

**THE PATENTS ACT 1970**

**(39 of 1970)**

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**THE PATENT RULES, 2003**

**COMPLETE SPECIFICATION**

**(See section 10 and rule 13)**

**1. TITLE OF THE INVENTION: -A METHOD FOR ACCELERATION OF LIGNOCELLULOSIC BIOMASS DEGRADATION USING MICROORGANISMS**

**2. Applicant(s)**

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**3. PREAMBLE OF THE DESCRIPTION**

**The following specification particularly describes the invention and the manner in which it is to be performed**

## FIELD OF INVENTION

This invention relates to a method for acceleration of lignocellulosic biomass degradation by using microorganisms.

## BACKGROUND OF INVENTION

The lignin is considered as the second most abundant biopolymer on this earth next to cellulose (Crawford and Crawford, 1978; Argyropoulous and Menachem, 1997). Lignin is widely distributed, but not universally throughout the plant kingdom. It is found in all vascular plants, where it acts as a structural component of support and conducting tissue (Crawford, 1981). It has been unambiguously identified in certain primitive groups such as ferns and club mosses but seems to be absent in bryophytes and lower plant groups (Miksche and Yasuda, 1978). According to an estimate, lignins constitute about 8.7% of wheat straw (Charaya and Singh, 2005; Singh *et al.*, 2015a; Singh *et al.*, 2017). A number of excellent and comprehensive reviews on different aspects of lignin and its breakdown have appeared from time to time (Huang, 2001; Dashtban *et al.*, 2010; Fisher and Fong, 2014; Gonzalo *et al*., 2016; Datta *et al*., 2017).

In nature large quantities of lignocellulogic biomass are generated every year from various sources including forest, agricultural, agro-municipal and food processing wastes. There are variety of options for it's use (Stainforth, 1979a; Anderson and Anderson, 1980):

**a.** It may be burnt in the soil itself thereby returning the minerals immobilized in it; however, it causes pollution and wastage of the valuable organic matter.

**b.** It may be chopped followed by ploughing to improve soil fertility (Mishra *et al.*, 1996), though sometimes shallow incorporation of straw may lead to the production of phytotoxic compounds resulting in poor establishment of subsequent crops (Kaur *et al.*, 2003).

**c.** It may be used to prepare compost manures including though aerobic composting (Singh and Sharma, 2002).

**d.** It may be used for growing microorganisms for proteins (Gao *et al*., 1997); for growing mushrooms and cucumbers (Allen, 1968); to produce xylitol (Rijkens,1977); methane biogas and alcohol (Stainforth, 1979b); for manufacturing particle board (Dalen and Sharma, 1996).

Proper well-planed strategies need to be evolved for the effective management of these highly useful resources. It is clear from above discussion that despite several uses of biomass,

the easiest method to manage this valuable resource, is the burning it in the field by farmers. However, it leads to several eco-societal problems. Burning of agricultural biomass residue, or Crop Residue Burning (CRB) has been identified as a major health hazard. In addition to causing exposure to extremely high levels of Particulate Matter concentration to people in the immediate vicinity, it is also a major regional source of pollution, contributing between 12 and 60 per cent of PM concentrations as per various source apportionment studies. In addition, it causes loss of vital components such as Nitrogen, Phosphorus, Sulphur and Potassium from the top soil layer, making the land less fertile and unviable for agriculture in the long run (Mukherjee, 2016).

On the other hands it may be chopped and followed by ploughing to improve soil fertility (Mishra et al., 1996). One may find references to "humus" and its importance in increasing soil fertility in the literature of 18th and early 19th century (Achard, 1786; Thaer, 1809). The origin of humus, however, was earlier believed to be only a chemical process, an oxidation and dehydration of plant materials (Detmer, 1871). Muller (1887) and Remann (1905) established that humus formation is not a purely chemical or physical process; rather, it is basically a biological one resulting from the diverse activities of microorganisms, protozoa and various other soil organisms like insects and worms. Though sometimes shallow incorporation of straw may lead to the production of phytotoxic compounds resulting in poor establishment of subsequent crops (Harper and Lynch, 1982)

Biological method is one of the best and ecofriendly method to manage agricultural residues, but problem is to decompose it in limited time so that, it converts into humus content of soil. This further leads to the establishment of subsequent crops. The aim of present investigation is to design a strategy, which can decompose valuable agricultural biomass within the limited time.

