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An Optimised Combination Approach of Physicochemical Methods for Separate Hydrolysis and Fermentation (SHF) Pretreatment of Wheat Straw for High Production of Bio-alcohol

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Production of bio-ethanol from lignocellulosic waste like wheat straw has been a very attractive approach since earlier. However, production of ethanol is compromised from lignocellulosic waste due to its tough and crystalline structures. To resolve this issue, the combination of physical methods (such as milling, irradiation, steam) and chemical methods (such as NaOH treatment) has been explored in our study for the pre- treatment of wheat straw during separate hydrolysis and fermentation (SHF) steps. Fungus VBVI (*Aspergillus terreus*) and *Saccharomyces cerevisiae* were used for the Microbial hydrolysis and Fermentation respectively for the optimization of alcohol production. Interestingly, we observed that content of xylan and lignin from wheat straw gradually decreases during pre-treatment events, which facilitates the overall enhanced alcohol production. Amount $10gL^{-1}$ of pre- treated wheat in nutrient media treated with *Aspergillus terreus* VB (VI) inoculum and was observed high cellulase and xylanase activity rapidly post 120 hrs of inoculation. Later on, *Saccharomyces cerevisiae* was inoculated for fermentation once the saturated microbial hydrolysis was achieved. After 192 hrs, $3gL^{-1}$ethanol was observed to be produced, indicating conversion rate and ethanol yield 45.6% and 18%respectively of the theoretical maximum values reported so far. Our study suggested that strain VBVI (*Aspergillus terreus*) has a very good potential for enhanced bio-ethanol production from wheat straw. Moreover, different steps of pre-

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treated method have also been optimized and explored in this study for the overall production of ethanol. Overall, this study reveals out an easier and cheaper approach to scale up the industrial bio-alcohol production.

Keywords: Wheat straw; bio-alcohol production; Separate Hydrolysis and Fermentation (SHF); microbial hydrolysis.

1. INTRODUCTION

Excessive and uncontrolled fossils fuel consumption and mounting of the pollution compel gradual transition of automobile fuel towards sustainable and renewable alternative sources. Use of alcohol based fuel engines is one of the attractive ways to prevent harmful greenhouse gas emissions. Among the alcoholic fuels, ethanol is best promising liquid fuel till date because of less toxicity, 0% net CO_2emission and most importantly, it can be produced through some renewable sources as well. Cellulosic ethanol production is one of the most commonly discussed and fascinated second-generation bio-fuel technologies worldwide [1]. Ethanol is produced now days through microbial process by using various kinds of feed stroke substrates such as molasses, corn and other lingocellulosic waste. From the above-mentioned substrates, wheat straw is one of the cheapest and abundant available raw materials for bio-alcohol production. Utilisation of lignocellulosic waste can replace equivalent to 40% of the gasoline used in US market (Yu Sen, 2001).

However, the primary limitation of bio-ethanol production from lignocellulosic is its high degree of complexity and crystalline structures. Lignocellulosic waste is a mixture of cellulose (30%), hemicellulose (29%) and lignin (30%) [2]. Complex structure of lignocellulosic material made ethanol production very less effective by SHF method irrespective of three major steps (pre-treatment, hydrolysis and fermentation). Pre-treatment process loosens texture of wheat straw and making them more accessible for the cellulase enzyme during the process of microbial hydrolysis. Various pretreatment methods have been used for optimization of scarification/ microbial hydrolysis of lignocellulosic waste namely milling [3], irradiation (Chunging et al. 2008), steam explosion [4], alkaline hydrolysis [5] and even sometimes combinational approaches were used also. So till now many approaches have been used for higher bio-alcohol production using above mentioned methods, however, still there is a need for further improvement.

The theme of this experiment is to reveal the outcome of enhanced alcohol production using SHF method by improving the pre-treatment process. In the previous report it was reported that steam treatment acts as swelling agent which helps in disruption of hardness and separates the individuals cellulosic fibers [6] while alkali pre-treatment has not only role in swelling but also helps the removal of inhibitory chemical like lignin and their byproduct from lignocellulosic waste and finally make cellulosic fibers more accessible for cellulase action during scarification process [7]. As we know that crystalline structure of lignocellulosic waste is one of the biggest hurdles for their microbial decomposition but use of radiation facilitates disruption of crystallinity and eventually decrease the degree of polymerisation of lignocellulosic waste. Recently, Ooshima et al. [8] reported an improvement in total reducing sugar production after irradiation pre-treatment of lignocellulosic waste. The objective milling is simple to increase the overall surface area of lignocellulosic waste of cellulase action.

Here, in our study, various combinations of radiation, steam and alkali were exposed to milled wheat straw for the development of more cheaper and efficient protocol for ethanol production. Our lab strain *Aspergillus terreus* VB (VI) and *Saccharomyces cerevisiae* were used for the microbial hydrolysis. Microbial hydrolysis was investigated using several parameters under ideal conditions.

2. MATERIALS AND METHODS

Fungal strains and culture condition: *Aspergillus terreus* (VBVI) and *Saccharomyces cerevisiae* were used respectively for microbial hydrolysis and further fermentation of lignocellulosic material i.e. wheat straw in this experiment. Both stock cultures were maintained on PDA slant at 4°C in dark room. Mycelium of culture from slant was transferred to PDA plates and incubated for atleast3 days. Mycelium discs from the edges were taken as inoculum during our study.

Treatment: Optimization of the cellulase activity of the substrate (i.e. treated wheat straw) was explored in the presence of NaOH or steam or radiation and in different combinations. Five grams of dried wheat straw powder (50-100 mesh size) and 50 ml of 1% alkali solution added to 300 ml Erlenmeyer flasks. After 30 minutes, wheat straw is washed with water to bring neutral pH and then air dried. Before using it, alkali pre-treated wheat straw was weighed to measure the lost weight after treatment. During steam treatment, Five grams of wheat straw was treated in steam exploded vessels at 120°C for 15 minutes. The compositions of raw and pre-treated wheat straw are as shown in Table 1. (Maria Carolina, 2013). For the NaOH and steam treatment together, straw were first soaked with NaOH and then later on pass through the steam. Physically treated wheat straw with various ways was irradiated with 100 KGY by nuclides of Cobalt-60.

Electron Microscopy: Wheat straws were embedded in polymethacrylate and sections were cut by an ultra-microtome. The embedding medium was then removed and sample was coated with gold. Samples were investigated and photographed using scanning electron microscope facility (JSM-6360) at North Eastern Hill University, Shillong.

