Biodegradation and delignification of sugar cane bagasse of pulp and paper mill effluent by *Cryptococcus albidus* for production of bioethanol

Ashwini Kumar Ray, Anjali singhal, Umesh C. Naik, Indu Shekhar Thakur

School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, 110067, India; E-mail: isthakur@hotmail.com, isthakur@mail.jnu.ac.in

**ABSTRACT**

The study aims to extend the range of resources from which ethanol may be made from sugar cane bagasse which is used primarily for preparation of pulp and paper. Seven fungal strains isolated from sediment and degraded wood of pulp and paper mill effluent in which one strain identified by 18S rDNA internal transcribed spacer (ITS) as *Cryptococcus albidus* had higher decolourization and delignification potency. The production of sugar by *Cryptococcus albidus* was increased (1.5 fold) in presence of sugarcane bagasse after optimization of process parameters by Taguchi approach. The sugar cane bagasse treated initially by fungus (saccharification) subsequently treated by *Saccharomyces cerevisiae* indicated production of ethanol (38.4g/L). However, an increase in 1.82 fold (70.0g/L) ethanol was measured after optimization of fermentation process by Taguchi approach where carbon (2%), nitrogen (0.24%), phosphate (0.24%) temperature (35°C), stirring (150) and pH (5.5) were used, indicated use of sugarcane bagasse of pulp and paper mill effluent for production of ethanol.

**Keywords**: bioethanol, *Cryptococcus albidus*, *Saccharomyces cerevisiae*, sugar cane bagasse, Taguchi approach

**INTRODUCTION**

Production of ethanol has been initiated by natural and potentially cheap and abundant polymer of agricultural waste (wheat straw, corn stalks, soybean residues and sugar cane bagasse), Bermuda grass, reed, rapeseed, industrial waste (pulp and paper industry), forestry residues and municipal solid waste [1]. Current ethanol production processes using lignocellulose are well established, however, utilization of a cheaper substrate such as sugarcane bagasse could make bioethanol more competitive with fossil fuel and also in mitigation of green house gases. The processing and utilization of lignocellulosic substrate is complex, differing in many aspects from crop-based ethanol production.

Cellulosic feedstock consists of lignocelluloses which are mainly comprised of cellulose, a polymer of six-carbon sugar, glucose; hemicellulose, a branched polymer comprised of xylose and other five-carbon sugars, and lignin consisting of phenyl propane units [2]. Lignocellulose such as sugar cane bagasse is difficult to hydrolyze using only cellulolytic enzymes due to its recalcitrant and heterogeneous nature. The chemistry and biotechnology of sugarcane bagasse has been reviewed [3]. Sugarcane bagasse has a complex structure, and is primarily composed of lignin (25%), hemicellulose (25%) and cellulose (40–50%) [3,4]. Conversion of sugarcane bagasse into fermentable sugars is possible through thermal, chemical or enzymatic hydrolysis [5]. Dilute sulfuric acid hydrolysis is thought to be one of the promising pretreatment methods and was extensively employed in industry. Pretreatment technology is important steps for improving the conversion of cellulose to glucose in following enzymatic hydrolysis [6]. Prior to ethanol
fermentation by a microorganism, the feedstock needs to be processed by saccharification technology in order to release fermentable sugars. There are three classes of enzymes acting synergistically in cellulose hydrolysis: endoglucanases, exoglucanases and β-glucosidases. Lignocellulose as a feedstock presents two major challenges for ethanol production due to recalcitrance to biodegradation and presence of pentose and hexose sugars [1,7].

