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Research Article

Amelioration of Bio-Ethanol Production by *TRICHODERMA REESEI* NCIM 992 using Saw Dust (An Industrial Waste) as a Carbon Source through Optimization

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ABSTRACT

The present study focuses on investigations pertaining to hydrolysis process development. The process of conversion of saw dust (carbon source) into ethanol carried out by *Trichoderma reesei* NCIM 992 and relative effect of physical (i.e. concentration of saw dust, pH, temperature and inoculum size) and chemical (i.e. nitrogen sources and surfactants) parameters have been investigated. For studied microbe pH 6.0 and temp 30^0C have been observed as optimum. Addition of yeast extract and beef extract significantly promoted the production of ethanol while both surfactants showed the negative impact on saccharification process.

Keywords Cellulase activity, Ethanol, Saccharification, Saw dust, *Trichoderma reesei*

INTRODUCTION

The demand of green fuel has increased recently due to high rise in oil prices and need for energy security (Pasha *et al.*, 2007; Kumar *et al.*, 2014). Several comprehensive reviews are available to fight with the economic challenge of ethanol production from lignocellulosic biomass as well as the use of ethanol as fuel and studies have shown that the production of ethanol is dependent on the availability of the sugars and the activity and concentration of cellulase enzyme produced by microbes (Eshaq *et al.*, 2011 and Rathnan and Ambili, 2011). An accountable study were carried out on the production of ethanol using *Trichoderma* sp. (Irfan *et al.*, 2010; Juwaied *et al.*, 2011; Maurya *et al.*, 2012; Hakkinen *et al.*, 2014) as a cellulase producing microorganism using different types of lignocellulosic materials as a substrate. The main object of the study

was to optimize the physical and chemical parameters for *Trichoderma reesei* NCIM 992 for maximum production of ethanol by using saw dust as carbon source (an industrial waste). In the present study, Separate Hydrolysis and Fermentation (SHF) method was adopted.

MATERIALS AND METHODS

Substrate: Sawdust was used as a carbon source collected aseptically from M/S Sharma Hardware Pvt. Ltd., District Alwar, Rajasthan, India-301001 and sun dried to reduce the moisture content.

Pretreatment of Substrate: Sawdust was soaked in 1% sodium hydroxide solution (NaOH) in the ratio 1:10 (sawdust: solution) for two hours at room temperature and autoclaved at 121^0C for one hour. The treated sawdust was then filtered and washed with distill water until the washed water become neutral **(**Soloman *et al.*, 1999 and G. Immanuel *et al.*, 2007) and then dried at 50^0C for overnight.

Microorganism: *Trichoderma reesei* NCIM 992 and *Saccharomyces cereiviase* NCIM 3494**,** used for the present study was procured from the National Collection of Industrial Microorganisms, Pune, India.

Maintenance of culture and Inoculum Preparation: Culture of *T. reesei* received in culture tube from NCIM, Pune was sub cultured on Sabouraud Dextrose Agar (SDA) plates at 28ºC for 7 days and stored thereafter in refrigerator at 4ºC till further use. Inoculum for fungus was prepared in Potato Dextrose Broth (PDB). About 100 ml of inoculum was prepared for fungal culture in 250 ml Erlenmeyer flask. Few mycelia were picked up from 7 days old culture and were inoculated at 28ºC on a rotary shaker (200rpm) for twenty four hours, before it was used for the saccharification process. *S. cereiviase* was maintained on Yeast Potato Dextrose Agar (YPDA) at 4^0C.

Minimum Culture Medium Preparation: The media contained following chemicals (g/l of distilled water); Na_2HPO_4 (1.4), KH_2PO_4 (2.0), $CaCl_2.2H_2O$ (0.4), $MgSO_4.7H_2O$ (0.3), $MnSO_4.7H_2O$ (0.016), $ZnSO_4.7H_2O$ (0.014) and $FeSO_4.7H_2O$ (0.05). The pH of culture medium was set as 5.62±0.2.

