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Bioconversion of Waste Paper into Bio-Ethanol by Co-Culture of Fungi Isolated from Lignocellulosic Waste

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ABSTRACT

In present study, examined the relative potentials of waste paper as microbial substrate for sacchrification using wild strains of Aspergillus sp., Penicillium sp. and Trichoderma sp. isolated from different degraded cellulose waste material. Office paper showed the strongest susceptible for hydrolysis by Aspergillus sp. (3.76mg/ml) followed Trichoderma sp. (3.26mg/ml) and Penicillium sp. (2.6mg/ml), On the other hand, news paper and filter paper showed least reduction of sugar by tested fungi. Saccharification of fungal cultures were mixed and incubated with all cellulosic materials. An equal mixture 2 (2:2:2) resulted in the highest increase in saccharification with office paper, filter paper as compared to mixture 1. The highest cellulase produced from office paper by Penicillium sp. followed by news paper and filter paper and highest cellulase also produced from office paper by mixed culture 2 followed by filter paper and news paper. The highest rate of protein were produced from news paper by Penicillium sp. and then Aspergillus sp., Trichoderma sp. and the highest rate of protein were also produced from news paper by mixed culture 2 than mixed culture 1 followed by office paper and filter paper. Office paper selected for the production of bioethanol for highest sacchrification by A. niger. The maximum amount of Ethanol estimated using Potassium dichromate method was 1.3ug/ml which was produced from 5gm office paper saccharified by Aspergillus sp.

Key words: *Waste paper, Saccharification, co-culture, Fungus, lignocellulosic waste*

INTRODUCTION

Environmental conservation and limiting of waste material by recycling it to useful product are currently topical aspects that should concern all sectors of the global community[1]. Fuel deficiency is a global issue due to exhaustion of fossil fuel and growing climate change[2]. This limitation along with the problem of Green House Gas (GHG) emissions leads findings for alternative energy that are environmentally and commercially feasible. Every day we are dumping lot of cellulosic waste materials into the environment[3].

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In order to overcome this, different types of techniques have been invented for the possible conversion of cellulosic waste material into glucose for the ethanol production, as an alternative way for fuel conservation[4]. The French chemist, Henri Braconnot, was the first to discover that cellulose could be hydrolyzed into sugars by treatment with sulfuric acid in 1819.The hydrolyzed sugar could then be processed to form ethanol through fermentation. The first commercialized ethanol production began in Germany in 1898, where acid was used to hydrolyze cellulose. In the United States, the Standard Alcohol Company opened the first cellulosic ethanol production plant in South Carolina in 1910. Later, a second plant was opened in Louisiana.

Lignocellulosic materials such as Waste Paper in large quantities are available in many countries with various climatic conditions, making them suitable and potentially cheap feedstock for sustainable production of fuel ethanol. Such materials are abundant and competitive in price with petroleum, and cellulosic biomass can provide a sustainable resource that is truly unique for making organic product[3].

Paper materials can be classified as multipurpose commodities as they are a medium to present information, can act as protective agents, serve as packaging materials, to name but a few of their uses. At present used paper products are recycled to a limited extent but eventually all are dumped or burnt, thus aggravating even further environmental pollution. The enzymatically catalyzed bioconversion of organic based waste such as used paper materials to soluble sugar like glucose is therefore an important step in the production of bioethanol and can be performed in an environmental- friendly way.

Bioethanol produced by fermentation of lignocellulosic biomass (second generation bioethanol), from paper material shows many potential advantages in comparison to sugar starch derived bioethanol (first generation bioethanol), from both energetic and environmental point of view. Lignocellulosic biomass such as paper material is the leafy or woody part of plants principally composed of the compounds cellulose, hemicelluloses, and lignin. Cellulose, a primary component of plant cell walls, is made up of long chains of glucose linked by (β-1, 4) - glycosidic bonds. These chains are arranged in bundles associated by means of hydrogen bonds forming a primary component of paper products. Cellulose molecules are interlinked by another molecule, hemicelluloses primarily composed of xylose. The structural strength of the plant and paper material is caused by lignin that exhibits a very complex molecular structure. Newspaper contains almost 61% cellulose and 16% hemicelluloses, Office paper contains 90% cellulose and Whatmann cellulose filters are manufactured from high-quality cotton linters which have been treated to achieve a minimum alpha cellulose content of 98% making these material good sources of sugars.