Industrial biocatalysts, such as the common yeast, *Saccharomyces cerevisiae*, rarely possess native pathways able to efficiently ferment both hexoses (such as glucose) and pentoses (such as xylose). Metabolic engineering through genetic modification may be an effective means of manipulating the capabilities of the microorganisms reported in *Escherichia coli*, *Klebsiella oxytoca*, *Zymomonas mobilis* and *S. cerevisiae* [7]. Enzymatic hydrolysis can be used for obtaining fermentable sugars from the polysaccharides contained in lignocellulosic biomass. However, since the access of native cellulose to the enzymes is poor, a pretreatment step is required to improve the enzymatic convertibility and to lower the cost of the process [8-15]. Enzymatic hydrolysis of such cellulosic material by cellulase enzymes is the most promising approach to get high product yields vital to economic success. The presence of lignin limits the usage of cellulose and hemicellulose which act as an inhibitor in formation of fermentable product. In order to convert these energy rich molecules into simpler forms, it is necessary to remove the lignin from lignocellulosic materials. Kaya et al. [16] reported that during enzymatic hydrolysis of lignocelluloses biomass, cellulase components, β-glucosidase and endoglucanase had more binding affinity towards lignin than to the carbohydrates, resulting in lower efficiency of saccharification. Hence, to achieve maximum hydrolysis of cellulosics, which is a prerequisite for ethanol fermentation, an appropriate delignification treatment of biomass is required. The cellulose and hemicelluloses sugars obtained through acid and enzymatic hydrolysis can efficiently be used for ethanol fermentation either by separate fermentation of individual hydrolysate or fermentation of mixed hydrolysate using co-culture. However, in co-culture cultivation, optimum growth conditions of the yeasts would be different and might result in lower efficiency and lower product yield. Therefore, proper optimization of fermentation processes is significant for ethanol preparation.

Tian et al. [17] reported *Saccharomyces cerevisiae* efficiently converts both glucose and mannose into ethanol, but is unable to convert xylose into ethanol. Other yeast species, e.g. *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus*, have been found to be highly efficient xylose-fermenting strains that can be used in ethanol production [18]. However, these yeasts have a relatively low ethanol yield and inhibitor tolerance. For enhanced production of sugar and ethanol, it is essential to optimize the composition of culture media and process conditions. There are several factors that affect enzymatic hydrolysis of cellulose including substrates, cellulase activity, and reaction conditions (temperature and pH). To improve yield and rate of the enzymatic hydrolysis, research has focused on the optimization of the hydrolysis process and enhancement of cellulase activity. In conventional methods, numerous experiments have to be carried out to optimize all the parameters (factors) to establish best possible culture conditions by interrelation of all the parameters. In these methods, studying one variable at a time is cumbersome and uneconomical. Another approach is to use statistical tools and experimental designs. Taguchi methods have been widely used to optimize the reaction variable by devising minimum number of experiments. This approach also facilitates to identify the impact of individual factor and find out the link between variables and operational conditions. Analysis of the experimental data using the ANOVA (analysis of variance) and factors effect, gives the output that is statistically significant [19,20]. In the present work, a lignin degrading fungus, *Cryptococcus albidus*, isolated from soil, sediment and degrading wood of pulp and paper mill effluent was applied for preparation of cellulolytic hydrolysate from sugarcane bagasse initially treated in alkaline condition. *Saccharomyces cerevisiae* optimized for production of ethanol by Taguchi method was subsequently applied for enhanced fermentation from cellulolytic hydrolysate of sugarcane bagasse.
MATERIALS AND METHODS

Sites for sample collection

For isolation of fungal strains, sediment and degraded wood in effluent was collected from two different places: Kaccha nala outside the Century Pulp and Paper Mill, Ghanshyam Dham, Lalkuan, Nainital, Uttaranchal, India, and a drain near Anand Tissue Paper Mill, Meerut, Uttar Pradesh, India. The samples were collected in clean plastic containers, immediately brought to the laboratory and stored at 4°C until further use. The effluent of pulp and paper mill collected was of pulping stage used for decolourization and delignification studies.

Sources of lignocellulose

Sugarcane bagasse suspended in industrial effluent were kindly provided by a medium scale pulp and paper mill, Anand Tissue Mill, Meerut, Uttar Pradesh, India, uses kraft pulping only (no bleaching). The sugarcane bagasse was recovered after filtration through cheese cloth, thoroughly washed with distilled water, dried in an oven at 70°C for 72h, and finally ground in a small disc mill.

