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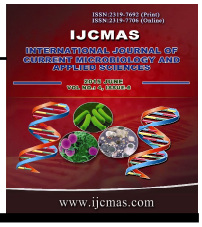

Original Research Article

Bioconversion of Waste Paper by Co Cultures of Fungi Isolated from Lignocellulosic Waste

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Keywords

Waste paper, Saccharification, Mixed culture, Optimization

A B S T R A C T

Waste papers were incubated with cellulolytic fungi *Penicillium citrinum*, *Aspergillus oryzae* and *Trichoderma viride* to hydrolyze its cellulose component and mixtures of these cultures were applied to increase the extent of degradation. Organisms were inoculated at equal concentrations and a ratio mixture of 2:2:2 resulted in the strongest increase of saccharification relative to the individual action of fungi on all substrates. Office paper resulted in the highest increase of saccharification at this microbial combination relative to the action of monocultures on saccharification of paper. *P. citrinum* showed the strongest hydrolysis on all substrates followed by *A. oryzae* and *T. viride*. The condition like temperature, pH, substrate concentration and incubation time for saccharification was optimized. The maximum saccharification was obtained with mixed cultures of fungi in 2:2:2 ratio and temperature 30^{o}C, pH 5.5, incubation time 10 days and substrate concentration 5%.

Introduction

The recycling of cellulosic waste is important not only to conserve the environment but it could also be applied to develop alternative and renewable energy resources. Cellulosic biomass constitutes the most abundant organic molecules on earth (Fan *et al.*, 1987). All cellulosic waste materials can be converted into commercially important products such as citric acid, ethanol, methane, glucose syrups and single cell proteins (Louime and Uckelmann, 2008). Bioconversion, particularly enzymatic hydrolysis, of these cellulosic materials into simple sugars, has been a subject of intensive research (Ahmadi *et.al*, 2010). The cellulose bioconversion would help to alleviate shortages in food and animal feeds and also reduce the problems of urban waste disposal and overdependence on fossil fuels.

Used paper materials constitute a major component of organic based waste dumped annually. The cellulose, polymer of glucose

is a major structural component of paper materials with its monomeric glucose units linked by means of b-1,4-glycosidic bonds. Cellulose is susceptible for acid- and cellulase catalyzed (Ja'afaru and Fagade,. 2007, Van Wyk and Botha, 1997.) hydrolysis producing reducing sugars such as glucose, but its association with hemi-cellulose and lignin results in a relative high resistance toward these ways of saccharification. The hydrolysis of cellulose is a complex process and attempts to improve it, receive currently attention (Baker *et al.,* 1998).

Cellulase, a group of hydrolytic enzymes which hydrolyze the B-glycosidic bonds of native cellulose and related cellooligosaccharides, is the key enzyme of potential use for industrial saccharification of cellulosic materials into simple sugars. Cellulase production was found to be the most expensive step, accounting for about 40% of the total cost, during the production of ethanol from cellulosic biomass. Cellulases are mostly manifested in fungi and bacteria (Chinedu *et al* 2010). Among the cellulolytic fungi, the genera *Trichoderma* sp., *Aspergillus* sp. *and Penicillium* sp. are notable cellulase producers.

In this study, we examined the relative potentials of waste paper as microbial substrate for saccharification using wild strains of *A. oryzae*, *P. citrinum* and *T. viride* isolated from different degraded cellulose waste material. Crystalline cellulose was used for comparative purposes to assess the relative effect of the various paper wastes on saccharification and cellulase production by the mono cultures and mixtures of cellulolytic fungi ***P. cirtinum***, ***T.viride*** and ***A. oryzae*** were used to increase degradation of cellulose present in filter paper, news paper, office paper and micro-crystalline cellulose.

Materials and Methods

A. Cellulosic materials

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.

B. Microbial strains

Fungi *P.citrinum NASC- 3, A.oryzae NASC - 2 and T.viride NASC- 6* were isolated from decayed lignocellulosic waste and they were identified by their morphological, colony and molecular characteristics. All the cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4 °C and the slants were subcultured once a month.

