Acetone-Butanol-Ethanol production from paper mill sludge by separate hydrolysis and fermentation

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Abstract
Paper Mill Sludge being a lignocellulosic waste serves the dual purpose of organic waste recycling as well as clean energy production. In this study, enhanced production of solvents from paper mill sludge has been carried out by a two-stage process involving separate hydrolysis and fermentation. Penicillium digitatum MG889480, isolated from the sludge sample, enzymatically hydrolyzed the PMS, thereby producing glucose.

The glucose obtained was utilized as the sole carbon source by Clostridium sporogenes NCIM 2337 for the generation of acetone, butanol and ethanol by anaerobic fermentation, exhibiting a total solvent yield of 1.462g/L as on 12th day of fermentation.

Keywords: Acetone-Butanol-Ethanol, paper mill sludge, separate hydrolysis and fermentation, anaerobic fermentation, pre-treatment.

Introduction
In response to constant hikes in fuel prices and increased demand for "Clean and Green" energy from renewable resources, most research is being focused on biofuels where valorization of waste biomass is a priority. Various bio-solvents like butanol and ethanol have emerged as an alternative to petroleum fuels owing to properties like higher calorific value, non-corrosive nature. However, the first challenge is the use of abundant feedstocks which can contribute to process profitability. Analogously, paper mill sludge (PMS) which is discharged annually by paper industries as waste is rich in valuable carbohydrates.

Considering this prognosis, in the future PMS will either be an alarming environmental problem or abundant raw material. The accessibility problem of the complex lignocellulosic structure of PMS by enzymes was overcome by optimization of various pre-treatment methods. Enzymatic hydrolysis of released cellulose into simple sugars using cellulase is exorbitant if commercially available enzymes are used.

However, operation expenses are curtailed if hydrolysis is done using cellulase producing organisms isolated from the sample effluent itself. The fermentable sugars obtained by enzymatic hydrolysis underwent separate fermentation as this process provides optimum conditions to both the microorganism and the enzyme causing a higher yield of solvents when compared to simultaneous saccharification and fermentation. This project deals with the production of ABE, using Clostridium sporogenes NCIM 2337, which anaerobically ferments the monosaccharide obtained from enzymatic hydrolysis of cellulose by Penicillium digitatum MG889480, isolated from the sludge sample procured from the effluent of a local paper mill.

Material and Methods
Screening and isolation of the cellulase-producing organism: Paper mill sludge was collected from the primary clarifier unit of a local recycling paper mill and characterization was performed. Congo red clearing zone assay was performed to isolate cellulose utilizing strains where microbes in the effluent were grown on nutrient medium plates containing 0.8 % agar and 1% CMC (Carboxy Methyl Cellulose). After an incubation period of 48 hours, agar plates were flooded with an aqueous solution of congo red(1%) and allowed to stand for 15 min at room temperature. The Congo red solution was then poured off and plates were counterstained by 1 M NaCl around growing colonies. The pH of the process was maintained at 5. Clear zones appearing around growing colonies and the fungal isolate is portraying largest clear zone selected for identification and was later ascertained by 18s rRNA analysis of the fungal genome.

Isolate identification and relatedness: Sequencing results were fed to the nucleotide blast tool through the NCBI database and to a sequence alignment program called ClustalX to identify isolate and determine the phylogenetic relatedness with different species. The given data was aligned by the UPGMA algorithm and the resulting alignment was visualized by a program entitled TreeView.

Aerobic batch fermentation study for cellulase activity: Batch fermentation studies were conducted in 250 mL Erlenmeyer flasks containing 100 mL medium comprising of corn steep liquor 2g/l, sodium molybdate 0.205g/l, magnesium sulfate 2.5g/l, ferrous sulphate1g/l, carboxy methyl cellulose 2g/l. The pH of the medium was adjusted to 5 and sterilization was performed by autoclaving at 121°C for 20 minutes.

The medium was inoculated with 5% (v/v) inoculum and the flasks were kept in an orbital shaker at 120 rpm at 50°C. The samples were withdrawn periodically for the measurement of optical density at 600 nm. The culture broth was centrifuged at 10,000 rpm, 4°C for 10 minutes and the supernatant was preserved for further analysis.
Enzymatic hydrolysis of pre-treated paper mill sludge:
Different pre-treatment methods namely thermal, sonication, acid and alkali were performed individually on the PMS. The pre-treated paper mill sludge underwent saccharification into fermentable sugar by the fungal isolate cultivated in the following medium composed of pretreated PMS, 50g/L; Corn steep liquor, 5g/L; sodium molybdate, 0.205g/L; magnesium sulfate, 2.5g/L; ferrous sulfate, 1g/L. The production medium for cellulase was inoculated with a 36th hour culture of Penicillium digitatum MG889480 (2% (v/v)). The flasks were incubated in a rotary shaker for 48 hours at an agitation speed of 150 rpm at room temperature.

