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## RESEARCH ARTICLE

### OPTIMIZATION OF CELLULASE AND BIOETHANOL PRODUCTION USING SAW DUST AS SUBSTRATE BY *BACILLUS SUBTILIS* MTCC 121

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#### ABSTRACT

Present study was aimed to determine the optimum conditions (Concentration, pH, Temperature and Inoculum size) of *Bacillus subtilis* MTCC 121 for cellulase enzyme production and to examine the effect of inorganic and organic nitrogen sources and surfactants on the saccharification process from saw dust. The highest cellulase activity was observed at 4% concentration of pretreated saw dust (lignocellulosic biomass). Optimum pH and temperature of the medium for cellulase production by *B. subtilis* MTCC 121 were 8.0 and 40°C. NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> and Urea were found to be best nitrogen sources for bioethanol production where as surfactants showed negative impact on saccharification process.

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#### INTRODUCTION

Lignocelluloses are sustainable sources of renewable bio-energy in the world (Joshi *et al.*, 2011). The major challenge of the production of second generation biofuels is to convert the lignocellulosic biomass into ethanol using cost effective, fast, and efficient methodologies (Laluce *et al.*, 2012). The main chemical components in lignocellulosic biomass that gives plants their rigidity are cellulose (40-50%), hemicellulose (15-25%), lignin (15-30%) and extractives (5-35%). The structure can be described as a skeleton of cellulose chains embedded in a cross-linked matrix of hemicellulose surrounded by a crust of lignin (Saxena *et al.*, 2009; Limayem and Ricke, 2012). The saccharification of the cellulose and hemicellulose components to fermentable sugars is a crucial step in the bioconversion of lignocellulosic feedstocks (Hu *et al.*, 2011). The cellulosic enzyme system consists of three major enzymes endo-glucanase, exo-glucanase and -glucosidase, which act in synergism and to convert cellulose into fermentable sugars (Rajoka *et al.*, 1998 and Devi and Kumar, 2012).

The main object of the study was to optimize the environmental parameters for *Bacillus subtilis* MTCC 121 for maximum enzyme and sugar production efficiency. To study the impact of inorganic and organic nitrogen sources on ethanol production, saccharification process was carried out by microorganism at optimized culture conditions (pH and temp) followed by fermentation process. In this present study, Separate Hydrolysis and Fermentation (SHF) method was adopted and saw dust was used as substrate.

#### MATERIAL AND METHODS

**Collection of 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:** The saw dust was sieved in muslin cloth to get powder. The fine saw dust powder was soaked in 1% sodium hydroxide solution in 1:10 ratio (saw dust: solution) for two hours at room temperature. Then, it was washed with hot water twice to remove chemicals and autoclaved. The treated saw dust was then filtered and washed with distilled water until the filtrate became neutral. This washed saw dust was dried and used for analysis. (Soloman *et al.*, 1999; Immanuel *et al.*, 2007)

**Organism and Inoculum preparation:** *Bacillus subtilis* MTCC 121 and *Saccharomyces cerevisiae* NCIM 3494, used for the present study were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India and National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India respectively. *B. subtilis* MTCC 4643 was maintained on Nutrient Agar (NA) plates at 4°C and inoculum was prepared in Nutrient Broth. *S. cerevisiae* NCIM 3494 was maintained on Yeast Potato Dextrose Agar (YPDA) at 4°C.

**Production media:** The media contained following chemicals (g/l) in distilled water; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1.3), KH<sub>2</sub>PO<sub>4</sub> (0.37), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.07), MgSO<sub>4</sub>·7 H<sub>2</sub>O (0.25), FeCl<sub>3</sub> (0.02), Yeast Extract (1.0) was prepared. The pH of culture medium was set on 7.2±0.2.

**Saccharification and Fermentation:** 100 ml of the production media was taken in 250 ml Erlenmeyer flask and sterilized at 121°C for 15 min. and cooled. Autoclaved sawdust was added in flask and inoculated with 2ml of inoculum of *B. subtilis* MTCC 121, under controlled conditions and incubated at 30°C for 24 h. This Culture was harvested after 24 hours under aseptic conditions and 4 ml (4%) inoculum of *S. cerevisiae* culture was added to all the flasks. This process was carried out for a period of 6 days at 28°C. Three replicates were set for each treatment.

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

**Effect of concentration of saw dust:** To optimize the concentration of saw dust, pretreated saw dust used 1, 2, 3, 4 and 5% w/v, in 100 ml of minimum culture medium. After inoculation, flasks were incubated at 30°C.

**Effect of pH:** To optimize the pH value of minimum culture medium, was adjusted by using 1% NaOH and concentrated HCl to 5.0, 6.0, 7.0, 8.0 and 9.0.

**Effect of Temperature:** Different temperatures were applied 25, 30, 35, 40 and 45°C for the optimization of temperature.