**WO2010094665A2**; Cathy Isop, Pascale Joseph, Jean-Paul Leonetti, Jacques Biton disclose composition and methods of producing bioenergy products and metabolites of industrial interest from lignocellulosic biomass. More specifically, the invention describes the identification, characterization and isolation of novel bacteria having the remarkable ability to transform lignocellulosic biomass into fermentable sugars and, even more remarkably, into bioenergy products and metabolites. The invention also discloses a method to isolate such bacteria, compositions comprising such bacteria, and their uses for the modification of lignocellulosic biomass, with a view to producing bioenergy.

**US6022419A;**Robert W. Torget, NandanPadukone, Christos Hatzis, Charles E. Wyman disclose a multi-function process is described for the hydrolysis and fractionation of

lignocellulosic biomass to separate hemicellulosic sugars from other biomass components such as extractives and proteins; a portion of the solubilized lignin; cellulose; glucose derived from cellulose; and insoluble lignin from said biomass comprising one or more of the following: optionally, as function 1, introducing a dilute acid of pH 1.0-5.0 into a continual shrinking bed reactor containing a lignocellulosic biomass material at a temperature of about 94 to about 160° C. for a period of about 10 to about 120 minutes at a volumetric flow rate of about 1 to about 5 reactor volumes to effect solubilization of extractives, lignin, and protein by keeping the solid to liquid ratio constant throughout the solubilization process; as function 2, introducing a dilute acid of pH 1.0-5.0, either as virgin acid or an acidic stream from another function, into a continual shrinking bed reactor containing either fresh biomass or the partially fractionated lignocellulosic biomass material from function 1 at a temperature of about 94-220° C. for a period of about 10 to about 60 minutes at a volumetric flow rate of about 1 to about 5 reactor volumes to effect solubilization of hemicellulosic sugars, semisoluble sugars and other compounds, and amorphous glucans by keeping the solid to liquid ratio constant throughout the solubilization process; as function 3, optionally, introducing a dilute acid of pH 1.0-5.0 either as virgin acid or an acidic stream from another function, into a continual shrinking bed reactor containing the partially fractionated lignocellulosic biomass material from function 2 at a temperature of about 180-280° C. for a period of about 10 to about 60 minutes at a volumetric flow rate of 1 to about 5 reactor volumes to effect solubilization of cellulosic sugars by keeping the solid to liquid ratio constant throughout the solubilization process; and as function 4, optionally, introducing a dilute acid of pH 1.0-5.0 either as virgin acid or an acidic stream from another function, into a continual shrinking bed reactor containing the partially fractionated lignocellulosic biomass material from function 3 at a temperature of about 180-280° C. for a period of about 10 to about 60 minutes at a volumetric flow rate of about 1 to about 5 reactor volumes to effect solubilization of cellulosic sugars by keeping the solid to liquid ratio constant throughout the solubilization process.

**10201803620W**; MEDOFF, Marshall, MASTERMAN, Thomas, Craig PARADIS, Robert disclose PROCESSING MATERIALS Biomass (e.g., plant biomass, animal biomass, and municipal waste biomass) is 5 processed to produce useful intermediates and products, such as energy, fuels, foods or materials. For example, systems and methods are described that can be used to treat feedstock materials, such as cellulosic and/or lignocellulosic materials, while cooling equipment and the biomass to prevent overheating and possible distortion and/or degradation. The biomass is conveyed by a conveyor, which conveys the biomass under 10

an electron beam from an electron beam accelerator. The conveyor can be cooled with cooling fluid. The conveyor can also vibrate to facilitate exposure to the electron beam. The conveyor can be configured as a trough that can be optionally cooled. FIG. 1