Microbial Hydrolysis and Fermentation condition: After pre-treatment, 0.3gm of pre-treated wheat straw and 30ml of basal media ($0.5gL^{-1}$yeast extract, 1.5 $gL^{-1,}$ peptone, 1.5 $gL^{-1}(NH_4)SO_4$, 4.0 gL^{-1} KH_2PO_4, 3.0 gL^{-1} K_2HPO_4, 0.3 $MgSO_4.7H_2O$, 0.3 $gL^{-}1$, CaCl2) (pH- 7.0) (references) were added to 100ml Erlenmeyer flask and autoclaved. Six- millimeter-diameter discs were punched from the peripheral region of the mycelium incubated on PDA plates. Two discs were placed into each of the 100ml Erlenmeyer flasks mentioned above and sealed with silicone plug stopper. Sealed culture was then incubated at 28°C in the dark for several days. *Saccharomyces cerevisiae* was inoculated for fermentation after 120hrs, after the saturation of microbial hydrolysis. Basal medium was used as control, where production of ethanol was not observed at all.

Enzyme production media were used for the analysis of cellulase activity. Various kind of extracellular enzymes such as Xylanse, Fpaseexo-glucanase, endo-glucanase and β-glucanase were qualitatively and quantitatively monitored.

Alcohol production assay: Ethanol content was estimated by using 1ml of cell-free supernatant to which 9ml of distilled water and 1ml of dichromate were added and heated for 10min in a boiling water bath. During heating, ethanol was converted to acid through a reaction with dichromate and resulted in a color change from orange to green [9]. The green colour was read at 660 nm by a spectrophotometer. Pure grade ethanol (Changshu chemical, China) was used as the control.

Analytical Methods: After the direct fermentation step, the remaining wheat straw and mycelium were removed by centrifugation at 10,000rpm for 10min (Remi group cooling centrifuge). The supernatant was used to measure the cellulase activity and ethanol concentration by above mentioned method. All experiments were conducted in triplicate.

Chemical composition of pre-treated wheat straw or initial wheat straw: Lignin and other monosaccharide levels of pre- treated and un-treated wheat straw were determined following the method described by the National Renewable Laboratory [10]. Briefly, sulphuric acid (72%) was mixed with treated and untreated wheat straw and incubated at 30°C for 1hr. After that, it was diluted up to 4% of sulphuric acid by adding water. Diluted sample was autoclaved at 121°C for 1hr. Soluble and insoluble fraction were collected and analyzed to determine the sugar composition by using HPLC system(Agilent Scientific system Hi-PexCa (8% cross linked 7.7x300mm,8µmp/n/n PL1170-6810) mobile phase 100%DI H_2O flow rate 0.6 ml/min, Temperature 85°C detector RI).

Cellulase Enzyme Assay

Preparation of inoculum for the production of enzyme: Strain VBVI was grown on potato dextrose agar plates. After full growth, an agar/ mycelium disc of 8 mm diameter was cut out from plate with the help of corn borer and transferred into 250 ml conical flask containing 30 ml of media. Media was supplemented with 10% (w/v) wheat straw as a carbon sources which was pre-treated in various ways.

Enzyme preparation: Samples (3ml) of culture broth were taken aseptically at regular intervals throughout the growth phase and subjected to centrifuge at 5000 x g for 8 minutes and the supernatant was used as extracellular enzyme solution.

β–1,4- glucan glucanodydrolase (Endo-glucanase or CMC cellulose): The enzyme activity was measured by adding 1.4 ml of 0.5 M citrate- phosphate buffer (pH 7.0) and 0.1ml of appropriately diluted enzyme to 0.5 ml of 1% aquous solution of CMC. The mixture was incubated at 50ºC for 20 mins and the amount of reducing sugar produced was determined (Ishaque et al. 1980). Absorbance was read at 540 nm against broth as reference and amount of reducing sugar was determined.

Reducing sugar Assay: Amount of reducing sugar was determined using DNSA method [11]. In brief 1.5 ml of culture media and 3 ml Dinitrosalicylic acid solution were mixed and placed in boiling for 5minutes, cooled at room temperature water bath and then Absorbance was read at 540nm. The absorbance values (after subtraction of reagent blank) were then translated into glucose equivalent and accordingly in standard graph plotted [12] amount of sugar produced was calculated from the standard curve of glucose.

Xylanase activity: The enzyme activity was measured by adding 1.4 ml of 0.5 M citrate-phosphate buffer (pH 7.0) with diluted enzyme (0.1ml of enzyme to 0.5 ml of 1% aqueous solution of Xylan). The mixture was incubated at 50^{o}C for 20 mins and then amount of total reducing sugar produced was determined by DNSA method [11]. Absorbance was read at 540 nm against broth as reference and amount of reducing sugar was calculated.

Filter paper activity (FP cellulase): The cellulose hydrolysis of filter paper test was tested by adding 1.4 ml of 0.05 M citrate-phosphate buffer (pH 5.6) and 0.1ml of supernatant to 50 mg of whatman filter paper (basis weight 87 gm/m^2) with thickness of 0.16 mm cut into 2mmx3mm strips. After incubation for 1 hour at 40^{o}C the reducing sugar of the supernatant was determined by DNSA method [11].

Unit of Enzyme: For Xylanse and FPase, the amount of reducing sugar was estimated from a glucose standard curve. oneunit of enzyme activity was expressed as the amount of enzyme which produced 1 µmole of reducing sugar per minute.

1 IU = 1 µmole/minute of glucose/xylose equivalent released

= 0.18 mg/minute of glucose

= 0.15 mg/minute of xylose

The conversion rate and ethanol yield were determined by the following equations:

Conversion rate (%) =
(Produced ethanol concentration gL^{-1}/Theoretical ethanol concentration gL^{-1}) X100

Ethanol yield (%) =
(Produced ethanol concentration gL^{-1}/ Sugar cane bagasse concentration gL^{-1})X100

Theoretical ethanol concentration gL^{-1} =
Glucan(Glucose+ Hemicellulose) X 1.1 X 0.51 + Xylan concentration 1.14 X 0.46

Here value 1.11is the coefficient of glucose obtained from Glucan, 1.14 is coefficient of xylose obtained from xylan, 0.51 is coefficient for ethanol conversion from glucose and 0.46 is the coefficient of ethanol from xylose [13].

2.1 Statistical Analysis

Each experiment was conducted in triplicate and represented as Mean Standard Deviation (SD). Statistical analysis was carried out by analysis of variance (ANOVA) in Microsoft Office2007, the probability values ($p < 0.05$) were considered as a statistically significant difference. The means and standard deviation of means (Mean SD) were computed for each treatment.