After incubation, the samples were centrifuged at 10,000 rpm for 15 minutes in a cooling centrifuge at 4°C. The collected supernatant was analyzed for reducing sugar produced from cellulose by DNS assay and cellulase enzyme activity was assessed by filter paper assay.

Culture maintenance and growth of Clostridium sporogenes NCIM 2337: Dried spores of Clostridium sporogenes NCIM 2337 obtained from NCIM Pune, India, were revived in Robertson’s Cooked Meat Medium and stored at -80 °C. The inoculums were prepared by transferring 1 ml of thawed spore suspension to 100 mL of Reinforced Clostridial Medium comprising of Glucose (5g/L), Yeast extract (3g/L), Starch (1g/L), Beef extract (10g/L), Peptone (10g/L), NaCl (5g/L), Sodium acetate (3g/L) and Cysteine HCl (0.05g/L). The cells were grown under anaerobic conditions at a pH of 6.8. The growth profile was studied for 96 hours with periodic measurement of OD at 600 nm.

Anaerobic batch fermentation for ABE production: ABE fermentation was pilot in 125 mL glass serum bottles containing 50 mL of the hydrolyzed supernatant along with 0.15% (NH₄)₂SO₄, 0.6% MgSO₄.7H₂O, 0.05% KH₂PO₄, 0.01% NaCl, 0.001% MnSO₄.H₂O, 0.001% FeSO₄.7H₂O, 0.15% yeast extract, 0.45% CaCO₃ at pH 6.7. The medium was deoxygenated by flushing nitrogen gas followed by sterilization at 121°C for 15 minutes. The batch culture was initiated by transfer of 10% (v/v) inoculum previously grown on RCM medium at the 18th hour of growth.

The serum bottles were incubated at 37°C in an anaerobic glove box. During the fermentation period of 15 days, aliquots of the sample were withdrawn at intervals for analysis of acetone, butanol and ethanol (ABE). Before analysis, the samples were centrifuged at 10000 rpm, 4°C for 20 minutes. The supernatant was filtered and stored at -4°C for further analysis.

Analysis of acetone, butanol and ethanol: The samples were filtered using a 0.45 μm syringe filter and tested for butanol by GC-MS (Agilent Technologies, GC- 7890B and MS- 5977A) analysis. The column used for detection in GC-MS was HP5MS (5%-Phenyl-methyl siloxane, 30 m x 250 μm x 0.25 μm) where the inlet temperature was 250 °C and the detector temperature was 290 °C. The following conditions were followed: Start at 40°C and increased to 200°C at 5°C per minute. Injection temperature was 220°C. M/z range is 20-200. Ethanol concentration was determined spectrophotometrically by potassium dichromate oxidation method in the presence of sulphuric acid. A standard graph was prepared using different concentrations of a stock solution of ethanol. The absorbance reading was taken at 578 nm. The concentration of acetone was analyzed using standard methods.

Results and Discussion
Characterization of PMS: The carbon, hydrogen and nitrogen content of PMS were found to be 57.51%, 9.72% respectively and hence it had low biological activity justifying the higher chemical oxygen demand of the sample. Cellulose content was found to be lesser than that of lignin as it is mostly present in the bound state as lignocellulose in the paper mill sludge. It needs to be separated into lignin and cellulose to ensure a better yield of glucose from free cellulose by naturally occurring cellulase producing organisms.