C. Saccharification of paper waste

Biodegradation of paper waste was studied in solid state in Erlenmeyer flasks (250 ml) using cellulolytic fungi isolated from natural sources. Five grams of paper waste containing 60 % moisture was taken in individual Erlenmeyer flasks (250 ml). The flasks were plugged with cotton and autoclaved at 121ºC for 15 mins. 1 ml of culture containing 2.7 X 10^7 spores /ml from seven days old cultures of the selected fungi was used as inoculum for monoculture experiments. For co culture studies, the spore suspension was taken as 1: 1: 1: as well as 2:2: 2 ratio as inocula. The conical flasks were incubated at 28 ± 2 ºC for a period of 30 days in the culture room. Separate flasks were maintained for studying the compositional changes in paper waste. At each 5 days interval of study, the entire content of each flask was withdrawn filtered and was used in the analyses for

measuring cellulase activity, protein content and total reducing sugars.

D. Determination of cellulase activity, total reducing sugars and protein concentration

Reducing sugars were determined with the DNS method (Miller *et al.,* 1960) using glucose as standard. For protein determinations BSA (fraction V, Sigma) was used as a standard (Lowry *et al.*, 1951).

Cellulase assay:

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 ^{0}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 1μmol of glucose per minute under the assay conditions.

Protein assay:

The Protein content of the crude enzyme preparations was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin (BSA) as standard.

E.Optimization of saccharification of paper

To select the suitable temperature, pH, incubation period and substrate concentration for saccharification of paper by mono and mixed fungal strain were cultivated with varying temperatures of 20°C-60°C, pH range 3-6.5, incubation period range of 2-30 days, and substrate concentration 1% - 10% by keeping all other parameters constant for 10 days.

F. Statistical analysis

Analysis of variance (ANOVA) was performed on all data using the SAS (1985) statistical package. The mean values were compared by the least significant difference (LSD) test at 5% level of confidence.

Result and Discussion

Table 1, 2 & 3 reflects the saccharification of all paper materials and CMC 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 and CMC. Office paper also exhibited the highest susceptibility towards hydrolysis by cellulase from *A. oryzae*. The highest reducing sugar was released from office paper by *P.citrinum* followed by news paper, filter paper and CMC. But the highest reducing sugar produced by *A.oryzae* is from news paper followed by office paper, filter paper and CMC.

To increase the degree of saccharification fugal cultures were mixed and incubated with all cellulosic materials (Figure 1). An equal mixture (2:2:2) resulted in the highest increase in saccharification with all substrates. Similar tendencies with cellulases from *P. funiculosum* and *Trichoderma reesei* on these cellulose materials were observe (Van Wyk, 1998).

The rate of saccharification can be increased by optimizing conditions for microbial growth. During growth of microorganisms in media containing paper waste, they utilize cellulose in paper as Carbon source. These organisms produce and secrete cellulase enzyme for the degradation of cellulose and release glucose. The process of

saccharification of paper can be increased by optimizing the conditions like Temperature, pH, incubation time and substrate concentration etc. The saccharifying media was prepared and cultures were inoculated in 2:2:2 ratio.

Figure 1 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 where as the maximum saccharification was observed at 10^{th} day in mixed cultures. Further increase in the incubation period however, resulted in the gradual decrease in the saccharification. Therefore, incubation period of 15 days for monoculture and 10 days for mixed culture was found to be optimal for saccharification.

The optimization of the time course is of prime importance for saccharification by fungi (Khud & Sing, 1993). The decrease in the saccharification 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 byproducts or catabolic repression of cellulase enzyme by released glucose.

The effect of incubation temperature (20-55ºC) on the saccharification of paper by mono and mixed cultures of fungi is shown in figure 2. There was a gradual increase in saccharification as the temperature was increased. But it showed maximum yield at 30ºC i.e., reducing sugar concentration for monocultures1.5 to 2.5 $\pm$ 2mg/ml and for mixed cultures 4.5$\pm$ 2 mg/ml. As the temperature was further increased, there was a gradual reduction in the saccharification. This may be due to the fact that higher temperature denatures the saccharifying enzymes mainly cellulase (Solomon, B.O.1999). High temperature may also lead to inhibition of microbial growth. Mekala *et al.*, (2008) showed that cellulases production and thus saccharification was maximum in flasks incubated at 33C and decreased with high temperature.