3. RESULTS AND DISCUSSION

3.1 Effect of Various Physio- chemical Pretreatment on Wheat Straw

To assess the effect of various pretreatments on wheat straw in SHF studied with fungal strain *Aspergillus terrus* VB (VI), chemical compositions of control and variably pretreated wheat straw were analyzed as mentioned in Tables 1 and 2. Information show chemical composition of each sample which are chemically different regarding their content of lignin, xylan, cellulose and hemicellulose and pre-treatment process shows great loss by alkali treatment respectively. Previous study also support that the pretreatment methods change the composition of agrowastes materials (Parveen Kumar et al. 2009). Statistical analysis shows the significant changes occured in sample after combination of pretreatment process on wheat straw. The data (Table 2) show significant weight loss of 37% and 41% under 1% NaOH treatment and 1% (OH-S-R) treatment respectively. Lignin content was drastically reduced by up to five fold after alkaline

treatment. A number of research works have been made by various researchers on different pretreatment methods and for removal of lignin to provide open end for enzymatic action [7]. Residual lignin content was decreased up to the level of 19% in case of OH-S-R treatment process. Initially the weight of lignin was 330mg/gram of wheat straw, around 12% weight loss after steam or radiation or steam plus radiation treatment while substantial weight loss in case of alkaline treatment either alone or with combination with radiation or/and steam was up to 85% .Such observation are also supported by the result according to him (Le Duy Khuong et al., 2014). Same trend was also observed in the case of xylan, The initially weight of xylan was 320 mg/gram of wheat straw and up to 21% weight lost after steam or radiation or steam plus radiation treatment while substantial weight loss in case of alkaline treatment either alone or with combination with radiation or/and steam was up to 71% during OH-S-R treatment process (Le Duy Khuong et al. 2014). Xylose is a pentose sugar which is not converting into alcohol by SC during fermentation therefore it is an unutilized residue in culture medium. Therefore it should be in fewer amounts in broth for any inhibition (Li J et al. 2014).

Radiation always increases in solubility of substances (Y.H Han, 1981). The effect of radiation on solubilization and scarification of wheat straw was apparent in steam treated method but increased when it was used in combination with alkali. Even alkali treatment alone was too effective on solubilization and scarification of wheat straw (Gasper et al. 2007). However, rate of increase in soluble amount of reducing sugar was also exponentially enhanced by the combination of alkali and radiation pre- treatment method. The result may be discuss as the alkali pretreatment along with radiation causes substantial change in configuration of lignocellulosic material (A. Kristaini 2014).

These weight losses are equivalent to the amount of dissolved carbohydrate along with other components after hydrolysis. Many studies have reported various means of pretreatment process for the hydrolysis of hemicellulose and released of their oligo-structures [14] Singh et al. 2015, Behera et al. 2014). Disadvantage of such pretreatment is the loss in total fermentable sugar but pretreatment of lignocellulosic makes itself a better substrate for alcohol production [14].

3.2 Structural Analysis

Alkali-pre-treated Wheat straw has a more porous and rougher surface with thinner fibres compared to untreated wheat straw Fig. 2B. This

Table 1. Chemical composition (%) of initial and pre- treated wheat straw (w/w)

Sample	Glucan	Xylan	Lignin
wt- C	33±0.3	32 ±0.3	33 ±0.2
wt- R	29±0.2	31±0.1	33±0.2
wt-st	30±0.5	26±0.1	29 ±0.8
wt-st-R	31±0.5	25±0.1	28 ±0.8
wt-OH	85 ± 2.1	10.3±0.1	6.6±0.1
wt-OH-R	85±3.0	10±0.2	6.5±0.1
wt- st-OH	88 ± 2.1	10±0.2	5.6±0.1
wt-st-OH-R	89±2.0	9.0±0.2	5.0±0.1

Table 2. The decrease in chemical components of pre-treated wheat straw. Residual components was calculated per 1 grams of initial wheat straw

Sample	Residual Glucan (%)	Residual lignin (%)	Residual xylan (%)	Coefficient of wt loss
wt- C	100	100	100	1
wt- R	97.00±0.1	99.0 ±0.5	99.0±0.4	0.98
wt-st	99±0.2	96±0.3	80±0.4	0.96
Wt-St-R	96±0.3	96±0.3	79±0.5	0.95
wt-OH	81±0.8	21±0.3	23±0.5	0.63
wt-OH-R	78±0.5	20±0.6	22±0.6	0.61
wt- St-OH	80±0.7	20±0.5	21±0.3	0.6
wt-St-OH-R	74±0.4	19±0.5	20±0.4	0.59

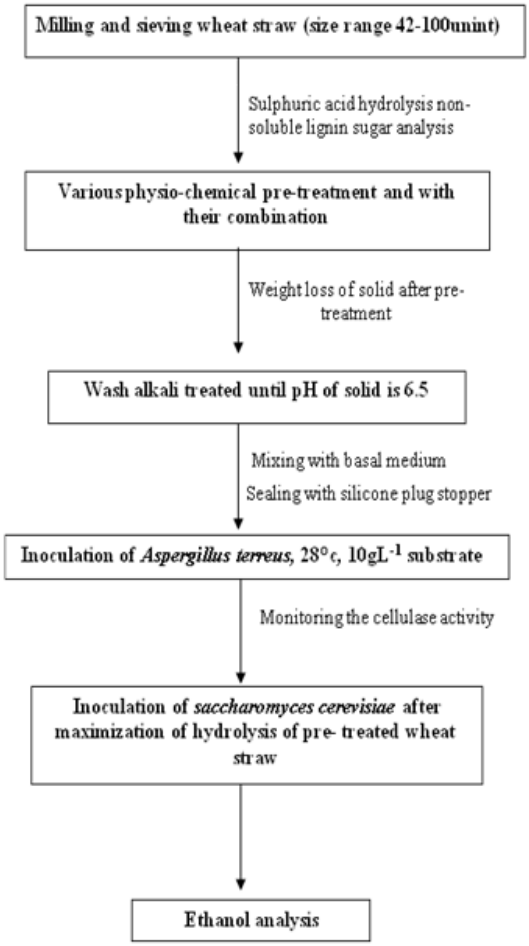
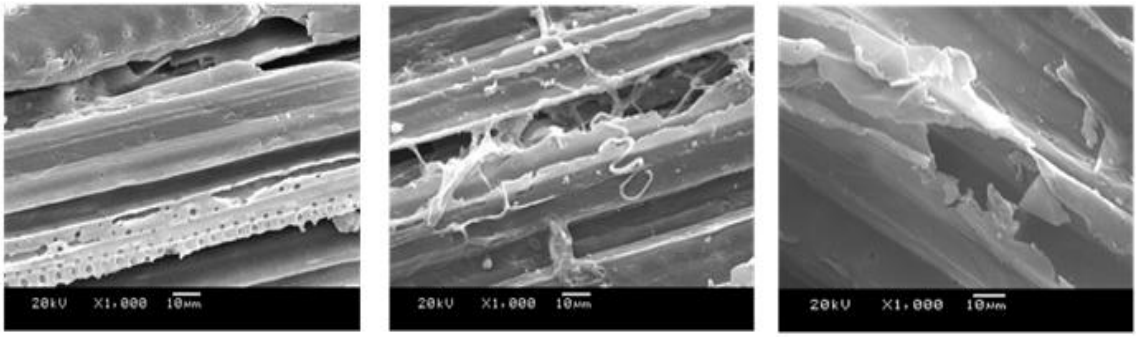