Cellulase, a group of hydrolytic enzymes which hydrolyze the β - glycosidic bonds of native cellulose and related cellooligosaccharides, is the key enzyme of potential use for industrial saccharification of cellulosic materials into simple sugars. Cellulases are mostly manifested in fungi and bacteria[5]. Filamentous fungi have been reported to be good producers of lignocellulolytic enzymes from industrial point of view due to extracellular release of the enzymes, higher yield compared to yeast and bacteria, and also the production of several auxiliary enzymes that are necessary for debranching of substituted polysaccharides[6].

Among the cellulolytic fungi, the genera *Trichoderma* sp. *Aspergillus* sp. and *Penicillium* sp. are notable cellulase producers. In present study, examined the relative potentials of waste paper as microbial substrate for sacchrification using wild strains of *Aspergillus* sp., *Penicillium* sp. and *Trichoderma sp.* isolated from different degraded cellulose waste material.

Trichoderma sp. is highly efficient producers of many extracellular enzymes. Since many species of *Trichoderma* are strongly cellulolytic i.e. they are capable of degrading cellulose since they produce large quantities of the enzyme cellulose. They are very common on cellulosic materials including decaying wood, wood products[7].

Aspergillus sp. is common mould is involved in many industrial processes. These organisms secrete acids and enzymes into the surrounding environment, breaking down polymeric molecules down into simpler once. For *Aspergillus* the process of degradation is the means of obtaining nutrients. When these moulds degrade human made substrates the process usually is called biodeterioration. Paper is particularly vulnerable to *Aspergillus* degradation[8].

Alexander Fleming was the first suggest the *Penicillium* mould in 1928. The name *Penicillium* comes from the resemblance of the spore producing structure (conidiophores) of the fungus to a paintbrush (Penicillus is the Latin word paintbrush). They are found in soil decaying vegetation such as decaying wood. The wide range of extracellular enzymes produced by species of *Penicillium* play an important role in the microbiological breakdown of cellulosic material. Notable examples of hydrolyses of *Penicillium* include various cellulolytic enzymes and other polysaccharases, such as α- and β- gluanases, hemicellulases, and pectin enzymes, together with a variety of lipases and proteolytic enzymes.

The bioconversion of waste paper into bioethanol is carried out under two step procedure: 1) The saccharifiction (hydrolysis) of cellulosic material such as waste paper into fermentable sugar. 2) Conversion of fermentable sugar into bioethanol .

The most commonly used microorganism for ethanol production solely utilizes saccharified sugar monomers for further fermentation purpose. The general requirement on an organism to be used in fermentation i.e. ethanol production is that it should give a high ethanol yield, a high productivity and be able to withstand high ethanol concentrations in order to keep distillation costs low. In addition to these general requirements, inhibitor tolerance, temperature tolerance and the ability to utilize multiple sugars. Tolerance towards low pH-values will minimize the risk of contamination. The work-horse in cellulose based ethanol production is the common Baker's yeast *Saccharomyces cerevisiae*[9].

MATERIALS AND METHODS

1. **Isolation of Fungi:**

Collection of Samples: - Samples were collected from sawdust, straw dust and decayed wood particles from the wood yard. Samples were collected into sterile containers and stored separately.

Initial culturing: - Potato dextrose agar (PDA), Czapek dox agar (CDA) and Sabouraud dextrose agar (SDA) medium were prepared and sterilized.T his PDA, CDA, and SDA were poured into previously autoclaved petriplate. Media were allowed to solidify. After solidification, samples were cultured by sprinkle method. Decayed wood particle were crushed properly by mortal- pestle. Crushed particle were sprinkled on the medium and petriplates were incubated in a dark place at room temperature. Visible colonies were observed after 4 days of incubation.

Identification of Fungi:-

In order to identify the fungal colonies, colony colour, shape, border and spots (if the spores are available) were recorded. The morphology of fungi was identified on the basis of microscopic examination. Lacto phenol cotton blue staining used for the identification of fungi. All the cultures were identified on the basis of the morphological characteristics and they were maintained on Potato dextrose agar (PDA) slants and stored at 4°C for further study and the slants were subcultured once a month.

2. **Collection and Preparation of Cellulosic Material:**

Filter paper (Whatman no 1), newspaper, office paper were used as substrate for saccharification and cellulase enzyme production. The materials were cut into small pieces Grinding was done using Mixer Grinder. The grind paper was used microbial substrates.