Isolation of fungi from the sediments and degraded wood

Fungi were isolated from soil and sediments and degraded wood; mixed with sterilized water in the ratio 1:10 w/v. It was kept standing at room temperature for 2 hrs. The supernatant was serially diluted with autoclaved double distilled water to $10^{-1}$, $10^{-3}$, $10^{-5}$ dilutions. Diluted sample (100µl) was spread on the potato dextrose agar plate and incubated at 30°C for 4 days. The microbial colonies (fungal) appeared on the PDA plates were then isolated and purified [21]. The fungal strains were observed under a microscope, Olympus and Magnus MLX-TR, at 40X and 100X having camera attached with it. Fungal mycelium, spores and the spore attachment was observed.

Identification of fungus and culture condition

Genomic DNA was extracted and internal transcribed spacer (ITS) regions were amplified using primers ITS1 having sequence 5’ TCCGTAGGTGAACCTGCGG 3’ and ITS4 having sequence 5’ TCCTCCGCTTATTGATATGC 3’ as described earlier for identification of fungi [21]. *Saccharomyces cerevisiae* was obtained from Institute of Microbial Technology, Chandigarh, India, was maintained on agar slants containing (g/L): glucose, 10.0; yeast extract, 3.0; peptone, 5.0; agar, 20.0 at pH 5.0 ± 0.2 and temperature 30°C. *Saccharomyces cerevisiae* inoculum was grown for 48h at 30°C in a culture medium as described above for fermentation processes.

Screening of potential fungal strains for decolorization and delignification of effluent

The fungal strains isolated were screened for their decolourization and lignin reduction potential. MSM-effluent i.e. MSM (in g/l: Na$_2$HPO$_4$.2H$_2$O, 7.8; KH$_2$PO$_4$, 6.8; MgSO$_4$, 0.2; Fe (CH$_3$COO)$_3$.NH$_4$, 0.01; Ca(NO$_3$)$_2$.4H$_2$O, 0.05) having 10% pulp and paper mill effluent, inoculated with individual fungal isolates, were incubated at 30°C, pH 7 in a rotary shaker, rpm 125 for 10 days. The parameter colour and lignin were estimated at an interval of 0, 1, 3, 5, and 10 day. The colour content was measured after centrifugation of samples at 10,000 rpm for 10 min. pH was adjusted to
7.6 and absorbance was determined at 465 nm [22]. Lignin in the effluent was estimated by reaction of effluent with acetic acid, sodium nitrite and ammonium hydroxide, and measuring the absorbance at 430 nm [22].

**Preparation of sugarcane bagasse for degradation**

The sugarcane bagasse was recovered after filtration through cheese cloth, thoroughly washed with distilled water, dried in an oven at 70°C for 72h, and finally grinded in a small disc mill. Steam treatment was applied to sugarcane bagasse. Sugarcane bagasse was mixed with distilled water (1:10 w/v). The mixtures were autoclaved at 121°C and 1.1kgcm⁻² for 4 h. The processed materials were dried in an oven at 80°C overnight. Sugarcane bagasse was initially inoculated with fungal inoculum (10%). Potato dextrose broth (PDB) was added to help fungus to establish on substrate. Fungus was mixed with sugarcane bagasse, incubated at 30°C and moisture content (40%) was maintained. After one month, bagasse was again washed with distilled water and mixed with white liquor (Na₂S 1.5%+NaOH 6.5%) in the ratio of 1:10 w/v, and autoclaved for 3 hrs. It was again washed with distilled water till maximum removal of lignin was achieved. Finally sugarcane bagasse was dried in an oven at 80°C for overnight.