Fig. 1. Experimental design



(A) **(B)** **(C)**

Fig. 2. Electron microgram (1000X) of cross section of wheat straw (A) before treatment and (B) after treatment with alkali (C) after treatment with steam- alkali and Radiation

is due to the removal of lignin content and reduction in cellulose crystallinity during alkali pre-treatment. Similar structural changes on lignocellulosic substrate have also been observed by other researchers when alkali pretreatment was employed (Jabasingh and Nachiyar, 2011). Unlike untreated wheat straw, the structure of alkali-pretreated wheat straw did

not vary significantly before and after SSF. The depolymerisation of untreated wheat straw might happen at a relatively rapid manner during SSF compared to the alkali-pre-treated wheat straw as deduced from the morphological analysis (Yoon et al. 2013). It is obvious that these pretreatment methods effect the molecular and supra molecular structure of agro-wastes materials which is reflects during microbial hydrolysis. Radiation on swollen wheat straw caused disintegration of fibers and promotes partial solubilization (Fig. 2C).

3.3 Production of Enzymes for Biomass Hydrolysis

The time course profile of endoglucanase, Fpase and xylanase activities under solid state fermentation condition is shown in Fig. 3, Fig. 4. During the investigation of the time profile activity of these enzymes, all three enzymes detected in the broth after 24hrs of solid state gradually it reach to high and maximum biomass scarification/ hydrolysis.

These graphs for enzyme activity indicating microbial scarification process in pretreated wheat straw with control. Endoglucanse, Fpase and xylanse were observed in 72hrs old culture filtrate as shown in Figs. 3 and 4. Enzymatic activities got saturated as culture become old. Perhaps it may be due to accumulation of microbial by products. Released Free sugar is one of the main products of microbial hydrolysis on which whole concept was developed. Estimation of Reducing sugar (RS) also observed at the interval of 24hrs Shown in Fig. 5. The maximum production RS occurred after 3rd day of incubation (almost 24mg/ ml). All variably treated wheat straw, highest RS yield was also reported in the medium containing wt-st-OH-R.

These results inferred that the combined effect of alkali and radiation treatment greatly enhanced the scarification process by *Aspergillus terreus* VB (VI). Fpase and xylanase enhanced by two and two and half fold respectively.

3.4 Biomass Saccharification/Hydrolysis

The major component of lignocellulosic materials is cellulose followed by hemicellulose and lignin. The proportions of these compounds vary between plants (John et al. 2006; Cara et al. 2007; Prassad et al. 2007; Ruiz et al. 2008). The heterogeneous nature of the carbon sources play an important role in the induction cellulase enzymes and release of sugar during

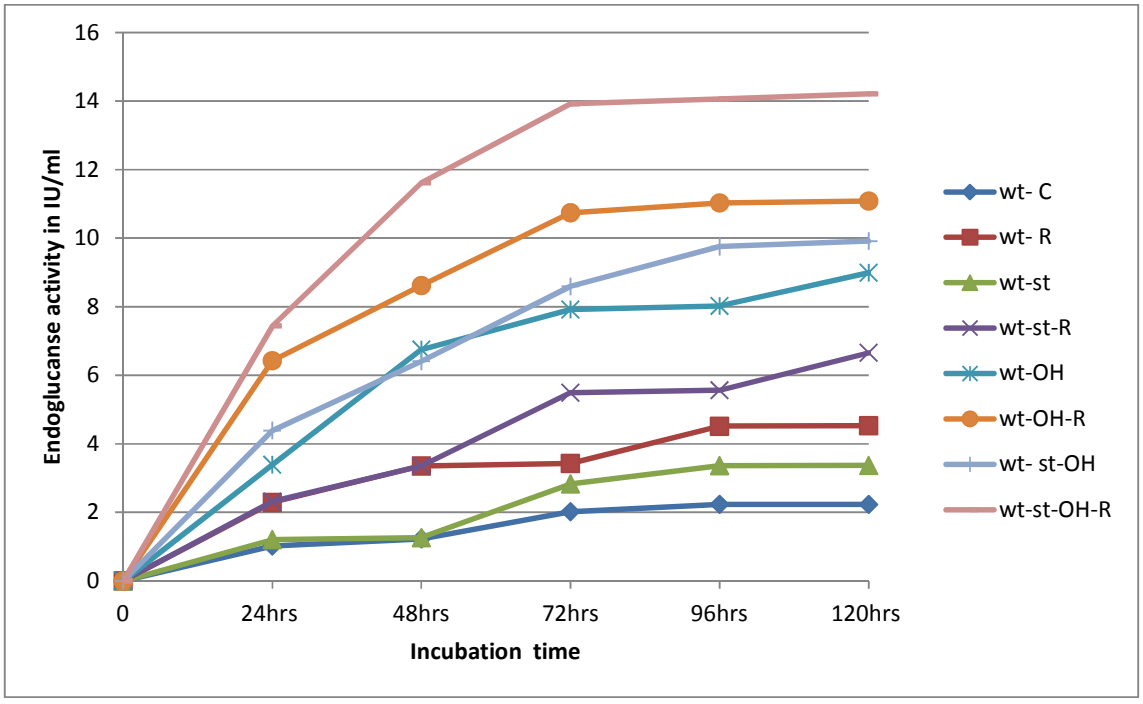


Fig. 3. Time course profile of endoglucanase activity under semi- aerobic condition