3. **Saccharification of waste paper :-**

Five grams of waste paper was taken in individual Erlenmayer flasks (250 ml) containing 100 ml distilled water. The flasks

were plugged with cotton and autoclaved at 121ºC for 15mins. 1 ml of fungal suspension from seven days old cultures of the selected fungi were used as inoculum for monoculture experiment. For co-culture studies, the spore suspension was taken as 1:1:1 as well as 2:2:2 ratios as consortia. The conical flasks were incubated at 28 ± 2 ºC for a period of 20 days in the culture room. Saccharification percentage was calculated as follows:

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugar (mg/ml)} \times 0.9}{\text{Substrate (mg/ml)}} \times 100$$

At each 5 days interval of study, some content of each flask was withdrawn filtered and followed by the centrifuged at 10,000 rpm for 5 min. Supernatant was used in the analysis for measuring cellulase activity, protein content and total reducing sugars.

4. **Determination of cellulase activity, total reducing sugars and protein concentration:**

Cellulase activity:- Cellulase activity was assayed by using Carboxymethyl-cellulose (CMC) as substrate. The reaction mixture contained 1 ml of 1.0% (w/v) CMC in 0.1M solution of sodium acetate buffer, pH 5.0, and 0.5 ml of the cell-free culture supernatant.The mixture was incubated at 50°C with for 30 to 60 minutes.The reducing sugar released by the enzyme was measured as glucose equivalent using dinitrosalicyclic acid reagent. A unit of activity was defined as the amount of enzyme required to liberate 1 mol of glucose per minute under the assay conditions.

5. **Reducing sugar assay: -**

Reducing sugars were determined by DNS method using glucose as standard. Place 1 ml sample in a test tube and add 3 ml DNS reagent. Place in boiling water for 5 minutes. Cool under tap water to room temperature. Measure the optical density of samples at 540 nm. The absorbance of the sample tube (corrected if necessary by subtraction of the enzyme blank) into glucose production during the reaction using a glucose standard curve.

6. **Protein assay: -**

The Protein content of the crude enzyme preparation was determined by the method of Lowry et al. using bovine serum albumin (BSA) as standard. Place 1 ml sample in a test tube and add 1 ml distilled water. Add 5 ml of Reagent I (**A**. 2% Na_2CO_3 in 0.1 N NaOH, B. 1% Na K Tartrate in H_2O, **C**. 0.5% $CuSO_4$.5 H_2O in H_2O D**. Reagent I**: 48 ml of **A**, 1 ml of **B**, 1 ml **C**) and incubate for 10 minutes. After incubation add 0.5 ml Reagent II (**1 part** Folin- Ciocalteau [2N]: 1 part water. Wait 30 minutes at room temperature and take the absorbance at 660 nm. Estimate the amount of protein present in the given sample from the standard graph.

7. **Optimization of saccharification of waste paper:**

To select the suitable temperature, pH, incubation period and substrate concentration for saccharification of waste paper by mono and mixed fungal strain were cultivated with varying temperature of 25°C-35°C, pH range 5-7, incubation period range of 5-20 days, and substrate concentration 3%,5%,7% by keeping all other parameters constant for 10 days. After 10 days, the entire content of the each test tube was withdrawn filtered and followed by the centrifuged at 10,000 rpm for 5 min and supernatant was used in the analysis for measuring total reducing sugars.

8. **Bioethanol Production:**

The reducing sugar was obtained with mono and mixed culture of fungi from waste paper which was used as substrate. The maximum reducing sugar produced from paper used for bioethanol production. After 15 days, entire content of the flask (those flasks which contain maximum reduced sugar) was withdrawn and

filtered by filter paper and then by Whatman No 1 filter paper. The filtrate was collected in the sterilized conical flask. The flasks were covered with foil and autoclaved at 80ºC for 10mins. After sterilization flasks were cooled to room temperature. 1 ml of activated yeast (*Sacchromyces cerevisiae*) was inoculated in conical flask containing fermentable sugar. All flasks were tightly packed and incubated at 25-28 ºC for 6 days.