**3265574;**DEL RIO LUIS FERNANDO, WAFA AL DAJANI WALEED, MAO CHANGBIN, YUAN ZHIRUN disclose La présente invention concerneunprocédéd' extraction de sucres à partird'unebiomasselignocellulosiqueprétraitée. Ce procédéconsiste à mettreen contact la biomasselignocellulosiqueprétraitée avec des charges faiblesd'une solution aqueuse de peroxyacide (PA) pour produireune fraction liquide (contenantune petite quantité de produits de dégradation de la lignine et l'hémicellulose) et une fraction solidecontenant de la cellulose, de l'hémicellulose et de la lignine. La fraction solidepeutensuiteêtresoumise à hydrolyse enzymatique avec unevariétéd'enzymesdégradant la paroicellulaire pour produire un résidu riche enlignine et une solution de sucre qui peutêtrefermentée pour obtenirunevariété de substances (bio)chimiques. (EN) The present disclosure relates to a process for extracting sugars from a pretreated lignocellulosic biomass. This process consists of contacting the pretreated lignocellulosic biomass with low charges of an aqueous peroxy acid (PA) solution to produce a liquid fraction (containing a small amount of lignin and hemicellulose degradation products) and a solid fraction containing cellulose, hemicellulose and lignin. The solid fraction can then be subjected to enzymatic hydrolysis with a variety of cell wall-degrading enzymes to produce a lignin-rich residue and a sugar solution that can be fermented to a variety of (bio) chemicals.

**Engineering in life science;Volume16, Issue1,January 2016,Pages 1-16**;GeaGuerriero, Jean-Francois, , Hausman Joseph Strauss HalukErtan, Khawar Sohail Siddiqui disclose Lignocellulose biomass derived from plant cell walls is a rich source of biopolymers, chemicals, and sugars, besides being a sustainable alternative to petrochemicals. A natural armor protecting living protoplasts, the cell wall is currently the target of intense study because of its crucial importance in plant development, morphogenesis, and resistance to (a) biotic stresses. Beyond the intrinsic relevance related to the overall plant physiology, plant cell walls constitute an exquisite example of a natural composite material that is a constant source of inspiration for biotechnology, biofuel, and biomaterial industries. The aim of the present review is to provide the reader with an overview of the current knowledge concerning lignocellulosic biomass synthesis and degradation, by focusing on its three principal constituents, i.e. cellulose, hemicellulose (in particular xylan), and lignin. Furthermore, the

current industrial exploitation of lignocellulose from fast growing fibre crops (such as hemp) is highlighted. We conclude this review by suggesting approaches for further research to fill gaps in our current knowledge and to highlight the potential of biotechnology and bioengineering in improving both biomass biosynthesis and degradation.

**Frontiers in Microbiology**; Larisa Cortes-Tolalpa, Joana F. Salles, Jan Dirk van Elsas disclose Lignocellulosic biomass (LCB) is an attractive source of carbon for the production of sugars and other chemicals. Due to its inherent complexity and heterogeneity, efficient biodegradation requires the actions of different types of hydrolytic enzymes. In nature, complex microbial communities that work efficiently and often synergistically accomplish degradation. Studying such synergisms in LCB degradation is fundamental for the establishment of an optimal biological degradation process. Here, we examine the wheat straw degradation potential of synthetic microbial consortia composed of bacteria and fungi. Growth of, and enzyme secretion by, monocultures of degrader strains were studied in aerobic cultures using wheat straw as the sole carbon and energy source. To investigate synergism, co-cultures were constructed from selected strains and their performance was tested in comparison with the respective monocultures. In monoculture, each organism – with a typical enzymatic profile – was found to mainly consume the cellulose part of the substrate. One strain, *Flavobacterium ginsengisoli* so9, displayed an extremely high degradation capacity, as measured by its secreted enzymes. Among 13 different co-cultures, five presented synergisms. These included four bacterial bicultures and one bacterial–fungal triculture. The highest level of synergism was found in a *Citrobacter freundii/ Sphingobacterium multivorum* biculture, which revealed an 18.2-fold increase of the produced biomass. As compared to both monocultures, this bacterial pair showed significantly increased enzymatic activities, in particular of cellobiohydrolases, mannosidases, and xylosidases. Moreover, the synergism was unique to growth on wheat straw, as it was completely absent in glucose-grown bicultures. Spent supernatants of either of the two partners were found to stimulate the growth on wheat straw of the counterpart organism, in a directional manner. Thus, the basis of the LCB-specific synergism might lie in the specific release of compounds or agents by *S. multivorum* w15 that promote the activity of *C. freundii* so4 and vice versa.