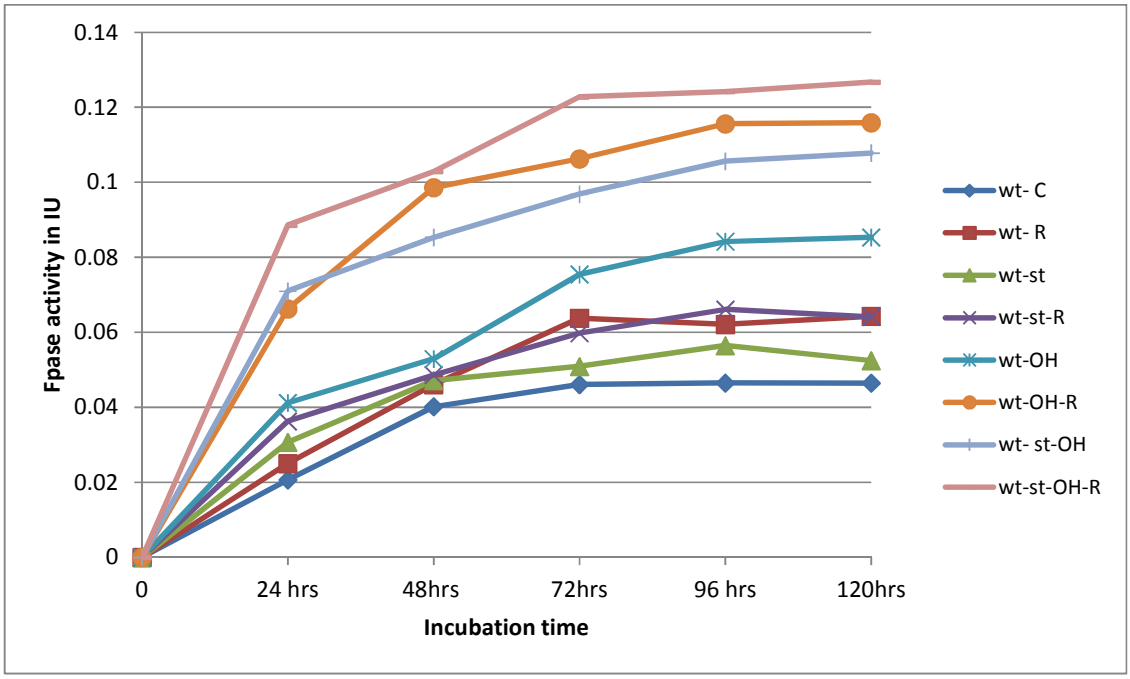


Fig. 4. Time course profile of Fpase activity under semi- aerobic condition

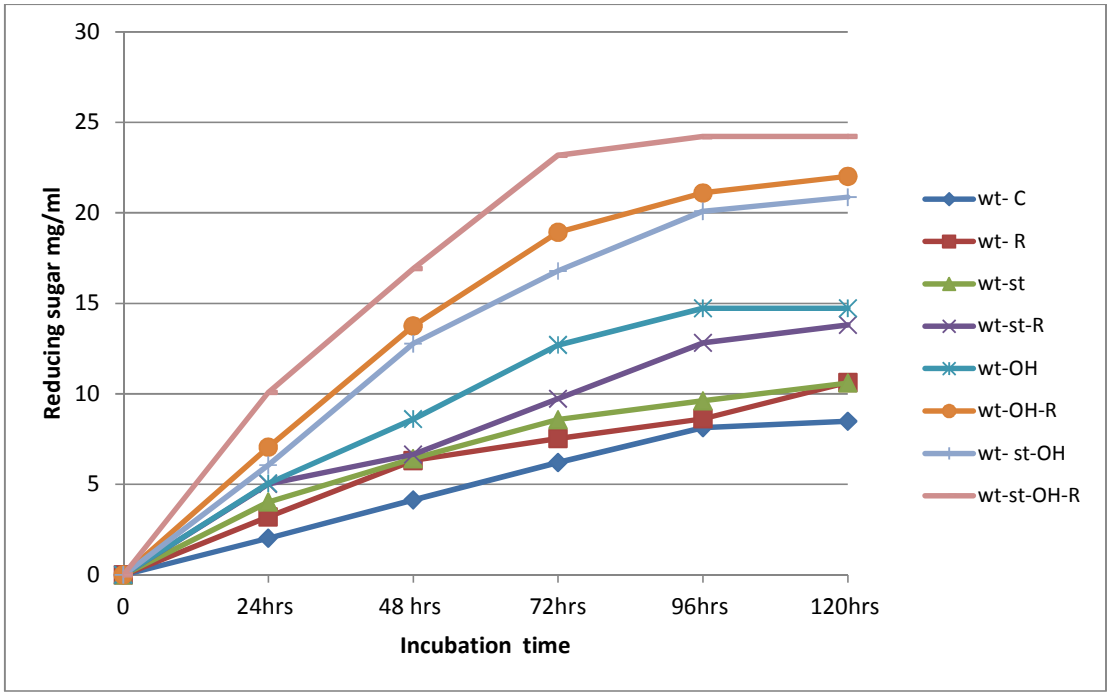


Fig. 5. Time course profile of reducing sugar production under semi- aerobic condition

fermentation (Kaur et al. 2006). The production of cellulases has been shown to be inducible and was affected by the nature of the substrates. Therefore, the choice of an appropriate inducible substrate is very important for production of optimum amount of sugar *Aspergillus terreus* VB (VI) was grown untreated and variably pretreated wheat straw and untreated wheat straw used as control substrates to determine their effect on the induction of cellulolytic enzymes.