Saccharification and Fermentation: 100 ml of the media was taken in 250 ml Erlenmeyer flask and sterilized by autoclaving 121°C for 15 min and cooled. Autoclaved sawdust was added in flask before inoculation then inoculated with 2ml of inoculum of *T. reesei* NCIM 992, under controlled conditions and incubated at 30ºC for 5 days. This culture was harvested after 48 hours under aseptic conditions and 4 ml (4%) inoculum of *S. cerevisiae* was added to all the flasks. This period was carried out for a period of 6 days at 28^0C. Three replicates were set for each treatment.

Biochemical assays: Ethanol estimation was performed by spectophotometric method (Caputi *et al*., 1968). The amount of total soluble sugar was estimated by phenol sulfuric acid reagent method and reducing sugars were determined by dinitro salicylic (DNS) method (Goel R.K., 2007). Cellulase activity of resultant enzyme was determined by using Carboxymethyl cellulose (CMC) as a substrate (Ghose, 1987).

Effect of Concentration of Saw dust: To optimize the concentration of saw dust, minimal culture medium was prepared in 250 ml conical flasks for the individual microbe by setting the different concentrations of saw dust such as 1%, 2%, 3%, 4% and 5% (w/v) respectively in triplet. The pH of minimal culture medium was set on 5.6±0.2. The optimum saw dust concentration that was efficiently utilized by microbe for total soluble sugar production was observed and the same concentration of saw dust was used for setting experiments for optimization of other factors.

Effect of pH: Minimal culture medium was prepared for and pH was set at different level such as 5.0, 6.0, 7.0, 8.0 and 9.0 by adding 1% NaOH and concentrated HCl respectively. They were tested for saccharification using optimum saw dust concentration.

Effect of Temperature: To optimize the saccharification temperature, process was carried out at 25, 30, 35, 40 and 45ºC respectively.

Effect of Inoculum Size: To study the effect of inoculum sizes of bacteria and fungi on saccharification process, different size of inoculum were set, such as 1%, 2%, 3%, 5% and 10% at pH 5.6±0.2 in triplet and the all flasks were placed in an incubator at 28ºC for 5 days.

Effect of nitrogen sources and surfactants: 1% concentration of different nitrogen sources i.e. ammonium nitrate, sodium nitrate, ammonium dihydrogen phosphate, ammonium sulphate, potassium nitrate, urea, peptone, yeast extract and beef extract were studied in triplet using 4% (w/v) optimum saw dust concentration, at optimized pH and temperature. 0.5 % concentration of surfactants (SDS and CTAB) was used to study the impact of surfactants.

Ethanol Production Ethanol yield was estimated using 4% (w/v) concentration of saw dust, 2% inoculum size with optimized pH and temperature of *Trichoderma reesei* NCIM 992.

Statistical Analysis The raw data obtained was further subjected to data analyses for mean (X), standard deviation (SD), strandard error (SE), correlation coefficient, percent of control and ANOVA

Results and Discussion

Optimization of concentration of saw dust: Hydrolytic formation of total soluble sugars, reducing sugars and cellulose activity using *Trichoderma reesei* is shown in Fig. 1. The highest yield of total soluble sugars (0.974 mg/ml), reducing sugars (0.519 mg/ml) and cellulase activity (28.27 U/g) was recorded at 5% w/v concentration of saw dust at 28^0C and 5.6± 0.2 pH. Maximum activity has been achieved at an optimal concentration 4% of various substrates using different fungal cells (Andrade *et al.*, 2011 from wheat bran using *Trichoderma* sp. and Gautam *et al.*, 2011 from municipal solid waste using *A. niger)*. Ramanathan *et al.* (2010) have recorded 1% concentration of CMC optimum for the enzyme activity by *T. harzianum.*

Optimization of pH: The data presented in Fig. 2 shows the effect of pH value by *Trichoderma reesei* on the production of total soluble sugars, reducing sugars and cellulase activity at different pH. Among different pH tested, pH 6.0 registered optimum for the highest yield of total soluble sugars (1.211 mg/ml), reducing sugars (0.825 mg/ml) and cellulase activity (38.36 U/g) at 5% (w/v) saw dust and 28^0C. Cellulase activity was obtained best at pH from 5.6 to 5.8 (Baig, 2005). Several researches have shown pH 5.0 as an optimum pH for *Trichoderma* sp. (Shiahmorteza *et al.*, 2003; Andrade *et al.*, 2011; Maurya *et al.*, 2012).