None of the prior art indicate above either alone or in combination with one another disclose what the present invention has disclosed. The objectives of present invention are

**i.** To utilize the lignocellulosic field crop residues for composting and accelerate rate of biomass decomposition with the help of different amendments.

**ii.** To discover effective decomposer for lignocellulosic biomass decomposition in the limited time.

**SUMMARY OF INVENTION**

Like many other agricultural wastes, the wheat straw also largely consists of lignified cell wall materials- cellulose, hemicellulose and lignin being the three structural components. Out of these, cellulose is most predominant constituent followed by hemicellulose and lignin (Singh *et al*., 2015b; 2016). The lignin together with the hemicellulose, encrust the cellulose chains forming a barrier which prevents wetting and access of cellulose-degrading enzymes (Krik and Haskin, 1973). To utilize the cellulose or hemicellulose components, this association must break first. However, to access the fermentable sugars of cellulose and hemicellulose, the highly recalcitrant lignin must be hydrolysed and removed. Usually thermochemical method used to treat the lignocellulosic biomass, it's very costly and harsh (Fisher and Fong, 2014). Biodegradation of lignin represents a key step for carbon recycling in land ecosystem, as well as for industrial utilization of plant biomass (Duenas and Martinez, 2009). Humification is a process of conversion of dead organic matter (leaves, twigs, etc.) into humus by the action of decomposers such as bacteria and fungi. Humification affects soil property and nature (Datta *et al*., 2017). Hence lignin biodegradation increases soil fertility.

There is increasing emphasis on the proper management of the straw for which various strategies have been proposed from time to time; a number of these strategies involved its decomposition by microorganisms (Charaya, and Mehrotra, 1998; Singh *et al.*, 2015a). Therefore, it would be worthwhile using selected micro-organisms for loosening the lignocellulosic bond (Eggins and Seal, 1978). There are large number of reports are available concerning biochemical changes during the decomposition of wheat straw (Charaya, 1985) as such; and of wheat stems and leaves separately (Robinson *et al*., 1994). **No such study seems to have been undertaken with respect to chaff. No comparable studies are available for separate components of wheat crop residues (internodes, leaves and chaff)**. The additions of phosphorus and nitrogen have been reported to enhance the rate of decomposition (Charaya *et al*., 1989; Singh, *et al*., 1995; Singh and Charaya, 2010). Therefore, it would be worthwhile to study the effect of addition of phosphorus and nitrogen containing compounds singly and in combination on the *in vitro* decomposition of straw and its components by

*Stachybotrys atra* and *Trichoderma lignorum* isolated from these substrates decomposing naturally in the field.

It is clear from above discussion that despite number of studies already undertaken on the fungal decomposition of wheat straw and its components, several aspects of this process still lie unresolved. The present study was therefore, undertaken to find out strategies to accelerate the decomposition of different components of wheat crop residues as lignin, is the hardest polymer to be decomposed.

The present invention can be claimed in following manner;

1. A method for acceleration of lignocellulosic biomass degradation comprising the steps of;

a. Separating wheat straw into internodes, leaves, chaff and straw and cutting in small pieces and air-dried;
b. Putting 2gm of each part into conical flasks;
c. Plugging the flasks and autoclaved at 15lbs/square inch pressure for 30 minutes and then again after 24hrs for 30 minutes;
d. Adding 10ml of Nitrogen source + phosphorous source + inoculum of Microorganisms to the flask so as to obtain desired product.

2. The method as claimed in claim 1, wherein said Nitrogen source is urea solution (0.21%).
3. The method as claimed in claim 1, wherein said phosphorous source is single super phosphate solution (1%).
4. The method as claimed in claim 1, wherein microorganisms used are *Stachybotrys atra* and *Trichoderma lignorum* ( 1% suspension).
5. The method as claimed in claim 1, wherein amount of microorganism used is 1% suspension of cultured growth.
6. The method as claimed in claim 1-5, wherein said method is used to decompose valuable agricultural biomass within the limited time on large scale also.