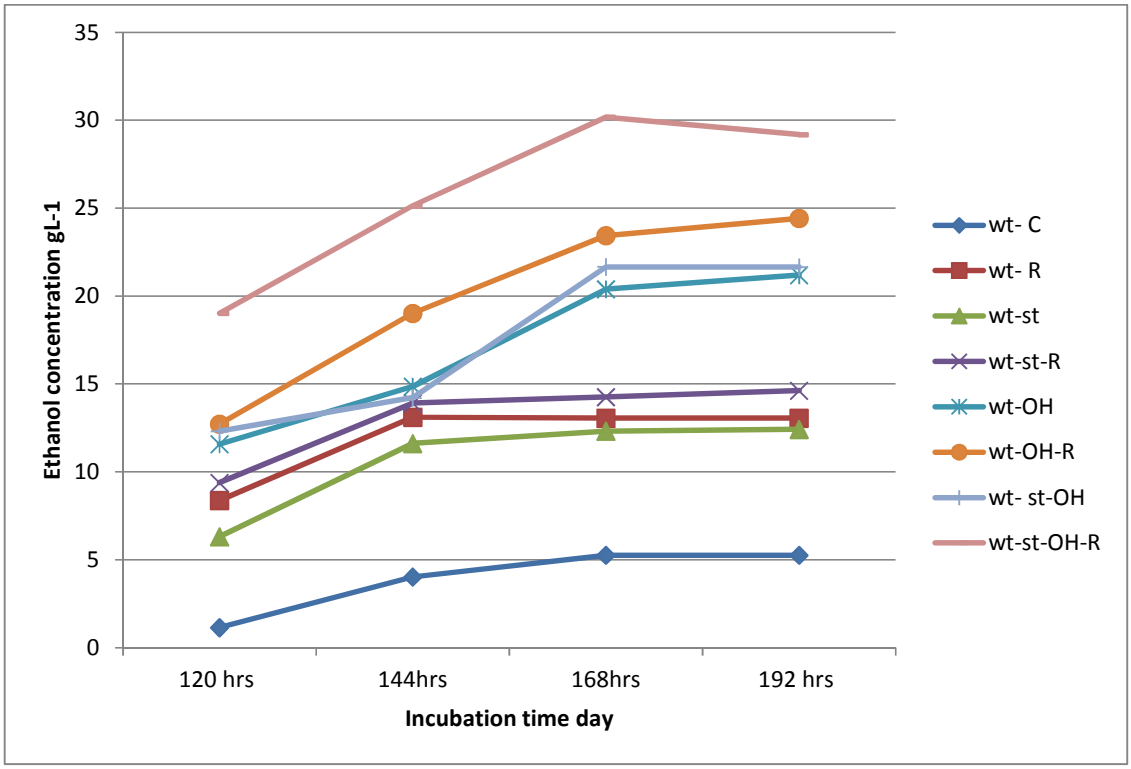


Fig. 6. Time course profile of ethanol production from pre-treated wheat straw by *Aspergillus terreus* and *Saccharomyces cerevisiae* under semi- aerobic condition

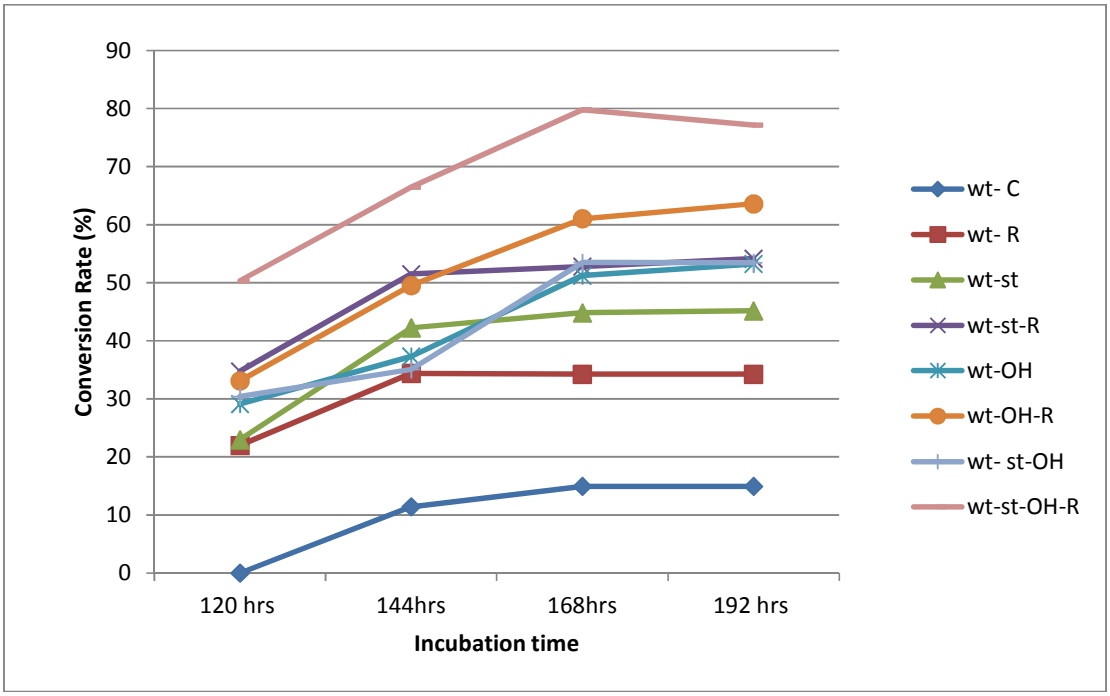


Fig. 7. Time course profile of ethanol conversion Rate (%) of various pre- treated wheat straw by using of *Aspergillus terreus* and *Saccharomyces cerevisiae.* Conversion rate was based on the untreated wheat straw

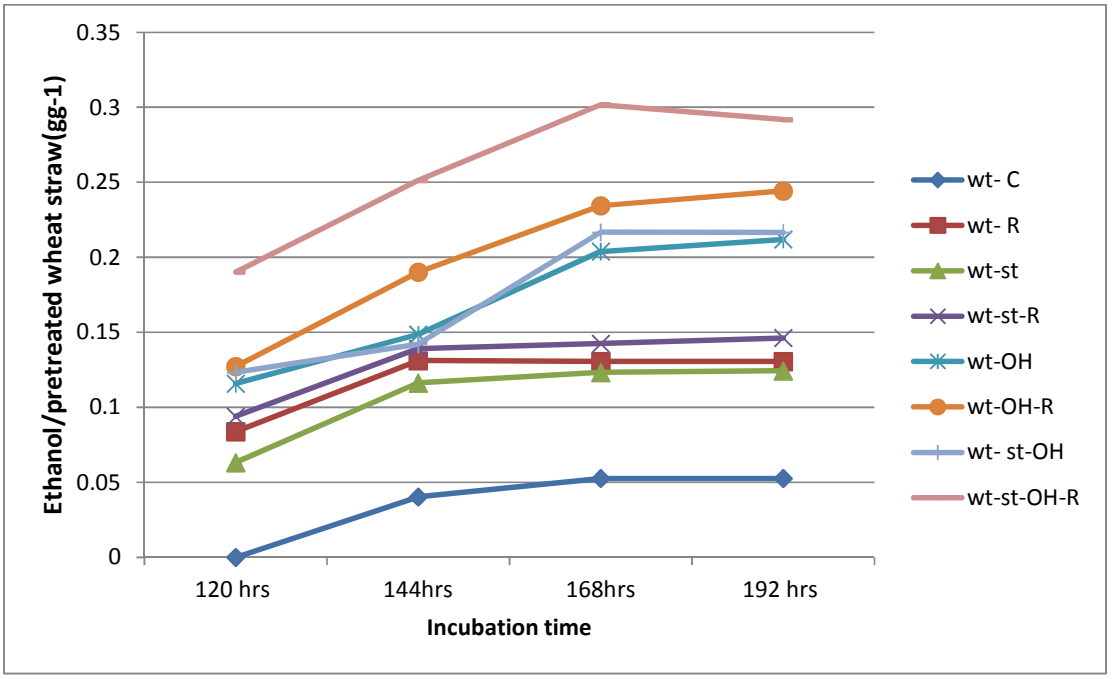


Fig. 8. Time course profile of ethanol production from pre-treated wheat straw by *Aspergillus terreus* and *Saccharomyces cerevisiae* under semi- aerobic condition