Optimization of temperature: The data on the effect of temperature on the production of total soluble sugars, reducing sugars and cellulase activity by *Trichoderma reesei* is shown in Fig 3. The optimum temperature was found 30^0C at 5% (w/v) saw dust concentration and 5.6±0.2 pH, the production of biochemicals decreased thereafter. At optimum temperature, TSS, RS and CA were recorded as 0.861 mg/ml, 0.452 mg/ml and 31.56 U/g, respectively. Further increasing in temperature up to 45^0C led to decrease in the production of total soluble sugar, reducing sugar and cellulase activity. This result was in correlation with the findings of many other authors. Maximum activity was achieved at an optimal temperature 30^0C (Liu & Yang, 2007; Maurya *et al*., 2012). However, the results appear to contradict previous findings in which optimum temperature was 50^0C for *T. reesei* NCIM 1052 when the Groundnut and soya bean meal was used. (Sathyavrathan and Krithika, 2013).

Optimization of inoculum size: Maximum production of total soluble sugars (0.926 mg/ml), reducing sugars (0.700 mg/ml) and cellulase activity (29.18 U/g) were achieved at 3% to 5% (v/v) inoculum sizes with optimum size 5% at 28ºC, 5% (w/v) saw dust concentration and 5.6± 0.2 pH as shown in Fig. 4. Thanapimmetha *et al.*, 2012, have been observed 10.5% inoculum size as an optimum for maximum cellulase activity with of *T. harzianum* using sweet sorghum bagasses as substrate. Leghlimi *et al.*, 2013, have reported that Higher concentration i.e. 10^5-10^8 spores/ml inoculum level of *T. longibrachiatum* (GHL)

found optimum for cellulase activity when cellulose avicel used as carbon source.

Impact of Nitrogen sources and surfactants: The data summarized in Fig. 5 and Fig. 6 indicated the impact of various inorganic and organic nitrogen sources and surfactants on production of total soluble sugars and ethanol by *Trichoderma reesei.* The results showed that yeast extract (111% and 113% of control) and beef extract (104% and 103% of control) led to maximum production of total soluble sugars and ethanol respectively while remaining nitrogen sources produced almost same and lesser quantity in comparison of control respectively and both surfactants (SDS and CTAB) showed the negative effect on production.

Ethanol Production Further ethanol yield has been estimated 1.12g/100g using 4% (w/v) concentration of saw dust, 2% inoculum size with optimized pH (6.0) and temperature (30^0) of studied *Trichoderma reesei* NCIM 992.

CONCLUSION

The present study points out that ethanol production from waste like saw dust is also promising. Nevertheless, edible sources like corn and sugarcane hold greater efficiency for ethanol production, at the same time these cash crops are highly valuable from food and fodder point of view. Ethanol production from saw dust though is not very efficient at the moment but still future research could provide some integrated mechanism to enhance the production from this waste biomass.