**Effect of inoculum size:** The inoculum size was optimized by measuring the enzyme activity at different inoculum sizes ranging from 1.0, 2.0, 3.0 5.0 and 10.0% v/v.

**Effect of nitrogen sources and surfactants:** Different inorganic nitrogen sources i.e. ammonium nitrate, sodium nitrate, ammonium dihydrogen phosphate, ammonium sulfate and potassium nitrate and organic nitrogen sources i.e. Urea, Peptone, Yeast extract and Beef extract were studied in triplet using 4% initial saw dust concentration at optimized pH and temperature (based on studied results). These all sources were used in 1% concentration. 0.5% concentration of SDS and CTAB was used as surfactants.

## RESULTS AND DISCUSSION

**Effect of Concentration of Saw Dust:** The optimum concentration of saw dust (4%) gave maximum production of total soluble sugar (0.433 mg/ml), reducing sugar (0.283 mg/ml) and cellulase activity (20.35 U/g) shown in Fig. 1. Kathiresan *et al.* (2011) investigated that *Pichia salcaria* exhibited maximum enzyme activity at 2% concentration of saw dust. However Shabeb *et al.* (2010) reported that *Bacillus subtilis* KO exhibited maximum activity at 10% concentration of molasses.

**Effect of pH:** As shown in Fig.2 the enzyme shows high activity over a wide range of pH 6.0-8.0 with maximum activity (12.08 U/g) at pH 8.0 and maximum production of total soluble sugar (0.564 mg/ml) and reducing sugar (0.344 mg/ml) was recorded at the same pH. Verma *et al.* (2012) and Shanmugapriya *et al.* (2012) reported that the *Bacillus* sp. exhibited maximum enzyme activity at pH 6.5-7.5. However Shankar & Isaiarasu (2011) and Ariffin *et al.* (2006) revealed that *B. pumilus* produced maximum cellulase at pH 6.0, where as in *B. circulans* pH 9.0 was found optimum for production(Nirmala & Sindhu, 2011).

**Effect of Temperature:** As shown in Fig. 3 initial temperature has profound effect on cellulase production. The highest cellulase activity (15.28 U/g) by *B. subtilis* MTCC 121 observed at 40°C, the maximum yield of total soluble sugar and reducing sugar were 0.499 mg/ml were 0.321 mg/ml respectively. Shankar & Isaiarasu (2011) and Samuel *et al.* (2011) reported that the *Bacillus* sp. exhibited maximum enzyme activity at temperature 37°C. On the other hand

Ariffin *et al.* (2006) revealed temperature 60°C optimum for cellulase production by *B. pumilus* EB3.

**Effect of Inoculum Size:** The data showed in Fig. 4 indicated that 2% inoculum size gave maximum yield of total soluble sugar (0.433 mg/ml), reducing sugar (0.251 mg/ml) and cellulase activity (17.31 U/g). Further increase or decrease in inoculum size affects the production. Azzaza *et al.*, (2012) reported that decrease in cellulase production with further increase in inoculum might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and enzyme release. Shankar & Isaiarasu (2011) reported that CMC had maximum enzyme production with 2% of inoculum size of *B. pumilus* which was in good agreement with our findings.

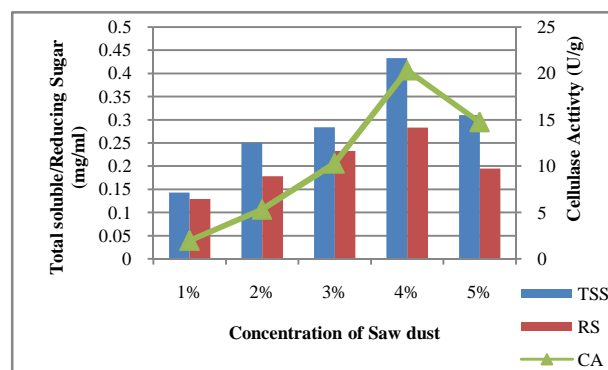


Figure 1 Effect of concentration of saw dust on Production of total soluble sugar, reducing sugar and cellulase activity (U/g) at pH7.0±0.2; 30 °C.

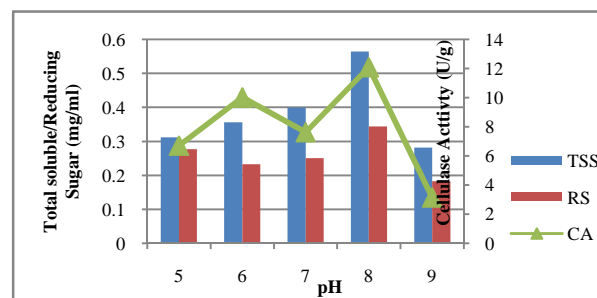


Figure 2 Effect of pH on production of total soluble sugar, reducing sugar and cellulase activity (U/g) at 30 °C using 4% substrate concentration.