9. **Estimation of Bioethanol by Biochemical method:**

For the quantity estimation of bioethanol, the standard curve was formed by using the pure ethanol with the series of 2-10 microliters of absolute ethanol was taken in different test tubes and the volume was made up to 1ml by adding distilled water in each test tube. Add 1 ml of Acid dichromate reagent. Examine the bioethanol production by Jones reagent [$K_2Cr_2O_7$ + H_2SO_4], 3ml of test sample was taken in the test tube and add 5ml acid dichromate. All the test tubes were incubated at 50 ºC for 30 minutes. Ethanol was oxidized into acetic acid with potassium dichromate in the presence of sulfuric acid converted to blue green color. Green color indicates positive test The absorbance was measured at 600 nm by using a colorimeter. Estimate the amount of ethanol present in the given sample from the standard graph[5,10].

RESULT AND DISCUSSION

Saccharification of Waste Papers:

Fig. 1, 2, 3, & 4 reflects the saccharification of all paper materials by *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. as well as consortia of them. Office paper showed the strongest susceptible for hydrolysis by *Aspergillus* sp. (3.76mg/ml) followed *Trichoderma* sp. (3.26mg/ml) and *Penicillium* sp. (2.6mg/ml), On the other hand, news paper and filter paper showed least reduction of sugar by tested fungi. Whereas office paper showed higher reduction in sugar by fungi in the ratio of 2:2:2 (6.8mg/ml) followed by filter paper (3.26mg/ml) and news paper (0.96 mg/ml). Rahna K. Rathnan *et al*. showed that different rate of the saccharification for all paper materials by *P.citrinum, A. oryzae and T. viride* as well as mixture of them. Office paper showed the strongest susceptible for hydrolysis by *P. citrinum* followed by news paper, filter paper. But the highest reducing sugar produced by *A. oryzae* is from news paper followed by office paper, filter paper[5].

To increase degree of saccharification fungal cultures were mixed and incubated with all cellulosic materials. An equal mixture 2 (2:2:2) resulted in the highest increase in saccharification with office paper, filter paper as compared to mixture 1. Beside this an equal mixture 1 (1:1:1) showed in the highest increase in saccharification with news paper as compared to mixture 2. While Rahna K. Rathnan *et al*. showed that different tendencies when organisms were inoculated at equal concentrations and a ratio mixture of (2:2:2) resulted in the strongest increase of saccharification with all substrates relative to the individual action of fungi[5]

These organisms produce and secrete cellulose enzyme for the degradation of cellulose and release glucose. The highest cellulase produced from office paper by *Penicillium* sp. followed by news paper and filter paper and highest cellulase also produced from office paper by mixed culture 2 followed by filter paper and news paper. (Fig.3). When compared with previous research of Mekala et al. showed that highest cellulase production by *Trichoderma reesei*[11].

The highest rate of protein were produced from news paper by *Penicillium* sp. and then *Aspergillus* sp., *Trichoderma* sp. and the highest rate of protein were also produced from news paper by mixed culture 2 than mixed culture 1 followed by office paper and filter paper. (Fig.4)

Optimization of saccharification of waste paper:

The rate of saccharification can be increased by optimizing conditions for microbial growth. Here the conditions were optimized for News

paper. During growth of fungi on media containing waste paper (News paper), they utilized cellulose in paper as Carbon source. The process of saccharification of paper can be increased by optimizing the condition like Temperature, pH, incubation time and substrate concentration etc. The saccharifying media was prepared and cultures were inoculated as mono and mixed in 1:1:1 and 2:2:2 ratios.

Fig. 5 shows the effect of incubation time on saccharification of paper waste by mono and mixed fungal cultures on media containing waste paper as carbon source. The saccharification increased with the increase in incubation period and reached maximum after 15 days of incubation for monocultures and mixed cultures. Further increase in the incubation period however, resulted in the gradual decrease in the saccharification. Therefore, incubation period of 15 days for monocultures and mixed cultures was found to be optimal for saccharification. Kund & Sing showed that optimization of the time course is of prime importance for saccharification by fungi. The decrease in the saccharifiction by monocultures and mixed cultures after 10 to 15 days of incubation period might be due to the depletion of the nutrients and accumulation of other byproduct or catabolic repression of cellulase enzyme by released glucose[12].