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Figures

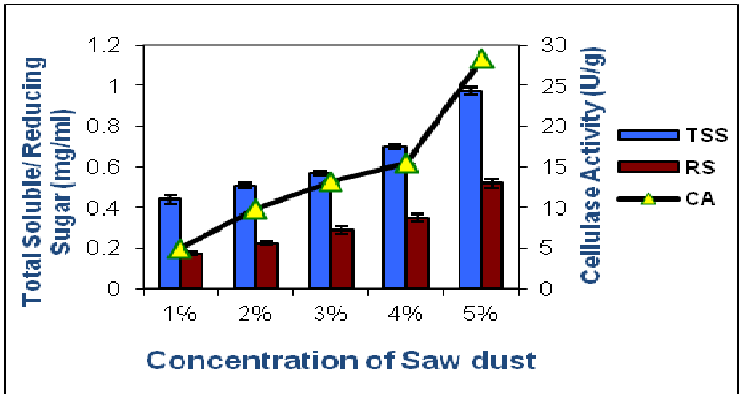

Figure 1: Effect of concentration of saw dust on production of total soluble sugar, reducing sugar and cellulase activity (U/g) at pH 5.6± 0.2; 28^0C.

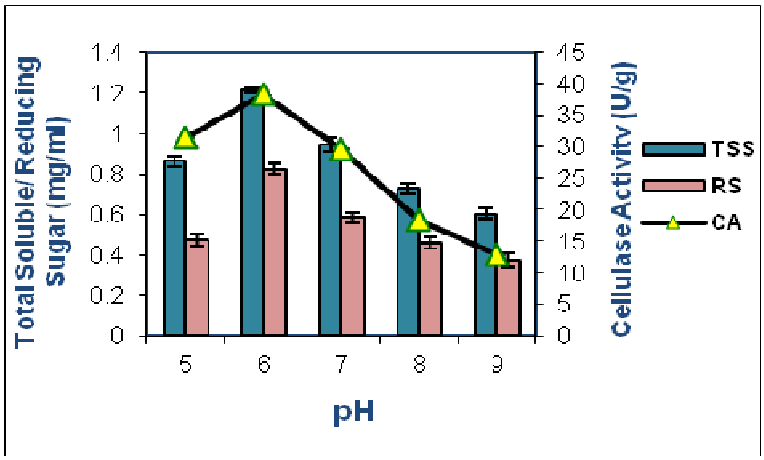

Figure 2: Effect of pH on production of total soluble sugar, reducing sugar and cellulase activity (U/g) at 28^0C using 5% substrate concentration.

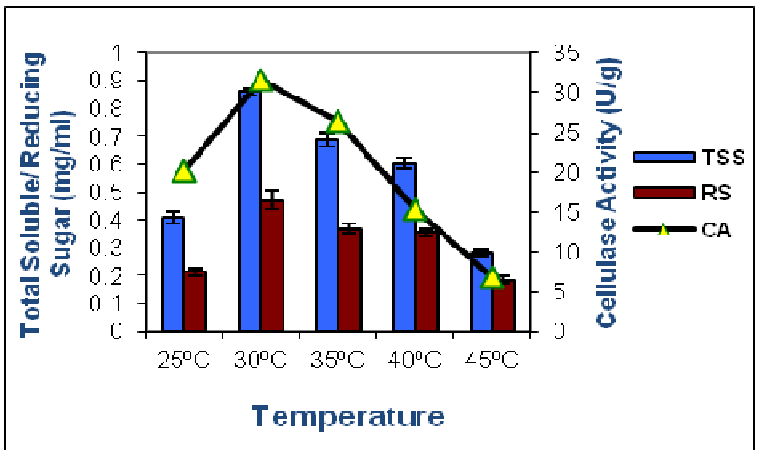

Figure 3: Effect of temperature on production of total soluble sugar, reducing sugar and cellulase activity (U/g) using 5% substrate concentration.