**Production and analysis of sugar**

For sugar analysis, bagasse (1% w/v) was suspended in MSM (in g/l: Na₂HPO₄.2H₂O, 7.8; KH₂PO₄, 6.8; MgSO₄, 0.2; Fe (CH₃COO)₃ NH₄, 0.01; Ca(NO₃)₂ 4H₂O, 0.05) and inoculated with fungus, and it was incubated in rotary shaker at 150 rpm, temperature 30°C and pH 5 as described earlier [21]. Fungus was also grown in presence of above culture medium with conditions: temperature (30–35°C); shaking condition (125 rpm); dextrose (1.0% w/v); tryptone (0.1% w/v); inoculum size (7.5% w/v); pH (5) and duration (48 h) optimized by Taguchi methods for decolourization of pulp and paper mill effluent as described by Singhal and Thakur [21]. The total sugar moiety in culture extract removed after 6, 12, 24, 48 and 72 hrs aseptically estimated by the anthrone method by formation of coloured product by measuring the absorbance of the resulting solution against a glucose standard curve [23]. GC-MS analysis was performed for identification of sugars. In this case the cell suspension (50 ml) was clarified by centrifugation at 7,000 rpm for 8 min. The cell free supernatant fractions were extracted thrice with an equal volume of ethyl acetate for shaking by 45 min. The organic layer was dried with anhydrous sodium sulphate. The ethyl acetate extracts residues were analyzed after derivatization by trimethylsilyl (TMS) after modification of methods [24]. In this method, 100 µl dioxane and 10 µl pyridine were added to the residues and silylated with 50 µl trimethyl silyl [BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide, and analyzed immediately on a GC–MS. The GC–MS analyses were performed in electron ionization (EI) mode (70 eV). The samples were analyzed in split mode (1:10) at anode. The analytic column connected to the system was a Restech capillary column (5% diphenyl 95% dimethylpolysiloxane; 30 m long 0.25 mm i.d., 0.25mm film thickness). Column was used at a temperature programme of 50°C for 0 min, increased to 180°C at 10°C/min for 5 min, and finally increased to 300°C at 15°C/min for 30 min Helium with flow rate of 1 ml /min was used as the carrier gas.

**Optimization of process parameters for enhanced production of bioethanol**

For the Taguchi design and analysis of results the Qualitek-4 version (7.6.0.3) software was used [25]. In this study we have used Taguchi approach of orthogonal array and experimental designs that help to gain more information about the optimum conditions (Table 2). Both orthogonal arrays and ANOVA were used for this purpose. Orthogonal arrays are well-defined method that is used to
reduce the number of experiments to be conducted. Taguchi’s L-8 orthogonal array method was used to carry out fermentation experiments by choosing seven parameters at two levels (Table 2 and 3) [25]. In the orthogonal array of L-8 type, L and subscript 8 means Latin square and the number of experiments respectively. Full factorial approach will require 128 experiments to be conducted for optimizing a process while in fractional factorial using L-8 orthogonal array the number of experiments reduces to eight [19]. After designing, experimental data was analyzed using ANOVA. Taguchi approach used ANOVA to statistically significant parameters in finding the optimum levels [20].

**Screening and optimization of growth factors for fermentation by Taguchi approach**

Screening experiments were performed to select most suitable carbon and nitrogen sources. Various carbon sources were used at an initial concentration of 1% (w/v). Culture extract of sugar was taken as control. Batch study was conducted in Erlenmeyer flasks containing culture extract for sugar along with inoculum supplemented with different carbon sources. Increase in fermentation was analyzed after 48 hrs. After selecting the most suitable carbon source, various nitrogen sources were screened at different concentration. After selecting various nitrogen sources, various phosphate sources were also screened at different concentration. Once carbon, nitrogen and phosphate sources were selected, the growth media was optimized for the optimum concentration of selected carbon and nitrogen sources i.e. carbon (%) and nitrogen (%) and phosphate (%). Other process parameters such as pH, inoculum size, temperature, revolution per minutes were also optimized. Table 2 shows the parameters and levels used in this experiment. All the experiments were done in triplicate to avoid experimental errors.

**Analysis of the data**

The optimum level for each factor (parameter) was derived by analyzing the data of the above mentioned eight experiments using Qualitek-4 software and the performance at optimum condition was predicted (http://Nutekus.com/wp-q4w.html). The contribution of each individual factor in fermentation and the interactions among various factors was also studied. The analysis was performed with ‘bigger is better’ quality characteristics. The bioethanol production was carried out at the optimum levels of process parameters and result was compared with the value predicted by the Taguchi model. This confirmatory experiment was repeated three times. Qualitek-4 software (Nutek Inc., USA) for automatic design of experiments using the Taguchi approach was used in this study [25]. This software is equipped to use L-4 to L-64 orthogonal arrays along with selection of 2 to 63 factors (parameters) with two, three or four levels. This is user-friendly Windows-based software that allows selecting the array used and assigning factors to the appropriate columns. In this study L-8 orthogonal array was used with seven parameters at two levels [26].