The effect of incubation temperature (25-35ºC) on the saccharification of waste paper by mono and mixed cultures of fungi is shown in (Fig.6). There was a gradual increase in saccharification as the temperature was increased. But it showed maximum yield at 25 ºC. As the temperature was further increased, there was a gradual reduction in the saccharification. While Mekala et al showed that higher temperature denatures the saccharifying enzymes mainly cellulase. High temperature may also lead to inhibition of microbial growth. They showed that cellulase production and thus saccharification was maximum in flasks incubated at 33 ºC and decreased with high temperature[11].

The effect of initial pH (5.0-7.0) of the culture medium on the saccharification by fungal cultures was studied (Fig.7). At the pH value of 5, there was very little saccharification of paper by mono cultures and mixed culture 2. However it started to increase as the initial pH of the growth medium was increased and reached maximum at pH 6 for monocultures, pH 5.5 for mixed culture1. But highest saccharification of paper by mixed culture 2 at pH 5. Further increase in pH resulted in a gradual reduction of saccharification by the fungi. Hence, pH of 6 was optimized for the maximum saccharification by monocultures and pH 5.5 for mixed culture 1 and pH 5 for mixed culture 2. Rahna K. Rathnan *et al*. showed that the initial pH of the growth medium was increased and reached maximum at pH 5.5. Further increase in pH resulted in a gradual reduction of saccharification by the organism hence; pH of 5.5 was optimized for the maximum saccharifiction by fungi[5] and Chandra et al showed that after pH value of 5.5, the production of cellulase decreased which might be due to the fact that cellulase are acidic proteins and are greatly affected by the neutral pH values[13].

Effect of substrate concentration on saccharification of paper waste by mono and mixed cultures were carried out (Fig. 8). It was observed that the highest saccharification was carried out 3% of substrate for *Penicillium* sp., *Trichoderma* sp. and mixed cultures 1&2 where as *Aspergillus* sp. showed highest saccharification obtained at 5%. This may be due to *Aspergillus* sp. produced highest amount of cellulase enzyme hence; they can convert high concentration of paper in to sugars. While Sivakumaran showed that highest saccharification was observed in *Trichoderma* sp. which is 0.857%, followed by *Aspergillus* sp[14].

Fig. 1: Total reducing sugar produced from waste paper at the optimum ratio mixture of *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. relative to the monoculture action

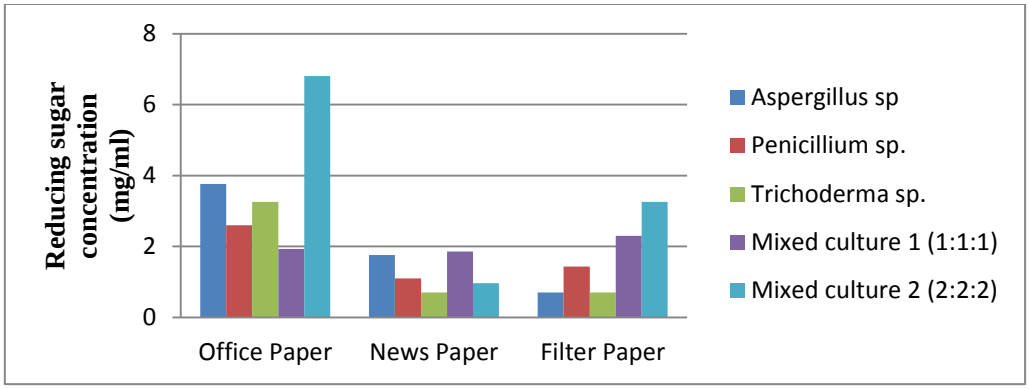


Fig. 2: Saccharification (%) of waste papers by monoculture and mixed cultures of cellulolytic fungi