Isolation, screening and identification of the microorganism: Cellulose utilizing fungal strain, isolated from paper mill sludge was identified and characterized as belonging to the genus Penicillium digitatum by 18S rRNA analysis of fungal genome which can be given as:

ACTGGCCCGCGGGGTGGTGACGCTCCCGGGCCCG
CGCCGGCCGAAAGACCCCGCACTTCGCTTGGAA
GATTGCACTCTGAGTGAAACCGAAATATTATTTAAAT
CTTCAACACCGAGTCTTTGTGGTCCCGGCACTGAT
GAAGAACGCAGCGAATGCAGATACGTAATGTGAA
TTGCAAAATTCAGTGAATCATCGACCGTTTGAACGC
ACATTGCGCCCCCTGTATTCCGGGGGGCATGCTT
GTCGAGCTGTATCGTCTGGCCCAAGCGGCGCTGGT
TGTGTTGGCCCGCCCTGCCGATCCCGGGGCAAGCG
GCCGGAAAGACGCAGCCGGCCACCCGCTCGCCTG
TCGAGCGTATGGGGGCTACCTATCCGCTGCTAGG
CCCGCCCGCCGCTCGGATCCACCCGAAATTTTT
AATCCAGGGTGACCTCCGAGTACGG

The nucleotide sequence obtained was allocated GenBank accession number MG889480. The phylogeny tree was obtained from the NCBI nucleotide database and the phylogenetic relations revealed that the given strain was Penicillium digitatum MG889480 (Figure 1). The mid-log phase determined for inoculation of this organism in the sludge sample corresponded to the time interval between 36-48 hours.

Cellulose utilization and cellulase activity: Cellulase activity of Penicillium digitatum MG889480 was studied for 24 hours at an optimal pH 5. This organism was found to act on carboxy-methyl-cellulose to produce glucose. From figure 2, it was observed that 2g/l of cellulose was to obtain
1.6g/l of glucose. On analysis of the curve, it can be inferred that, with the decrease in substrate (cellulose) concentration, the concentration of glucose increases simultaneously over a period. The cellulase activity showed an increase with increasing substrate utilization and was estimated to be around 1.22 (IU/ml.min) (Figure 3).

**Pre-treatment of PMS:** It is well known that the presence of lignin and hemicellulose intertwined together in PMS makes it difficult for cellulase attack. To facilitate the conversion of cellulosic biomass to ABE, various pre-treatment methods were tried before enzymatic hydrolysis of PMS by *Penicillium digitatum* MG889480. Enzymatic hydrolysis requires lesser energy, mild conditions with few inhibiting products released. Alkaline pre-treatment proved to be most effective as glucose released in this system was found to be maximum, indicating the disruption of the complex lignocellulose network characteristic of the paper mill sludge, thus increasing the efficiency of hydrolysis of carbohydrate to fermentable sugars.

The alkali treated sample must have loosened, thereby increasing the surface area for enzyme activity. The other pre-treatment methods such as sonication and thermal degradation did not solubilize the complex cellulose fibers as efficiently as the alkali treatment which is evident by the low glucose release portrayed in figure 4. Sonication and thermal pre-treatment are likely to have disengaged the fibers of the lignocellulose compound. Higher acidic concentration also happens to damage the sludge sample and produces toxins that negatively affect biomass growth and metabolism.

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**Fig. 1:** Phylogeny tree analysis of *Penicillium digitatum* MG889480

**Fig. 2:** Time profile of cellulose consumption and glucose release
**ABE Production:** The GC-MS chromatogram obtained for butanol is shown in fig. 5. The retention time for butanol is found to be 2.361 minutes. The standard curves for solvents were plotted to calculate the amount of solvents produced during fermentation. The concentration of biological solvents produced by *Clostridium sporogenes* NCIM 2337 is demonstrated in fig. 6. To study the effect of fermentation time on the production of ABE, fermentation culture was collected at different periods and subjected to analytical assays.

Fig. 6 reveals that the maximum concentration of total solvents produced was 1.462 g/l on the 12th day of fermentation implying that the microbial pathway of solventogenesis intensifies at the stationary phase of growth. It can be seen that in contrary to our previous research, separate hydrolysis and fermentation portrayed an increment in total solvent yield compared to simultaneous saccharification and fermentation of the PMS³.

![Cellulase activity curve of *Penicillium digitatum* MG889480](image1)

![Enzymatic hydrolysis of pre-treated sludge](image2)
Fig. 5: GC-MS analysis
Conclusion

The enzymatic hydrolysis of PMS using isolated organism provides for major cost-reduction. PMS, being a non-food lignocellulosic source, serves as a viable substrate for ABE production without competing for food reserves as well as provides for a partial solution to industrial waste management thereby proving its value as a desirable source for ABE production. It can be inferred from this project that separate hydrolysis and fermentation is a superior approach than simultaneous saccharification and fermentation due to higher yields showcased when PMS is used as a substrate.

However, to achieve the commercialization of lignocellulose-derived ABE, efficient optimization of process parameters can be performed to increase the overall yield of by-products of Clostridial fermentation.

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References


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