**Production and analysis of ethanol**

The fermentation was carried out by *Sacharomyces cereviceae* under optimized conditions and samples were removed after 6, 12, 24, 48 and 72 h for estimation of ethanol. Production of ethanol was determined by spectroscopic and gas chromatography methods after samples were centrifuged at 13,000 rpm for 2 min. and the supernatant filtered through a 0.45 lm filter to remove solids. The ethanol produced was determined by spectroscopic method against different concentration of ethanol standard curve [27]. The ethanol was also determined by gas chromatography using a
chromatograph (Shimadzu GC-2010) with an FID detector. The inlet temperature was 250°C, the mode was split flow and the carrier gas was helium. The column was 30.0 m long with a diameter of 0.25mm, with film thickness of 0.25µm. The oven was warmed to an initial temperature of 40°C, held for 3 min. and then raised to 70°C at a rate of 3°C/min for 5 min. The oven temperature was then raised to 250°C at a rate of 10°C/min for 30 min.

RESULTS AND DISCUSSION

Four fungal strains PF1, PF2, PF3 and PF4 isolated from Anand Tissue Paper Mill, Meerut Uttar Pradesh, India, and three fungal strains PF5, PF6 and PF7 were isolated from Century Pulp and Paper Mill, Lalkuan, Uttarakhan, India (Table 1). The fungi (PF1 to PF7) were tested for their ability to remove colour and lignin from the pulp and paper mill effluent. Initial colour was 6521 CU and lignin was 16161 ppm. Among seven fungi tested most efficient strain was PF4 followed by PF7>PF1>PF6>PF2>PF3 and finally PF5. Maximum efficiency was shown by PF4 30% (4565.6 CU) colour and 24% lignin (12282 ppm) reduction on 3rd day. Second best strain was PF7 showing 27% colour reduction (4757.6 CU) and 24% (12347 ppm) lignin reduction on 5th day. The fungus (PF4) showed higher decolourization and delignification was subjected to amplification of ITS1 and ITS2 region of genomic DNA showed 99% homology with Cryptococcus albidus (GenBank database accession no. EU839451) was applied for preparation of lignocellulose hydrolysate.

Table 1. Isolation and characteristics of fungal strains isolated from Anand paper mill sediments and Century pulp and paper mill sediments.

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>Source</th>
<th>Growth</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF1</td>
<td>Anand paper mill</td>
<td>Slow</td>
<td>White mycelia, light green spores</td>
</tr>
<tr>
<td>PF2</td>
<td>Anand paper mill</td>
<td>Slow</td>
<td>White mycelium with dark colour spores, mycelia becomes dark from behind</td>
</tr>
<tr>
<td>PF3</td>
<td>Anand paper mill</td>
<td>Fast</td>
<td>White cottony mycelia with green spores</td>
</tr>
<tr>
<td>PF4</td>
<td>Anand paper mill</td>
<td>Slow</td>
<td>Dirty white mycelia with green spores. Gives purple colour on maturation</td>
</tr>
<tr>
<td>PF5</td>
<td>Century paper mill</td>
<td>Fast</td>
<td>White mycelia with excess spores production of green colour</td>
</tr>
<tr>
<td>PF6</td>
<td>Century paper mill</td>
<td>Fast</td>
<td>White mycelia with light brown spores produced in excess</td>
</tr>
<tr>
<td>PF7</td>
<td>Century paper mill</td>
<td>Medium</td>
<td>White snowy cottony mycelia with yellow-brown pigmentation</td>
</tr>
</tbody>
</table>

The total sugar moiety in culture extract prepared in presence of MSM and bagasse (1%) was determined after removal of culture medium after 6, 12, 24, 48 and 72 hrs aseptically. Results of the study indicated maximum production of sugar at 48 h by Cryptococcus albidus (Figure 1a). However, after optimization of process parameters by Taguchi approach for decolourization of pulp and paper mill effluent indicated increase in sugar formation (1.5 times) in this studies (Figure 1b). GC-MS was performed to analyze the type and nature for sugar after saccharification of lignocellulose present in sugarcane bagasse. Our result indicated formation of pentose (RT 9.52) and hexose (12.62) in culture medium (Figure 2).

