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# Induction and optimization of cellulases using various agro-wastes by *Trichoderma virdii*: Effect of alkali pretreatment

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**This study presents optimization of various lignocellulosics and alkali pretreatment for maximum cellulase production by *Trichoderma virdii* sp. Maximum endoglucanase (642 IU/L) and exoglucanase (187IU/L) activity was achieved with maize straw at 5% concentration. Oat hay was the most suitable agro-waste for β - glucosidase (7100 $IUL^{-1}$) production followed by maize straw (6500 $IUL^{-1}$). Maize straw was chosen in an effort to enhance cellulase production with 0.1 N NaOH, 0.5 N NaOH and I.0 N NaOH pretreatment. 0.1 N NaOH produced desirable results showing 2.5, 1.6, and 1.7 fold increase in endoglucanase, exoglucanase and β-glucosidase activity. This 0.1 N pretreated straw produced reducing sugars 3.5 times more than untreated straw.**

**Key words:** Lignocellulosics, maize straw, cellulase production, *Trichoderma virdii*, alkali pretreatment

## INTRODUCTION

Lignocellulosic biomass is the most abundant organic raw material in the world (Singh et al., 2006). The recent thrust in bioconversion of agriculture waste to chemical feed stock has led to extensive studies on cellulolytic enzymes produced by fungi and bacteria. Low cost of enzyme production improves the economics, as the cost of enzyme constitutes a major part of the total cost of hydrolysis (Baig et al., 2004). Successful utilization of abundantly available lignocellulosics as an alternate carbon source for cellulose production can lower the cost of enzyme production.

India is a land of agriculture. After harvesting and processing of various cereal crops, about 300 million tons of straw is produced annually. These agro- wastes can effectively be utilized for the production of cellulases which can be used in the saccharification of these wastes. The ability of various fungal spp. to produce cellulases on various lignocellulosics has been reported (Baig et al., 2003; Singh et al., 2010).

This study presents a comparison of various lignocellulosic wastes (maize straw, Jowar straw, Bajra straw, wheat straw, oat hay and berseem hay) for efficient on-site production of cellulase by a local soil isolate of *Trichoderma viride* (S 34). A methodology is formulated for maximum cellulase production by manipulating medium components and with alkali pretreatment. The cellulolytic enzymes will be used for saccharification of pretreated straw.

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**Table 1.** Effect of incubation period on endonuclease activity ($IUL^{-1}$) by *Trichoderma viride* (S34) at 25°C using various carbon sources at 1% level.

| Carbon source | Incubation days | | | | |
|---|---|---|---|---|---|
| | 7 | 11 | 14 | 17 | 21 |
| CMC | 628 | 692 | 607 | 500 | 482 |
| Maize straw | 26 | 143 | 160 | 197 | 187 |
| Jowar straw | 66 | 162 | 106 | 106 | 82 |
| Wheat straw | 23 | 28 | 53 | 17 | 10 |
| Bajra straw | 44 | 51 | 51 | 106 | 82 |
| Oat hay | 87 | 99 | 120 | 85 | 73 |
| Barseem hay | 13 | 21 | 32 | 32 | 9 |

## MATERIALS AND METHODS

### Substrates

Natural lignocellulosics namely maize straw, Jowar straw, Bajra straw,wheat straw, oat hay and berseem hay were dried in an oven and ground in a Wiley Mill to pass through I mm screen and utilized as substrate for submerged fermentation (SmF). All the lignocellulosics were passed through same mash size to provide equal surface area for fungus to grow and do not differ due to difference in oxygen diffusion, nutrient absorption and assimilation by mycelia.

### Liquid state fermentation

*T. viride* (S34) was inoculated in Erlenmeyer flasks (250 ml) containing sterilized enzyme production medium with 1-5% of different lignocellulosics as sole carbon source. The broth culture was incubated at 25°C up to 21 days. The supernatants, collected after centrifugation of contents, were used for assaying cellulase.