Table.1 Total reducing sugar produced from waste paper and Carboxy methyl cellulose at the optimum ratio mixture of *P. citrinum A. oryzae and T.viride* relative to the monoculture action.

Substrate	**Total reducing sugar concentration (mg/ml)**				
	P.citrinum	***A.oryzae***	***T.viride***	**Mixed culture 1**	**Mixed culture 2**
OP	1.3	1.2	1.0	1.88	2.56
NP	1.1	1.35	0.94	1.46	2.37
FP	0.7	0.99	0.58	1.20	1.52
CMC	8 0.8	0.60	0.56	0.93	1.09

OP-Office paper, NP-News paper, FP-Filter paper, CMC-Carboxymethyl cellulose, Mixed culture 1-1:1:1 ratio of three culture, Mixed culture 2-2:2:2 ratio of cultures

Table.2 Cellulase enzyme produced during Saccharification of different waste papers and Carboxy methyl cellulose by monoculture and mixed cultures of cellulolytic fungi

Substrate	Cellulase enzyme activity (U/ml)				
	P.citrinum	*A.oryzae*	*T.viride*	Mixed culture 1	Mixed culture 2
OP	1.2	1.16	0.98	1.59	2.17
NP	0.99	1.23	0.75	1.36	2.09
FP	0.63	0.82	0.49	1.15	1.39
CMC	0.75	0.53	0.45	0.856	0.95

OP-Office paper, NP-News paper, FP-Filter paper CMC-Carboxymethyl cellulose, Mixed culture 1-1:1:1 ratio of three culture, Mixed culture 2-2:2:2 ratio of cultures.

Table.3 Protein produced during Saccharification of different types of waste paper and Carboxy methyl cellulose by monoculture and mixed cultures of cellulolytic fungi

Substrate	Protein concentration (mg/ml)				
	P.citrinum	*A.oryzae*	*T.viride*	Mixed culture 1	Mixed culture 2
O P	1.5	1.4	1.15	2.12	3.26
N P	1.2	1.5	1.02	1.86	2.91
F P	0.9	1.0	0.88	1.52	1.84
CMC	0.79	0.7	0.71	1.121	1.13

Figure.1 Effect of incubation time on saccharification of paper waste by *Trichoderma viride*, *Aspergillus oryzae*, *Penicillium citrinum* and mixed culture

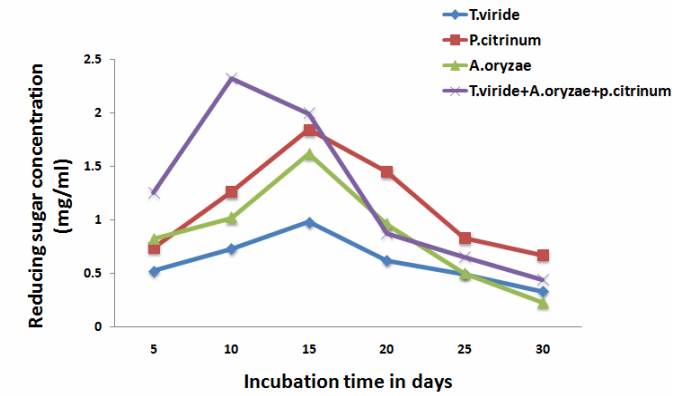


Figure.2 Effect of Temperature on saccharification of paper waste by *Trichoderma viride*, *Aspergillus oryzae*, *Penicillium citrinum* and mixed culture