## DETAILED DESCRIPTION OF INVENTION

**Methodology:** To find out the effect of urea (nitrogen) and Single Super Phosphate (SSP) on the ability of the selected fungi- *Stachybotrys atra* and *Trichoderma lignorum* to decompose different compounds of wheat crop residues *in vitro* (Singh *et al*., 2015c). The wheat internodes, leaves, chaff as well as straw were cut in to small pieces and were air-dried.

About 2g of wheat internodes were placed in 27 conical flasks each (250ml). Same procedure was done with wheat leaves, straw and chaff. Thus, total 108 flasks were used. The flasks were plugged and autoclaved at 15lbs/square inch pressure for 30 minutes and then again after 24hrs for 30 minutes. Each set of 27 flasks was treated as given below:

**a.** 10ml of sterilized water were added to each of the three flasks.

**b.** 10ml of sterilized water + inoculum of *Stachybotrys atra* (1% suspension) were added to each of set of 3 flasks.

**c.** 10ml of sterilized water + inoculum of *Trichoderma lignorum* (1% supension) were added to each of set of 3 flasks

**d.** 10ml of urea solution (0.21%) + inoculum of *Stachybotrys atra* were added to each of a set of 3 flasks.

**e.** 10ml of urea solution (0.21%) + inoculum of *Trichoderma lignorum* were added to each of a set of 3 flasks.

**f.** 10ml single super phosphate solution (1%) + inoculum of *Stachybotrys atra* were added to each of a set of 3 flasks.

**g.** 10ml single super phosphate solution (1%) + inoculum of *Trichoderma lignorum* were added to each of a set of 3 flasks.

**h.** 10ml of urea solution (0.21%) + single super phosphate solution (1%) + inoculum of *Stachybotrys atra* were added to each of a set of 3 flasks.

**i.** 10ml of urea solution (0.21%) + single super phosphate solution (1%) + inoculum of *Trichoderma lignorum* were added to each of a set of 3 flasks.

**Note:** To prepare 1% suspension of inoculum, 1gm. cultured growth were collected from petridishes with the help of spatula and mixed in 100 ml. aquous solution of Nitrogen (0.21%) and Phosphorus (1%).

The urea solution (0.21%) was used because *Stachybotrys atra* and *Trichoderma lignorum* can grow at very low nitrogen level. These fungi were earlier shown to exhibit significant increase in the rate of decomposition of paddy straw amended with 0.21% urea solution (Charaya, 1985; Jain, 1989). The single super phosphate solution (1%) amendment was used for cereal straw treatment under Karnal process (Ramachandran, 1989).

**Results** (Table 1 and Table 2)

**Table 1.** Loss in lignin fraction (% initial dry wt.) in unamended wheat crop residues and those amended with urea and single super phosphate or both, and inoculated with *Trichoderma lignorum.*

| Wheat crop residues | Lignin fraction | | | | |
|---|---|---|---|---|---|
| | Fresh biomass | Control (W+$F_1$)* | Urea + $F_1$ | SSP** + $F_1$ | Urea + SSP + $F_1$ |
| **Internodes** | 13.17 | 12.17 | 11.94 | 12.07 | 11.30 |
| **Leaves** | 4.85 | 3.14 | 3.02 | 3.10 | 2.46 |
| **Chaff** | 3.68 | 1.89 | 1.78 | 1.82 | 0.77 |
| **Mixed (Straw)** | 7,55 | 4.29 | 3.28 | 3.17 | 2.96 |

*W, Water; $F_1$, *Trichoderma lignorum*. **SSP, Single Super Phosphate.

**Table 2.** Loss in lignin fraction (% initial dry wt.) in unamended wheat crop residues and those amended with urea single super phosphate or both, and inoculated with *Stachybotrys atra.*

| Wheat crop residues | Lignin fraction | | | | |
|---|---|---|---|---|---|
| | Fresh biomass | Control (W+$F_2$)* | Urea + $F_2$ | SSP** + $F_2$ | Urea + SSP + $F_2$ |
| **Internodes** | 13.17 | 11.82 | 11.30 | 4.53 | 3.76 |
| **Leaves** | 4.85 | 2.93 | 2.19 | 2.78 | 2.61 |
| **Chaff** | 3.68 | 2.79 | 2.64 | 2.66 | 2.35 |
| **Mixed (Straw)** | 7,55 | 6.26 | 6.18 | 5.23 | 5.18 |

*W, Water; $F_2$, *Stachybotrys atra*. **SSP, Single Super Phosphate.