### Enzyme assay

Activity of cellulases was assayed by reported methods (Ray et al., 1993). For endoglucanase activity, suitably diluted enzyme solution was incubated with 1% CMC and 0.5 M citrate buffer (pH 4.8) in a total volume of 2 ml at 50°C for 30 min. For exoglucanase activity, 0.1-0.5 ml of enzyme solution was incubated with Whattman filter paper strip (1 x 10 cm) and 0.5 M citrate buffer (pH 4.8) in a total volume of 2 ml at 50°C for 60 min. β-glucosidase activity was measured with 0.05-0.1 ml of enzyme solution in a reaction mixture of 2 ml containing 1 ml of 1% cellobiose and 0.5 M citrate buffer (pH 4.8). The reaction mixture was incubated for 15 min at 50°C. The liberated sugars in the above assays were estimated by Nelson method (Nelson, 1944) and the activity was expressed in International Unit (IU). One unit of cellulose is defined as the amount of enzyme that released one micromole of reducing sugar per minute under the assay conditions (pH 4.8, 50°C).

### Optimization of culture condition

Optimum culture conditions including incubation period (7, 14, 17, 21 days), carbon source (maize straw, Jowar straw, wheat straw, oat hay and berseem hay), concentration of lignocellulosics (1, 2, 3, 5%), pH (4 - 6.5), temperature (20, 25, 30 and 35°C) and nitrogen (ammonium chloride, sodium nitrate, ammonium nitrate, ammonium sulphate, urea and peptone; 0.05% w/v) were determined for maximum growth of *T. viride* and cellulase activity was recorded.

### Pretreatment of lignocellulosics

Overnight dried lignocellulosics were autoclaved with 10 ml of different concentrations of alkali (0.1, 0.5 and 1.0 N) for 30 min in 150 ml Erlenmeyer flasks. After cooling, the alkalinity was neutralized with acid and stock production media was added to make final volume (30 ml) having desired concentrations of nutrients and pH 4.0. The fermentation was carried out with *T. viride* (S 34) for 14 days at 25°C. The filtrate was used for the estimation of cellulase.

### Saccharification of agro wastes

Saccharification experiment was performed with crude enzyme preparation obtained by fermenting 5% alkali treated and untreated Jowar for 14 days using *T. viride* (S34). The enzyme concentration used was 0 (control), 0.5 and 1 ml/g of substrate in the reaction mixture containing 50 mM sodium acetate buffer (pH-5.0) in a total volume of 30 ml and 3% of lignocellulosics in each flask. Each treatment was taken in triplicates. The reaction mixture was incubated at 45°C under shaking conditions at 100 rev $min^{-1}$ up to 72 h and samples were drawn for the determination of reducing sugars by Nelson (1944) method.

## RESULTS AND DISCUSSION

### Optimization of medium constitutes

The carbon source of the medium was affected considerably in the synthesis of cellulolytic enzymes by *T. viride* (S 34) in liquid cultures. Maize straw, jowar straw, wheat straw, oat hay and berseem hay invariably affected the synthesis of cellulolytic enzymes in the medium. Maximum induction of endoglucanase (Table 1) and exoglucanase (Table 2) activity among agro-wastes was achieved by maize straw at 17th day followed by jowar straw at 11 day incubation period. Oat hay produces maximum β-glucosidase at 14 day incubation period (Table 3). Very low level of induction was seen with berseem hay and wheat straw. The variability in the production as well as incubation period on different lignocellulosics could be attributed to various factors such

**Table 2.** Effect of incubation period on exonuclease activity ($IUL^{-1}$) by *Trichoderma viride* (S34) at 25°C using various carbon sources at 1% level.

| Carbon source | Incubation days | | | | |
|---|---|---|---|---|---|
| | 7 | 11 | 14 | 17 | 21 |
| CMC | 53 | 199 | 67 | 50 | 32 |
| Maize straw | 4 | 5 | 10 | 30 | 21 |
| Jowar straw | 11 | 13 | 12 | 6 | 9 |
| Wheat straw | 3 | 3 | 7 | 4 | 4 |
| Bajra straw | 7 | 5 | 2 | 9 | 3 |
| Oat hay | 5 | 6 | 14 | 1 | 10 |
| Barseem hay | 5 | 6 | 8 | 10 | 2 |