In present work, cellulosic hydrolysate of sugarcane bagasse subsequently fermented with Saccharomyces cerevisae. The result of the study indicated production of 38.4 g/L ethanol with a yield of 3.9 g/g after 48h. The Taguchi method was used to identify the optimum culture conditions and to achieve maximum fermentation for bioethanol production by Saccharomyces cerevisae.
Figure 1. Formation of sugar in cellulolytic hydrolysate of sugarcane bagasse of pulp and paper mill effluent by *Cryptococcus albidus* in minimal salt medium (a), and sugar recovered after optimization of process parameters by Taguchi approach (b).

The process parameters such as temperature, RPM, pH, inoculums size, quantity of carbon and nitrogen ammonium hydrogen orthophosphate sources were assigned two levels: minimum and maximum values (Table 2 and 3). The relative differential influences of each factor on fermentation level two and level one against average value is shown in table 3. The inoculum size of the experiment was found to have the highest impact on the process of fermentation by the organism,
whereas pH of the medium had the least impact among all the selected optimization parameters. Various parameters, apart from having an individual effect on the process, also interacted among them. These interactions may be independent of individual effect.

Table 2. Overall eight experiments (run) designed on the basis of L-8 orthogonal array.

<table>
<thead>
<tr>
<th>Run</th>
<th>Temperature</th>
<th>Rpm</th>
<th>Sucrose</th>
<th>Yeast extract</th>
<th>Phosphate</th>
<th>pH</th>
<th>Inoculum size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3. Parameters and levels used for optimization of the experiment.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>RPM</td>
<td>130</td>
<td>150</td>
</tr>
<tr>
<td>pH</td>
<td>4.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Carbon (%)</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Nitrogen (%)</td>
<td>0.08</td>
<td>0.24</td>
</tr>
<tr>
<td>Ammonium hydrogen orthophosphate (%)</td>
<td>0.24</td>
<td>0.30</td>
</tr>
<tr>
<td>Inoculum size (%)</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4 gives the interactions among various factors. It was observed that the most significant interaction was between pH and ammonium hydrogen orthophosphate (Significance Index, S.I. 87.71), though these two parameters were not significant contributors, in terms of individual effect in fermentation. The least significant interaction was between rpm and inoculum size with SI of 37.54 whereas per individual effect they were the two most influential parameters. The data of this study implied that though inoculums size had the highest impact at the individual level, it did not show much effect at the interaction level. Thus the interactive effect of various parameters was entirely different from their individual effect. Analysis of variance was calculated to find out the significance of individual factors on fermentation.

The data revealed that inoculum size of the experiment had maximum effect, contributing 53.905% in fermentation, followed by nitrogen which contributed about 19.317% Parameter pH in the analysis, as the percent contribution by the factor was deemed insignificant. On the basis of the present results revealed by the Taguchi software, the total contribution of all the selected parameters was 25.23% and the current grand average of performance was 18.25%. The optimum conditions along with the level description for maximum fermentation is shown in table 3 and figure 3, which shows that when these conditions were selected then total fermentation was achieved 9% v/v in culture. Before optimizing the process parameters the average fermentation was about 5% v/v in culture. After optimization by the Taguchi approach the total fermentation was increased up to 9% v/v. Thus the data in this study indicated an effective influence of Taguchi L-8 orthogonal array for fermentation and the imperative role of optimization of all parameters in achieving the best possible
results with increased in fermentation from sugarcane bagasse. Fermentation of cellullosic using \textit{S. cerevisiae}, gave maximum ethanol after optimization was (70.00g/l) with yield (7.0 g/g).

Table 4. Interactions between different parameters along with Severity Index (SI).