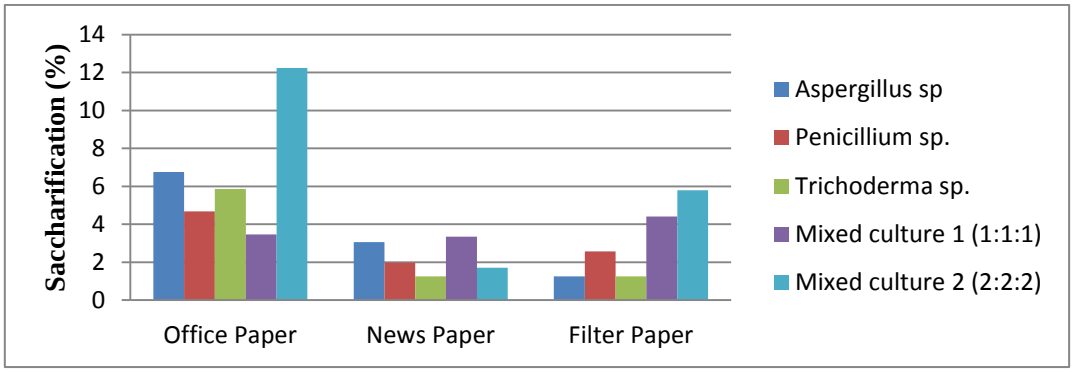


Fig. 3: Cellulase enzyme produced during saccharification of different waste papers by monoculture and mixed cultures of cellulolytic fungi

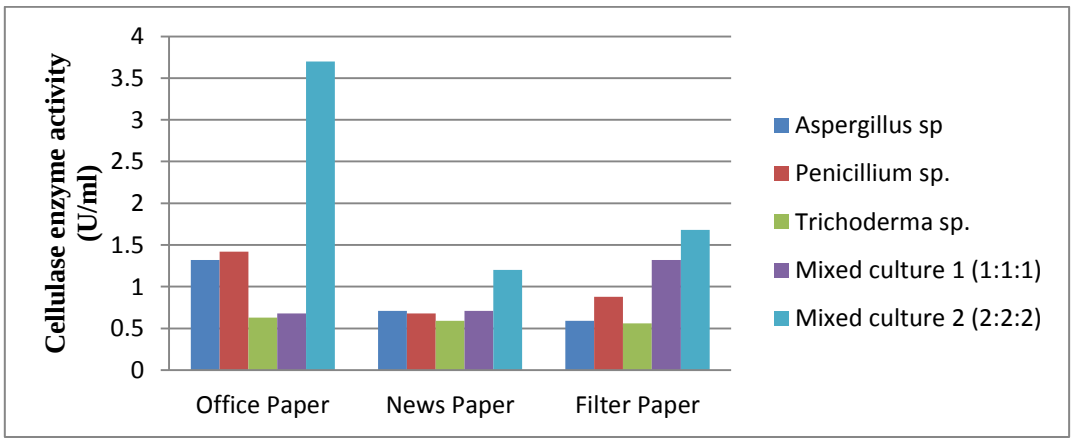


Fig. 4: Protein produced during saccharification of different types of waste paper by monoculture and mixed cultures of cellulolytic fungi

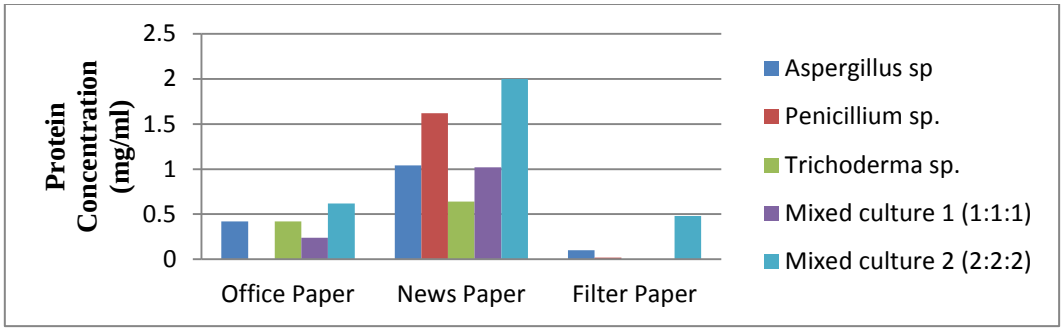


Fig. 5: Effect of incubation time on saccharification of waste paper (News paper) by *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp. and Mixed culture 1&2