The findings were evaluated and validated by the application of numerical parameter ANOVA and found to be significant ($p$ 0.05 and 0.005 for treatment and substrate respectively).

The study of tables no.1 and 2 reveals that the treatments with urea and Single Super Phosphate or the combination of both of these elicited positive response with respect to the decomposition of the substrates by the selected fungal species- *Stachybotrys atra* and *Trichoderma lignorum* .There were significant differences in the responses from treatment to treatment; and from substrate to substrate.

The crop residues inoculated with *Trichoderma lignorum,* the addition of urea resulted in greater loss of lignin except in case of straw, where addition of SSP was found to be more effective for the degradation of lignin. In the case of the internodes and straw inoculated with *Stachybotrys atra* also, the addition of SSP resulted in greater decomposition of lignin than

the addition of urea. However, urea was found to be more effective in the case of leaves and chaff inoculated with *Stachybotrys atra*. The very strong promoting effect of SSP on lignin decomposition was observed in the internode inoculated with *Stachybotrys atra.* The combination of urea and SSP was always more effective than either urea or phosphorus alone in all the cases- the combined effect in many cases exceeding the additive effect.

**WE CLAIM;**

1. A method for acceleration of lignocellulosic biomass degradation comprising the steps of:

a. Separating wheat straw into internodes, leaves, chaff and straw and cutting in small pieces and air-dried;

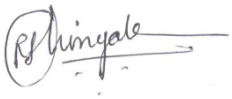
b. Putting 2gm of each part into conical flasks;

c. Plugging the flasks and autoclaved at 15lbs/square inch pressure for 30 minutes and then again after 24hrs for 30 minutes;

d. Adding 10ml of Nitrogen source + phosphorous source + inoculum of Microorganisms to the flask so as to obtain desired product.

2. The method as claimed in claim 1, wherein said Nitrogen source is urea solution (0.21%).
3. The method as claimed in claim 1, wherein said phosphorous source is single super phosphate solution (1%).
4. The method as claimed in claim 1, wherein microorganisms used are *Stachybotrys atra* and *Trichoderma lignorum*.
5. The method as claimed in claim 1, wherein amount of microorganism used is 1% suspension of cultured growth.
6. The method as claimed in claim 1-5, wherein said method is used to decompose valuable agricultural biomass within the limited time on large scale also.

Dated this 24 th day of October, 2018



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Authorized Agent for the Applicant

## ABSTRACT

### A METHOD FOR ACCELERATION OF LIGNOCELLULOSIC BIOMASS DEGRADATION USING MICROORGANISMS

This invention relates to a method for acceleration of lignocellulosic biomass degradation using microorganisms. Lignin is the second most abundant aromatic biopolymer next to cellulose constituent of cell wall of vascular plants, where it acts as a structural component of support and conducting tissue. It is recalcitrant to degradation, and creates a barrier towards enzymatic attack by any microbes. It has been identified in primitive groups of plants such as ferns, club mosses and gymnosperms but absent in bryophytes and lower plants. To improve the processing of lignocellulosic feed stocks, humic compound in soil and $CO_2$ Concentration in the environment, it's required to develop eco-friendly strategies. Lignin degradation has found in nature through the lignolytic enzymes of microbes. In the present study we discuss about the different components of wheat crop residues *viz*. internodes, leaves. chaff and combined straw, their biochemical nature and *in vitro* decomposition by *Stachybotrys atra* and *Trichoderma lignorum* fungi. Authors focus on the effect of urea and single super phosphate on degradation of lignin through these fungi found in the wheat crop residues and soil that are capable of producing lignolytic enzymes, which in turn release lignin fractions in soil, hence increase soil fertility through humification. Studies reveal that the treatments with urea and Single Super Phosphate or the combination of both of these elicited positive response with respect to the decomposition of the substrates by the selected fungal species.