<table>
<thead>
<tr>
<th>Interacting factor pair</th>
<th>Columns</th>
<th>SI (%)</th>
<th>Col.</th>
<th>Optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH × ammonium hydrogen orthophosphate</td>
<td>3x7</td>
<td>87.71</td>
<td>4</td>
<td>2,1</td>
</tr>
<tr>
<td>Temperature × ammonium hydrogen orthophosphate</td>
<td>1x7</td>
<td>69.14</td>
<td>6</td>
<td>2,2</td>
</tr>
<tr>
<td>Temperature × Carbon</td>
<td>1x5</td>
<td>67.24</td>
<td>4</td>
<td>1,2</td>
</tr>
<tr>
<td>RPM × Nitrogen</td>
<td>2x6</td>
<td>62.55</td>
<td>4</td>
<td>2,1</td>
</tr>
<tr>
<td>Temperature × PH</td>
<td>1x3</td>
<td>60.45</td>
<td>2</td>
<td>2,2</td>
</tr>
<tr>
<td>PH × Carbon</td>
<td>3x5</td>
<td>55.13</td>
<td>6</td>
<td>2,2</td>
</tr>
<tr>
<td>RPM × ammonium hydrogen orthophosphate</td>
<td>2x7</td>
<td>54.39</td>
<td>5</td>
<td>1,2</td>
</tr>
<tr>
<td>Carbon × ammonium hydrogen orthophosphate</td>
<td>5x7</td>
<td>45.6</td>
<td>2</td>
<td>2,2</td>
</tr>
<tr>
<td>PH × Nitrogen</td>
<td>3x6</td>
<td>44.86</td>
<td>5</td>
<td>2,1</td>
</tr>
<tr>
<td>RPM × PH</td>
<td>23</td>
<td>39.54</td>
<td>1</td>
<td>1,2</td>
</tr>
<tr>
<td>RPM × Innoculum size</td>
<td>24</td>
<td>37.54</td>
<td>6</td>
<td>2,2</td>
</tr>
</tbody>
</table>

The fermentative product of cellulolytic hydrolysate was subjected to determination of ethanol by spectrophotometer and gas chromatography against a standard curve prepared by different concentration of ethanol. Result of the study indicated maximum production of ethanol at 48 h (Figure 4). Data of GC indicated production of ethanol. Results from chromatographic studies indicated that one sharp peak in the figure 5. The sharp peak implies the presence of alcohol detected using a standard at RT 4.5. A sharp peak is present at RT 3.8 which implies the presence of ethyl acetate used as the solvent. Other peaks which are present in figure 5 showed the presence of other impurities besides the peak of alcohol at RT 4.5. This was confirmed by the chromatograms of standard for ethanol and solvent ethyl acetate used in this study.

The pulp and paper mill uses sugarcane bagasse for preparation of pulp contains debris of bagasse which is waste product which can be used for bioethanol production after saccharification and fermentation. The major problem in production of ethanol is end product inhibition and formation of inhibitory products due to presence of lignin in the raw material. Fungus, \textit{Cryptococcus albidus}, isolated from degraded wood and sediment of pulp and paper mill effluent has decolourisation and delignification efficiencies [21]. The process parameters optimization by Taguchi approach had significantly increased removal of colour and lignin from pulp and paper mill effluent [21]. Therefore, in this study, \textit{Cryptococcus albidus} was used for saccharification of sugarcane bagasse. However pretreatment of bagasse is necessary for maximum saccharification. Alkaline treatment of sugarcane bagasse digests lignin matrix and makes cellulose and hemicellulose available to enzyme degradation [3]. Similar treatment of sugarcane leaves enhanced subsequent hydrolysis by a cellulolytic enzyme complex [3]. Alternatively, biological delignification of bagasse is possible using selected strains of white rot fungus.

In this study the optimum conditions for growth of fungus for preparation of cellulolytic hydrolysate was performed in minimal salt medium supplemented by bagasse (1.0%) and pH (5.0). Taguchi approach was used to optimize the growth media for five factors i.e., pH, copper sulphate, carbon, nitrogen and inducer at four levels using M-16 orthogonal array was used for production of sugar as described earlier [21,28]. In earlier studies after optimization of process parameters there was seven fold increases in laccase production from 32U/mg to 219±8U/mg. Inducer (bagasse) had the maximum effect, contributing 52% while pH had the minimum effect (7%) [21]. Therefore, in this study experiment was set up for production of sugar in similar condition for degradation of lignin molecules and production of sugar and ethanol. Commercial enzyme preparations have been
used to convert sugarcane bagasse to fermentable sugars. Krishna et al. [29] used *Trichoderma reesei* cellulase and cellobiase to hydrolyze sugarcane leaves after alkaline delignification. Martin et al. [30] used a mixture of endo-glucanases and cellobiases to saccharify steam pretreated sugarcane bagasse. Adsul et al. [31] treated sugarcane bagasse chemically with varying quantities of lignin and hemicelluloses for the production of cellulase and xylanase enzymes by *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051 in the production medium and recovered higher xylanase and β-glucosidase activities.