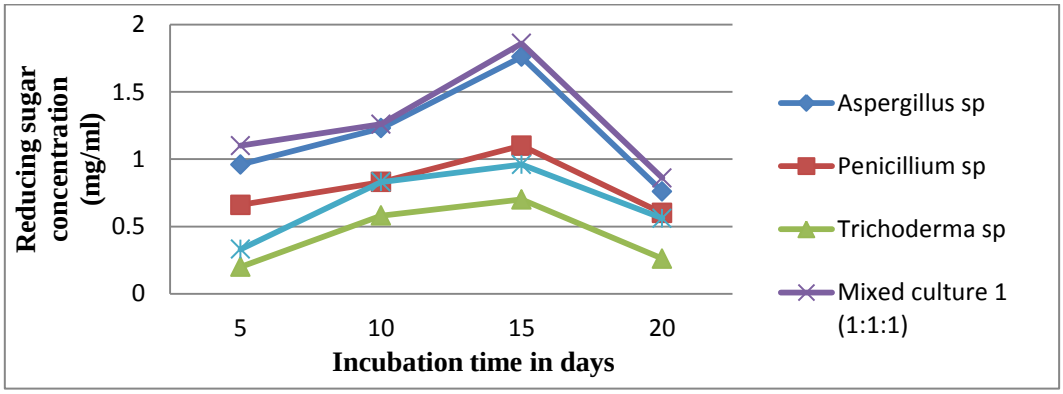


Fig. 6 Effect of Temperature on saccharification of waste paper (News paper) by *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp. and Mixed culture 1&2

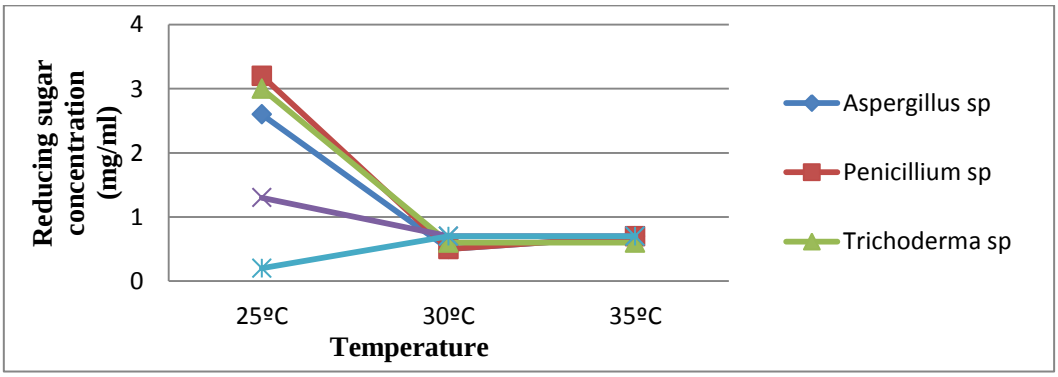


Fig. 7: Effect of pH on saccharification of waste paper (News paper) by *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp. and Mixed culture 1&2

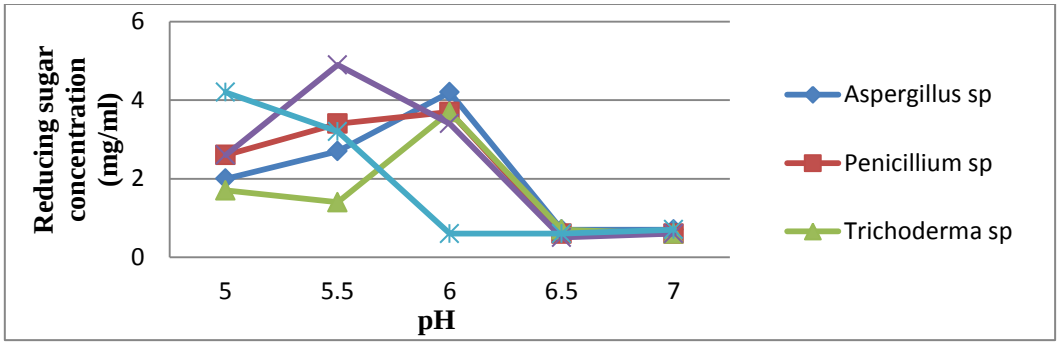


Fig. 8: Effect of substrate concentration on saccharification of waste paper (News paper) by *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp. and Mixed culture 1&2