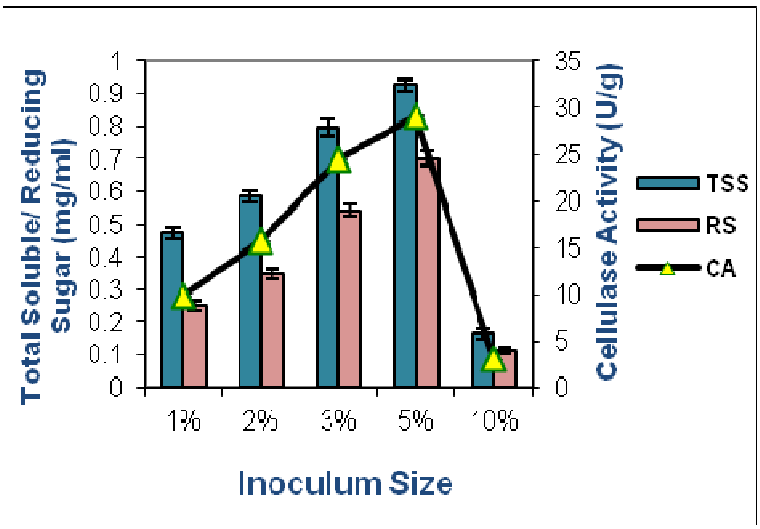

Figure 4: Effect of inoculum size on production of total soluble sugar, reducing sugar and cellulase activity (U/g) at pH 6.0± 0.2; 30^0C and 5% substrate concentration.

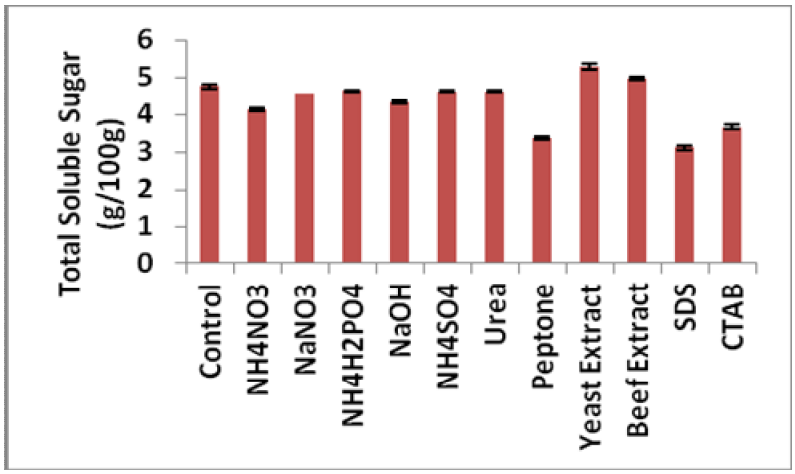

Figure 5: Effect of nitrogen sources and surfactants on production of total soluble sugar using 5% concentration of saw dust at pH 6.0± 0.2 and temp. 30^0C.

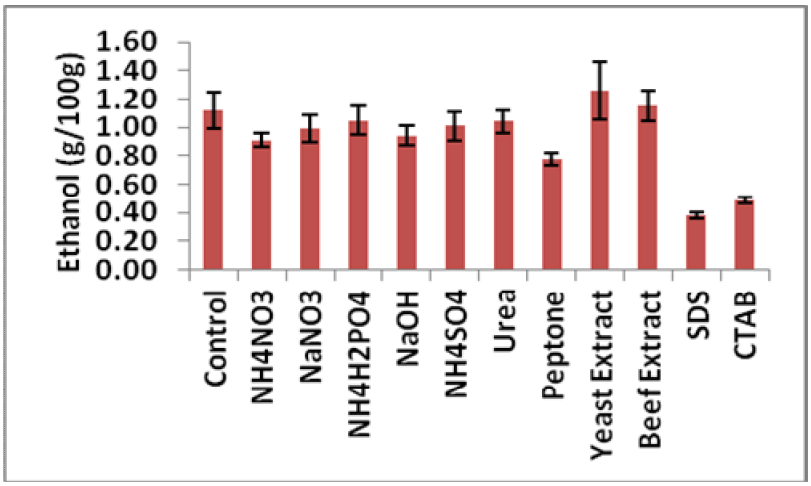

Figure 6: Effect of nitrogen sources and surfactants on production of ethanol using 5% concentration of saw dust at pH 6.0± 0.2 and temp. 30^0C.