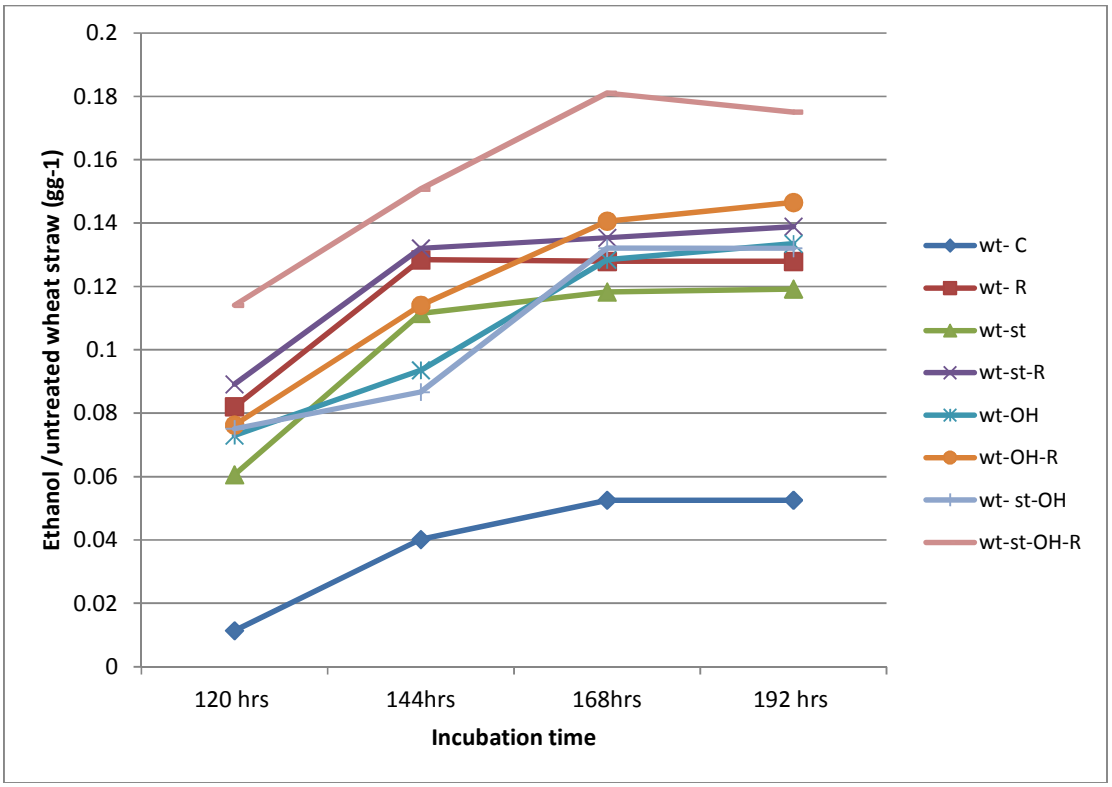


Fig. 9. Time course profile of ethanol yield (%) of various pre- treated wheat straw by using of *Aspergillus terreus* and *Saccharomyces cerevisiae.* Conversion rate was based on the original untreated wheat straw

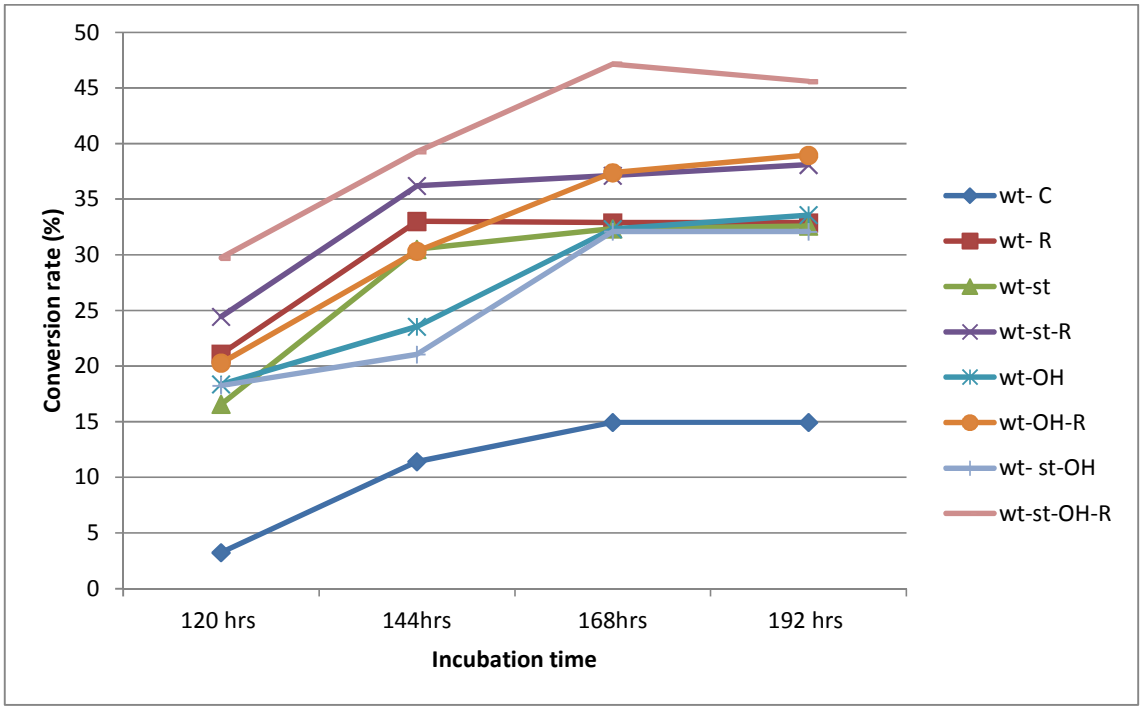


Fig. 10. Time course profile of ethanol conversion Rate (%) of various pre- treated wheat straw by using of *Aspergillus terreus* and *Saccharomyces cerevisiae.* Conversion rate was based on the original untreated wheat straw

Reducing sugar time course profile indicates the amount of released simple sugar by the Microbial hydrolysis in Fig. 6. Graph indicates the cumulative effect of irradiation, alkali and steam treatment on wheat straw ligno cellulosic materials on the production of reducing sugar in a medium contain wt- st-OH- R as substrate got saturated 3rd day from incubation. Total reducing sugar production was significantly high in medium having wt-OH-R and wt-st-R in comparison to the untreated wheat straw. Reducing sugar production got saturated after 96 hrs.