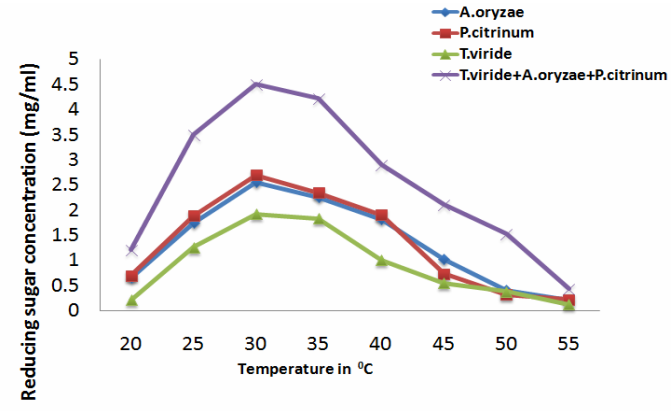


Figure.3 Effect of pH on saccharification of paper waste by *Trichoderma viride*, *Aspergillus oryzae*, *Penicillium citrinum* and mixed culture

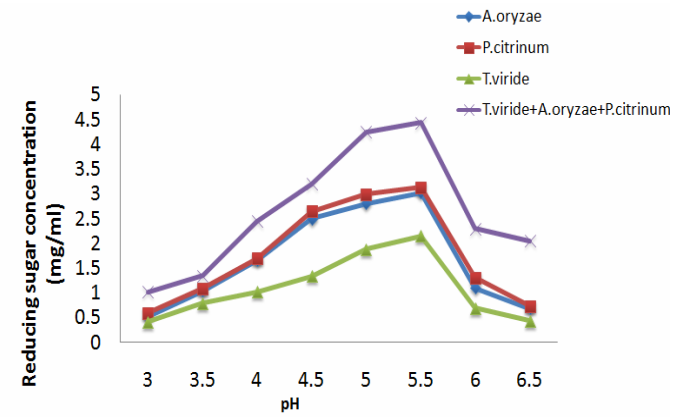
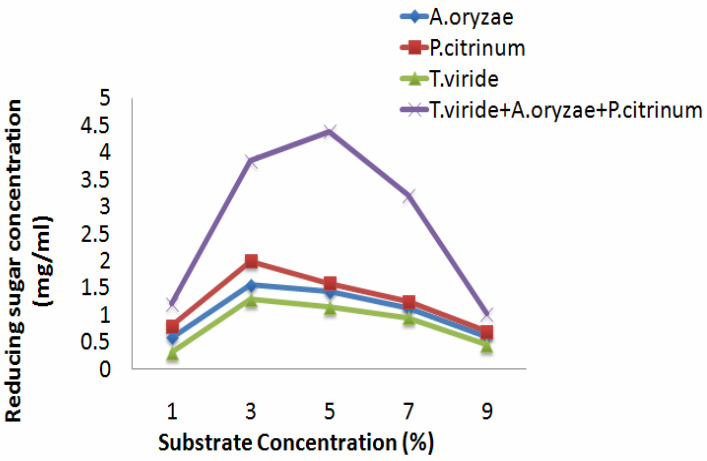


Figure.4 Effect of substrate concentration on saccharification of paper waste by *Trichoderma viride*, *Aspergillus oryzae*, *Penicillium citrinum* and mixed culture



The effect of initial pH (3.0-6.5) of the culture medium on the saccharification by fungal cultures was studied (Fig. 3). At the pH value of 4.5, there was very little saccharification of paper 1 to 2.4 mg/ml by mono cultures and mixed cultures, however, it started to increase as 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 saccharification by fungi. After pH value of 5.5, the production of cellulases decreased which might be due to the fact that cellulase are acidic proteins and are greatly affected by the neutral pH values (Chandra *et al.*, 2009).

Effect of substrate concentration on saccharification of paper waste by mono and mixed cultures were carried out (Figure 4). It was observed that the highest saccharification was carried out 3% of substrate for monocultures where as mixed cultures highest saccharification obtained at 5%.This may be due to mixed culture contain all the enzyme complex of cellulase enzyme hence, they can convert high concentration of paper in to sugars.

In this research work, from above findings it may be concluded that strains *A. oryzae*, *T.viride* and *P.citrinum* showed good performance for the 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. The maximum saccharification was obtained with mixed cultures of fungi in 2:2:2 ratio and temperature 30^{o}C, pH 5.5, incubation time 10 days and substrate concentration 5 %.The reducing sugar produced from paper may be used in future for ethanol production or others. This research may be meaningful both in the conversion and utilization of renewable biomass, and in the reduction of environmental pollution.

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