**Table 3.** Effect of incubation period on β-glucosidase activity ($IUL^{-1}$) by *Trichoderma viride* (S34) at 25°C using various carbon sources at 1% level.

| Carbon source | Incubation days | | | | |
|---|---|---|---|---|---|
| | 7 | 11 | 14 | 17 | 21 |
| CMC | 6400 | 7467 | 4053 | 1705 | 1560 |
| Maize straw | 4693 | 5100 | 5808 | 6200 | 5700 |
| Jowar straw | 1599 | 3541 | 1400 | 1386 | 1251 |
| Wheat straw | 1636 | 1386 | 2418 | 355 | 482 |
| Bajra straw | 1209 | 1920 | 2453 | 3840 | 3203 |
| Oat hay | 3200 | 4551 | 6187 | 5227 | 5183 |
| Barseem hay | 1000 | 1067 | 1972 | 2150 | 1505 |

as variable cellulose content, heterogeneity of structure and accessibility of cellulose for microbial attack leading to difference in the lag period of growing fungus. A number of reports are available for the use of lignocellulosics by *Trichoderma* spp. (Muthuvelayudham and Viruthagir, 2006; Kubicek et al., 2009). However most of the *Trichoderma* spp. described in the literature are deficient (Duff, 1985) or low (Tiwari et al., 2013) in the production of β-glucosidase. Therefore cellobiose accumulates and represses the enzyme biosynthesis. These rate-limiting steps in the bioconversion of lignocellulosic residues to industrially important products remain one of the most significant hurdles in saccharification and producing economically feasible cellulosic ethanol.

In the present study, *T. viride* (S 34) produces a good amount of β-glucosidase enzyme on all type of lignocellulosics especially on oat hay (7100 $UL^{-1}$). Higher induction of cellulase with maize straw could be attributed due to more cellulose content compared to other lignocellulosics (Goyal et al., 2008).

Cellulase induction was maximized with CMC as carbon source but the use of purified cellulose as substrate is uneconomical for large scale production of enzymes. High cost of cellulases production hindered use of this enzyme in industry. Therefore efforts were made to optimize cellulase production with cheaply available agricultural lignocellulose waste. Increased concentrations of all lignocellulosics favored higher induction of cellulase complex with differences in the yield of enzyme. Maize straw at 5% level was effectively utilized by fungus to increase the production to 3 times for endoglucanase (Figure 1a) and about 6 times for exoglucanase (Figure 1b) from that at 1% level. Increased production of cellulases by raising lignocellulosic concentration was also observed by Jadhav et al. (2013).Oat hay proved to be maximum β-glucosidase producer at 5% level (Figure 1c).

Alkali pretreatment of lignocellulosics remove acetyl and uronic acid substitution on hemicelluloses increasing accessibility of hemicellulose and cellulose for enzyme attack (Chang and Holtzapple, 2000). Therefore, pretreatment of 5% maize straw with different concentrations of NaOH (0.1 - 1.0 N) and subsequent fermentation was tried. Mild treatment (0.1 N NaOH) showed desirable results with higher production of cellulase than produced with CMC (Figure 2). This induction might be attributed due to changes in the structure of maize biomass with increased solublization of hemicelluloses, reduced crystallinity and increased available surface area and pore volume of substrate (Singh et al., 2010). More severe alkali treatment might have resulted into the production of toxic compounds like furfurals inhibiting microbial metabolism and thus decreasing the