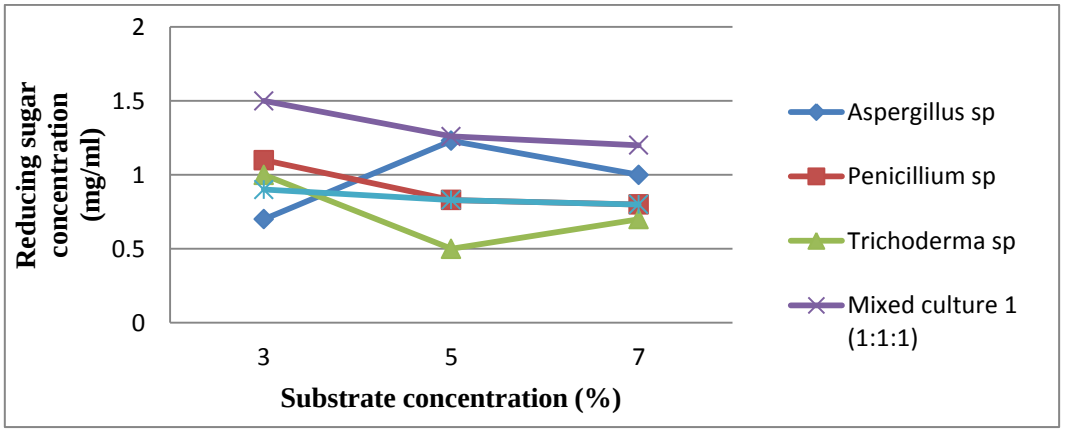
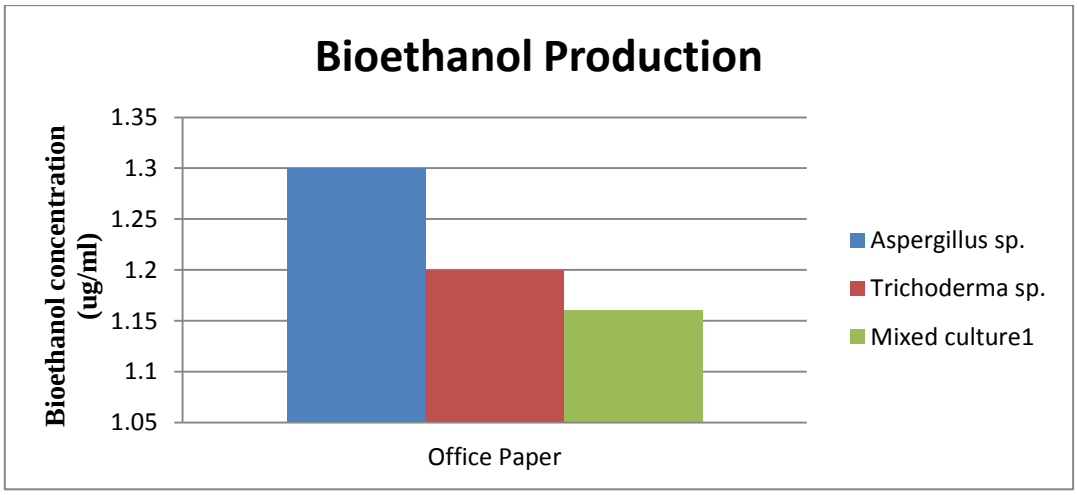


Fig. 9: Bioethanol produced from maximum reducing sugar obtained during saccharification of different waste papers by monoculture and mixed cultures of celluloytic fungi



In present research work, from above findings it concluded that strains *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp showed saccharification of paper. Among the tested substrates, office paper was the best inducer of cellulase enzyme and found to be the best for releasing maximum reducing sugar and saccharification. News paper was second inducer of cellulase enzyme and found to be the best for releasing maximum reducing sugar and saccharification. The maximum saccharification was obtained with mixed cultures of fungi 1:1:1 ratio and temperature 25 ºC, pH5.5, incubation time 15 days and substrate concentration 3%. The maximum reducing sugar produced from waste paper used for Bioethanol production.

Estimation of Bioethanol by Biochemical method

The enzymatic hydrolysis of different lingo-cellulosic waste (Office paper, News paper & Filter paper) to convert into reducing sugar had shown positive results. The maximum amount of sugar was produced from hydrolyzed office paper. The released sugars were fermented by *S. cerevisiae* for 6 days for the ethanol production. After 6 days Ethanol estimation was done by Potassium dichromate method after bioethanol was successfully produced from Office paper saccharified by *Aspergillus* sp. followed by *Trichoderma* sp. and mixed culture 1(1:1:1 ratio of three cultures).

The maximum amount of Ethanol estimated using Potassium dichromate method was 1.3ug/ml which was produced from 5gm Office paper saccharified by *Aspergillus* sp. (Fig. 9) Thus; waste paper can be economically used for ethanol production. This research is meaningful both in the conversion and utilization of renewable biomass, and in the reduction of environmental pollution.

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