![Figure 2. GC-MS profile for sugar analysis in cellulolytic hydrolysate of sugarcane bagasse of pulp and paper mill effluent after optimization of process parameters by Taguchi approach.](image)

Fermentation of cellulolytic hydrolysate from lignocellulosic residues is to produce alcohol. Both pentose-utilizing yeast strains (*P. stipitis*), and non-pentose-utilizing yeast strains (*Saccharomyces cerevisiae*) have been used in earlier studies [29]. Recombinant strains of *Escherichia coli*, *Zymomonas mobilis* and *S. cerevisiae* capable of hexose and pentose catabolism and high ethanol production have also been constructed. The production of ethanol is higher than
earlier report of sugarcane leaves and from the hydrolyzate of sugarcane depthed (32.6%) bagasse [29]. Huang et al. [32] reported ethanol production by fermentation of NaOH-neutralized hydrolysate without detoxification using the adapted *P. stipitis* and compared to fermentation of detoxified hydrolysate. The bioethanol yield using the adapted *P. stipitis* with both types of hydrolysate at pH 5.0 achieved 87% of the maximum possible ethanol conversion.

Figure 3. Significant factor and interaction influences for production of ethanol from cellulolytic hydrolysate of sugarcane bagasse of pulp and paper mill effluent prepared by *Cryptococcus albidus* subsequently fermented by *Saccharomyces cerevisiae*.

Figure 4. Production of ethanol from cellulolytic hydrolysate of sugarcane bagasse of pulp and paper mill effluent subsequently fermented by *Saccharomyces cerevisiae*. Ethanol was estimated by spectrophotometer method against known concentration of ethanol.
Mielenz et al. [33] used soybean hulls for production of ethanol by the simultaneous saccharification and fermentation (SSF) process with *Saccharomyces cerevisiae* D5A and recovered 31.2±0.3 (flask B) g/L ethanol after thirteen days. Even insertion and expression of *Z. mobilis* genes encoding essential enzymes involved in the fermentation pathway, alcohol dehydrogenase II (adh II) and pyruvate decarboxylase (pdc), into *E. coli*, resulting in increased cell growth and ethanol production of 30 g/L [7]. Oleskowicz-Popiel et al. [34] pretreatment anaerobically digested (AD) manure for the simultaneous saccharification and fermentation (SSF) recovered 30.8 kg ethanol per 100 kg dry mass of maize silage. Gupta et al. [35] used *Prosopis juliflora* as raw material for acid pretreatment, delignification and enzymatic hydrolysis and recovered 18.24 g/L and 37.47 g/L sugars and 7.13 g/L and 18.52 g/L of ethanol with *Pichia stipitis* and *Saccharomyces cerevisiae* respectively. Martín et al. [30] used clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) mixtures as raw materials for ethanol production. The simultaneous saccharification and fermentation of the pretreated material yielded cellulose conversions of 87.5 and 86.6%, respectively, with *Saccharomyces cerevisiae* and the filamentous fungus *Mucor indicus*. Li et al. [36] used Bermuda grass, reed and rapeseed for ethanol production by means of simultaneous saccharification and fermentation (SSF) with a batch and fed-batch mode. When the batch SSF experiments were conducted in 3% low effective cellulose, about 16 g/L of ethanol were obtained after 96 h of fermentation. However, after optimization of process parameters by Taguchi approach it is possible to produce more ethanol as reported in this study. Statistical methods like Plankett-
Burman design or central composite design/response surface methodology as well as the Taguchi approach are available for optimizing fermentation conditions. However, in this study we have used the Taguchi approach and the results indicated that the application of these statistical methods not only helped us in establishing the optimum levels of the most important factors considered with minimum amount of manpower and time, but also proved to be useful and satisfactory in optimizing the fermentation medium for the production of bioethanol.

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