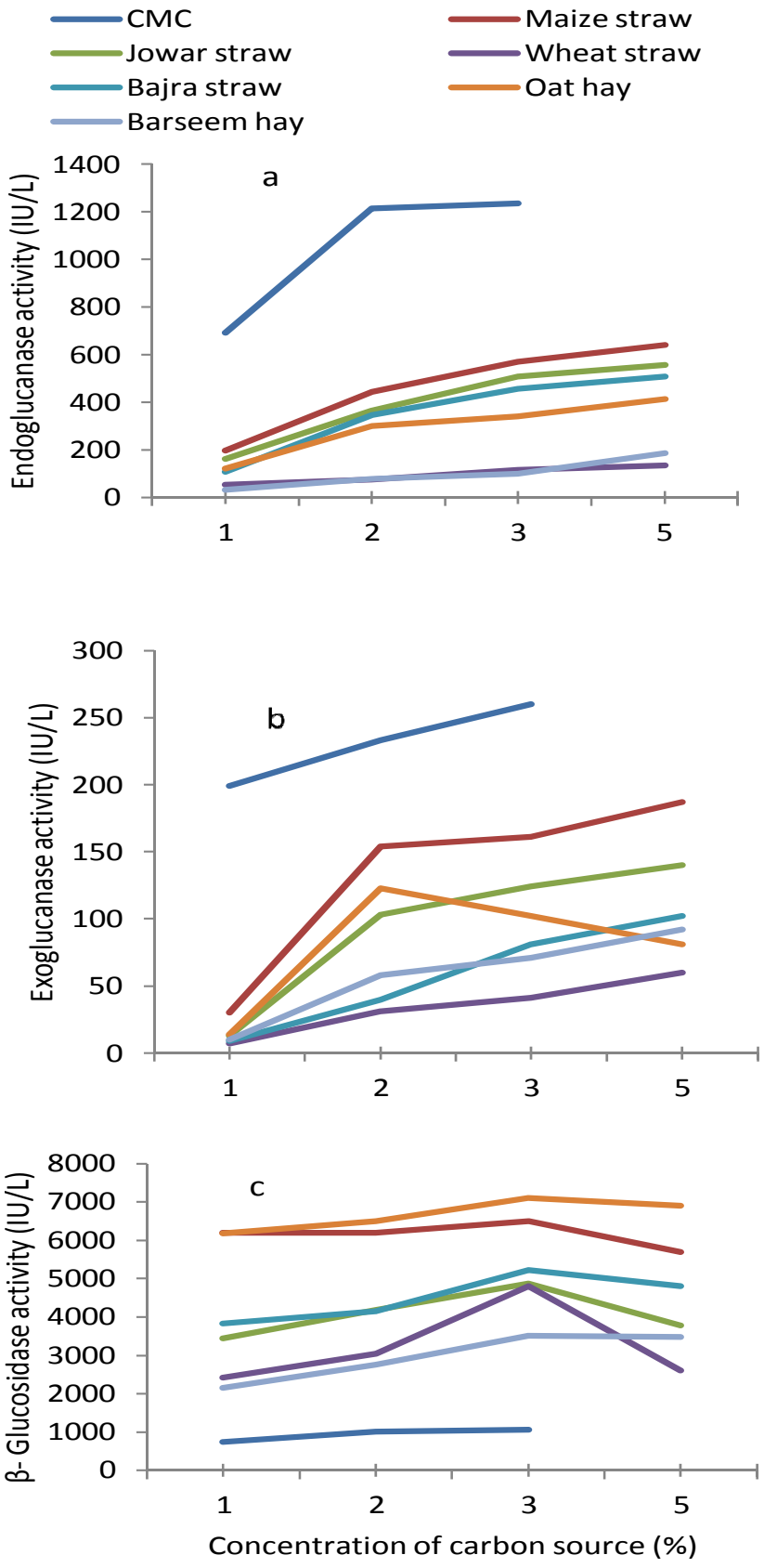


**Figure 1.** Effect of different carbon source concentration on (a) endoglucanase, (b) exoglucanase and (c) β-glucosidase activity.