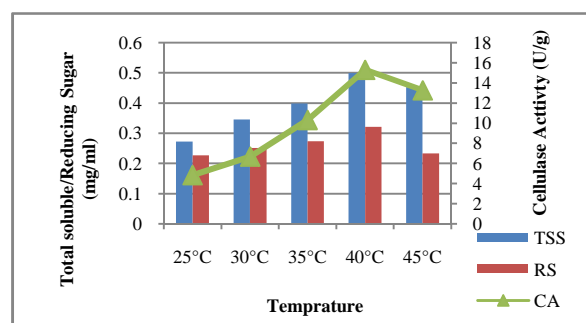


Figure 3 Effect of temperature on production of total soluble sugar, reducing sugar and cellulase activity (U/g) using 4% substrate concentration.

**Effect of Nitrogen sources and Surfactants:** The data shown in Fig. 5 and Fig. 6 revealed that among nitrogen sources tested, Urea and NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> served as intensive source to *B.subtilis* MTCC 121. The total soluble sugar and ethanol increased by 107% and 121% of control and 102% and 112% of control respectively. Both SDS and CTAB showed adverse effect to *Bacillus subtilis*. The results recorded by Shabeb *et*

al. (2010) revealed that the addition of  $(\text{NH}_4)_2\text{PO}_4$  and tryptone to molasses medium enhanced cellulase productivity by *B. subtilis* KO. In another study, Shankar & Isaiarasu (2011) reported that malt extract and ammonium molybdate supplemented medium help to improve enzyme production and inhibited with use of urea, glycine and SDS when CMC used as a substrate by *B. pumilus*. Shanmugapriya et al. (2012) found the maximum enzyme activity with yeast extract as nitrogen source by *Bacillus* sp. when sawdust was used as a substrate.

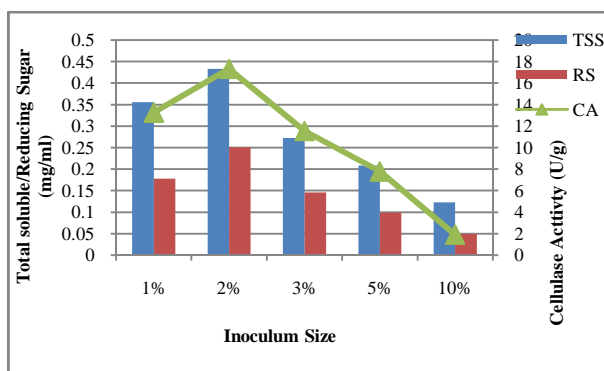


Figure 4 Effect of inoculum size on production of total soluble sugar, reducing sugar and cellulase activity (U/g) at pH8.0±0.2; 40 °C and 4% substrate concentration.

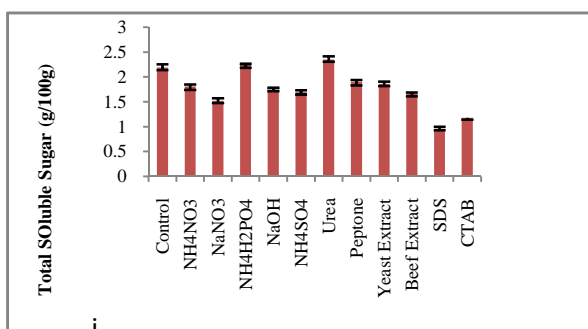


Figure 5 Effect of nitrogen sources and surfactants on production of total soluble sugar using 4% substrate concentration at pH8.0±0.2; 40 °C

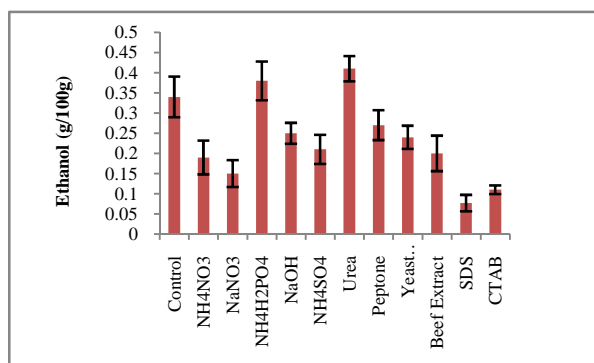


Figure 6 Effect of nitrogen sources and surfactants on production of Ethanol using 4% substrate concentration at pH8.0±0.2; 40 °C.

## CONCLUSION

The results of present study have shown potential of *Bacillus subtilis* MTCC 121 to produce significant amount of bioethanol using saw dust as a substrate. It can be concluded that saw dust is beneficial raw material as it is available abundantly and more importantly it is renewable.

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