Endoglucanase activity of Strain VBIV on different pretreated substrate in Fig. 10 indicates the combination of pre- treatment method makes the wheat straw as a better substrate for endoglucanase activity. Media contains wt-st-OH-R as substrate shown maximum endoglucanase activity maybe due to swelling and delignification effect of alkali and negative effect of radiation on cellulose crystallinity and degree of cellulose polymerization. By the action of endoglucanse number of cellulose end chain upraise and subsequently increased amount total reducing sugar. Significant improved Endoglucanse activity was ordered of wt-st-OH-R > wt-OH-R > wt-st-OH.

3.5 Effect of Pretreatment Process on SHF Using *Aspergillus terrus* VB (VI) and *Saccharomyces cerevisiae*

3.5.1 Ethanol production from wheat straw hydrolysate

Major constrains in biomass to ethanol conversion is the cost of cellulase enzyme and any strategy which can bring down the production cost of cellulase can significantly reduce the cost of bio-ethanol. Therefore, microbial hydrolysates were further fermented with the use of *Saccharomyces cerevisiae* MTCC174 to produce ethanol. Different types of lignocellulosic materials produced reducing sugar in the range of 8.0 to 24.12 mg ml^{-1}. The yields of ethanol from seven different pre- treated plus contrl lignocellulosic materials with respective reducing sugar yield are given in Fig 10. The maximum ethanol yield (1.8gL^{-1}) was obtained after 168 h from hydrolysate of 10gL^{-1} pulp with production 2.5gL^{-1} reducing sugar. In wheat straw hydrolysate having initial sugar

concentration of 0.25%, the minimum ethanol yield (1.90 g/L) was obtained. The time course of ethanol production from wheat straw is shown in Fig 6. The rate of ethanol production was $0.015gL^{-1}h^{-1}$ for the initial 168h and then saturates while the yield of ethanol peaked at 72h after inoculation of *Saccharomyces cerevisiae*. There was no significant increase in the production after 72h and the ethanol concentration remained at about $3.0gL^{-1}$. Karimi et al. (2006) have used commercial cellulase for hydrolysis of rice straw along with three different microorganisms for fermentation of hydrolysate. The yield of ethanol was in the range of $0.59gL^{-1}$ to$2.91\ gL^{-1}$ after 192hrs.

Performance of *Aspergillus terrus* VB (VI) *and Saccharomyces cerevisiae in SHF* pretreated wheat straw for bioethanol production was studied. Time course of ethanol production from $10gL^{-1}$ of each pretreated wheat straw by *Aspergillus terrus* VB (VI) *and Saccharomyces cerevisiae* under semi- aerobic condition was analyzed as shown in Fig 6. Almost in each treated sample, ethanol production reaches its maximum value after 7 days of inoculation of *Aspergillus terreus* VB(VI) or after 2nd days of *Saccharomyces cerevisiae* inoculation.

3.6 Maximum Ethanol Production Time Profile and Different Substrate

3.6.1 Conversion factors

As seen in the Fig. 2, untreated wheat straw shows $0.05\ gL^{-1}$production of ethanol. The highest ethanol production of $3.01\ gL^{-1}$was observed in wt-st-OH-R treated wheat straw which corroborates 79%conversion rate of theoretical maximum value (Fig. 7).

However ethanol yield calculated according to the polysaccharide composition of initial wheat straw was 30% (Fig. 8). wt—OH-R treated wheat straw also shown forty time more bio- ethanol production then the control. Interestingly, it is observed that in all those samples which were alkali treated, the alcohol production got saturated after 3rd day while non- alkali treated samples got saturated on2nd day of fermentation. Other combination of pre-treatment method also promotes the substantial rise in bio- ethanol production.

However, It is noted that, when ethanol yield was calculated on the basis of initial wheat straw weight, highest yield was reported in wt-st-OH-R treated substrate as $296mg\ L^{-1}$ (Fig. 6) which is equivalent to18.1% of its theoretical maximum value (Fig. 9) and along with 45.6% conversion rate of its theoretical maximum (Fig. 10). Statistical analysis revealed the significance of combination of pre- treatment process for overall ethanol yield even though ethanol production is based on the original weight. However the weight loss of initial wheat straw cannot be nullified. These all results indicate that st-OH-R is most effective for improving the hydrolysis process by *Aspergillus terreus* VB(VI) on wheat straw.

Overall, upon observing the effect of various pre-treatment of wheat straw, it is detected that pre-treatment of alkali increases the substantial amount of solubilization and hence increased the production of bio-ethanol during fermentation process. However, the rate of solubilization and fermentation yield was enhanced further in combined NaOH and radiation pre- treated wheat straw. Combination of both these causes complete disintegration of fibers physically leading to enhance solubilization and fermentation process. NaOH acts asswelling agents whereas radiation reduces the crystallinity of ligno-cellulosic matrix. The relation between reduction of lignin content and increased cellulase activities in SSF by *Aspergillus terreus* VB (VI) indicates that the removal of lignin content increases the accessibility of cellulose fibers of lingo-cellulosic waste and also promotes the cellulase production. By the combination of NaOH and radiation pre- treatment increased the cellulase activity and promoted the process of solubilization. The enhanced process of solubilization increased the amount of reducing sugar which gets converted into ethanol by the fermentation process. st-OH-R pre- treated wheat straw shows 45.6% of conversion rate and 18% of ethanol yield of its theoretical maximum i.e. 180mg alcohol per gram of original wheat straw. It corroborates significant increase of bio-ethanol production in comparison to the untreated wheat straw.

4. CONCLUSIONS

This study suggested that combination approach of pre- treatment processes augment the overall hydrolysis of cellulosic material and eventually the production of ethanol production by using *Aspergillus terreus* VB (VI) and *Saccharomyces cerevisiae.* Combination of physio-chemical methods as a pre-treatment is very effective in order to make cellulose more accessible while

the process of microbial hydrolysis causes significant increase of reducing sugar under the action of strains VB (VI) *Aspergillus terreus* and increasing reducing sugar turn into alcohol by fermentation process. Production of 180mg per gram original wheat straw which makes developed approach of pre-treatment process Accountable and strains VB(VI) *Aspergillus terreus* relevant for industrial production of 2nd generation ethanol production. The huge difference in result of control and pre-treated wheat straw indicates that inferred that pretreatment method described above is promising approach for bio-ethanol production. Hence applying developed pre- treatment method with other previously reported strains may give better ethanol yield.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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