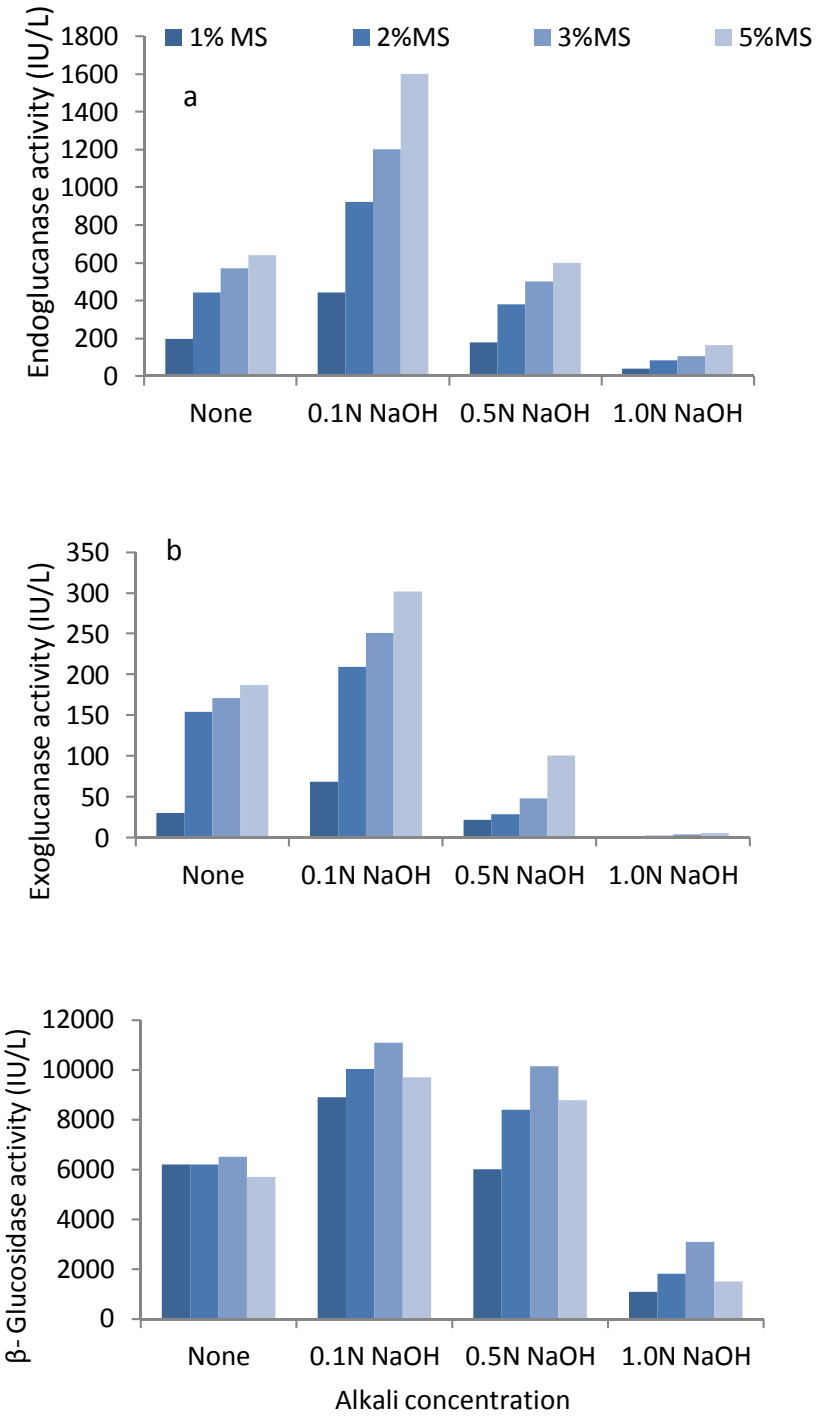


**Figure 2.** Effect of alkali pre treatment on (a) endoglucanase, (b) exoglucanase and β-glucosidase activity.

production of cellulase to many fold.

**Effect of N source, temperature and pH on cellulase production**

Enzyme activity got influenced when $NaNO_3$ as sole nitrogen source was used (Table 4). Maximum enzyme activity was recorded at 25°C (Table 4). A *Trichoderma* strain in a recent paper preferred temperature 35°C for maximum growth (Leghlimi et al., 2013).

At pH 4, the fungal strain showed heavy growth and higher cellulase enzyme activity. Similar pH dependency was shown by strain of *Aspergillus* grown on sawdust

**Table 4.** Effect of nitrogen source, incubation temperature and initial pH on cellulases activity ($IUL^{-1}$) by *Trichoderma viride* (S34) using carbon source at 1% level.

| Nitrogen source | Enzyme activity($IUL^{-1}$) | | |
|---|---|---|---|
| | Endoglucanase | Exoglucanase | β-Glucosidase |
| **0.05% N (w/v)** | | | |
| $NH_4Cl$ | 128.0 | 10.2 | 2346.6 |
| $NaNO_3$ | 165.6 | 15.0 | 3540.9 |
| $NH_4NO_3$ | 106.6 | 8.7 | 2986.6 |
| $(NH_4)_2SO_4$ | 136.0 | 11.4 | 2026.6 |
| $NH_2CoNH_2$ | 45.3 | 4.1 | 3199.9 |
| Peptone | 15.9 | 3.2 | 3201.0 |
| **Incubation Temperature (°C)** | | | |
| 20 | 152.5 | 11.2 | 3224 |
| 25 | 188.7 | 16.2 | 3504.8 |
| 30 | 106.5 | 7.1 | 2124.6 |
| 35 | 51.0 | 2.4 | 1202.6 |
| **Initial pH** | | | |
| 4.0 | 195.8 | 1.05 | 3504.0 |
| 4.5 | 174.2 | 10.2 | 1802.0 |
| 5.0 | 124.6 | 7.6 | 1002.6 |
| 5.5 | 109.6 | 5.2 | 804.6 |
| 6.0 | 50.5 | 2.1 | 502.8 |
| 6.5 | 44.8 | 1.8 | 214.7 |

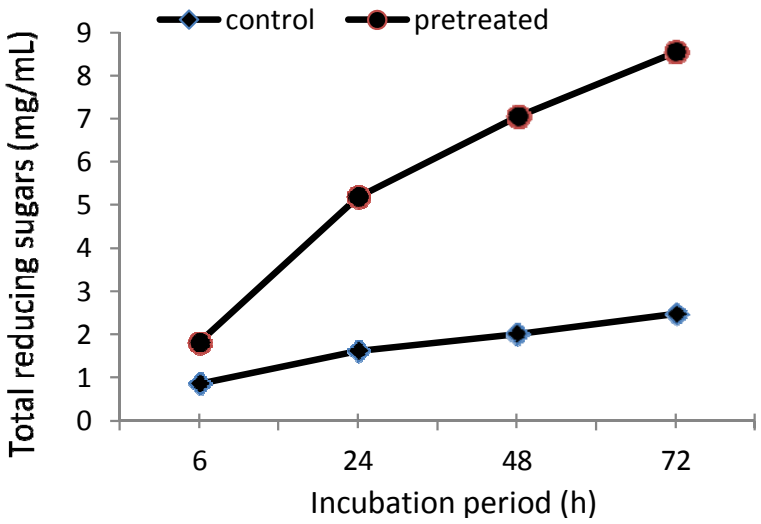


**Figure 3.** Effect of pretreatment on total reducing sugars.

(Milala et al 2009) and CMC (Oyeleke et al., 2012).

## Saccharification of pre-treated maize straw

Cellulases produced from *T. viride* were used for saccharification of maize straw at pH 5.0 and temperature 45°C (Figure 3). The results show highest level of total reducing sugars with the pretreated agro- waste. Release of reducing sugar increased with increase of incubation period.

At the end of 72 h reducing sugars were released 3.5

fold more with alkali treated maize straw than produced with nontreated straw (Milala et al., 2009).

## Conclusions

Maize straw could provide an economical advantage as carbon source for production of cellulase enzymes by using *Trichoderma viride* as fungal source. Factors [pH (4), temperature (25°C) and nitrogen source($NaNO_3$)] were optimized for maximum cellulase production. In optimum conditions production of endoglucanase, exoglucanase, β-glucosidase and reducing sugars in hydrolysis process showed best results with mild alkali pretreatment.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENT

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