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Bioethanol production from cellulolytic wastes: A comparative study on acid and enzymatic hydrolysis pretreatments for enhanced saccharification and fermentation

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Abstract: **Due to the limited capacity of landfills, cellulosic waste is one of the main components of municipal solid waste has become a serious disposal issue in both developed and developing nations. The task of managing solid waste is crucial and difficult. Newspaper, tissue paper, cardboard, wood powder, and dry leaves, which are cellulosic waste, are becoming a more appealing choice for the generation of bioethanol due to its diminished feedstock costs, increased potential for substituting fossil fuels, and decreased releases of greenhouse gases. The study framework followed three steps which included the isolation of cellulose-degrading microorganisms, acid (1% H_2SO_4), and/or enzymatic hydrolysis of substrates followed by fermentation by yeast (*Saccharomyces cerevisiae*). The highest cellulase enzyme activity of isolate BY7 was 2.004±0.03 U/mL. The optimized condition for acid pretreatment was 1% H_2SO_4 at 121 °C for 30 minutes heating period. The bacterial isolate (BY7) was used for the enzymatic hydrolysis which helps to convert the cellulose into fermentable sugars. The acid and enzymatic hydrolysates were analyzed by the DNS method. The highest yield of sugars from acid and enzymatic hydrolysis was about 11.8 ± 0.01 g/L and 15.44 ± 0.04 g/L respectively from dry leaves. The fermentable sugars were fermented to produce bioethanol using *Saccharomyces cerevisiae* and yield was estimated using the Dichromate method.**

Keywords: **Cellulosic waste; Enzyme activity; Acid and enzymatic pretreatments; Hydrolysates; Fermentation.**

1. INTRODUCTION

The present scenario is an increasing population worldwide which leads to a rise in the demand for energy and transportation fuels [22]. By 2030, it is projected that worldwide consumption of energy is expected to have increased by 50% [29]. The Middle East is home to almost two-thirds of the world's known petroleum reserves, and these reserves are running low. The world transportation sector puts up around 19 % of carbon dioxide (CO_2) which is about 8 kg CO_2 per gallon, and more than 70% of carbon monoxide (CO) [2]. Currently, the world is searching for nonpetroleum-based energy sources due to the inevitable depletion of petroleum resources in the coming years. The primary energy sources at the moment are petroleum and oil, but these are rapidly depleting on a worldwide scale [24]. In today's world, the growing need for energy and the depletion of renewable resources have become progressing issues. However, there are restrictions on the availability and storage of renewable energy sources, such as hydroelectric, solar, and wind power. This has prompted decision-makers in government and business to look into alternative energy sources and more effective ways to use energy [15]. According to Nguyen and Kakinaka, there are multiple expectations for alternative fuels, such as a significant decrease in greenhouse gas emissions, global accessibility of raw materials, and the ability to be produced using renewable feedstocks [22].

Notwithstanding their advantages for the environment, renewable energy sources still require extensive analysis and study to completely grasp their effects before being widely used [30]. Furthermore, renewable energy offers a special chance to lower carbon emissions and prevent global warming. Additionally, to meet the increasing requirement for energy, new energy-saving technologies must be established [1]. One kind of biofuel that is used as a substitute for fossil fuels is bioethanol, which is produced from various organic biomass and crops [6]. Because of the structural complexity of agriculture-based lignocellulosic materials, a pretreatment procedure is required to decompose their structure and increase the accessibility of the carbohydrate polymers to hydrolytic enzymes. Up until now, a variety of pretreatment techniques have been employed, such as liquid hot water treatments, ammonia, steam explosion, acid, alkali, organic solvent, and alkaline hydrogen peroxide [8]. The selection of the pretreatment process affects waste treatment, cellulose conversion rates, and the performance of hydrolytic enzymes, which makes it potentially the most significant factor in the economics of the ethanol production process [12]. When compared to chemical conversion routes, the use of enzymes for lignocellulose hydrolysis is thought to be the most practical approach because it offers advantages such as higher yields, less by-product formation, low energy requirements, mild operating conditions, and environmentally friendly processing [10]. Either simultaneous saccharification and fermentation (SSF) or separate hydrolysis and fermentation (SHF) are effective methods for fermenting ethanol from cellulosic sugars produced by acid and enzymatic hydrolysis [2].

In this study, we have isolated the cellulase-producing microorganism and subjected it to cellulase production in a lab-scale fermenter. Reducing sugar concentrations was estimated using various cellulosic substrates pretreated with acid and/or enzymatic hydrolysis. Further fermentation of the hydrolysate for ethanol production was also investigated using *Saccharomyces cerevisiae*.

2. MATERIAL AND METHODS

Microorganisms and cultivation

For the study of the proposed research work following types of soil which contain Sugarcane farm, home backyard, kitchen waste, sawmill, dumping site, and cow dung were selected. Identification of cellulase producer was done using CMC agar plate and cultivation of cellulase producer was done in culture medium containing (gm/L distilled water): Carboxy-Methyl Cellulose salt (10), peptone (5), yeast extract (5), $MgSO_4$ (0.25), K_2HPO_4 (1), $FeCl_3.6H_2O$ (0.25), $MnCl_2$ (0.5), pH 7.0 and sterilized at 121°C for 15 minutes [20]. The preserved culture of yeast *Saccharomyces cerevisiae* was taken from the microbiology laboratory of TMES, Mandvi, Gujarat, India. The yeast culture was maintained in a YEM medium containing 0.7% (w/v) glucose, 0.4% (w/v) yeast extract, and 1% (w/v) NaCl and incubated at 30 °C for 48 h.

Primary screening of cellulase-producers

According to the Teather and Wood primary screening of different isolates was done using Congo red as an indicator dye for the production of the CMCase (carboxymethylcellulose) enzyme, specifically endo-β-1,4-D-glucanase [27]. The bacterial isolate that formed the clear zone (positive isolate) was chosen to determine their cellulase activity or cellulolytic index (CI) [26].

$$CI=\frac{\text{Diameter of zone of cellulose hydrolysis}}{\text{Diameter of colony}}$$

Cellulase enzyme activity determination

The method of determining endoglucanase activity is based on the amount of reducing sugar released by the action of endoglucanase (0.5 mL) on a cellulose substrate (0.5 mL). Following a 30-minute incubation period at 50°C in a water bath, 2 mL of DNS (Dinitrosalicylic Acid) reagent was added to halt the enzyme-substrate reaction. The treated sample was placed in a boiling water bath for 10 minutes before cooling to room temperature. Enzyme activity was measured at 540 nm maxima using spectrophotometer [23].

$$\text{Enzyme activity (U/mL)} = \frac{\text{Reducing sugar conc.}\left(\frac{\text{mg}}{\text{mL}}\right)\times 1000 \times \text{Reaction volume (mL)}}{\text{Molecular weight of Glucose} \times \text{Incubation time (minute)}}$$

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Substrates collection

Newspapers, tissue paper, cardboard (household), wood powder (sawmill), and dry leaves (college garden) were used as substrates for the production of bioethanol. The substrates were collected in a dust-free, fungus-free state followed by drying, blending, and stored in sealed plastic bags. The composition of different lignocellulosic materials used for bioethanol production is shown in Table 1.

Table 1. Composition of Cellulose, Hemicellulose, and Lignin for different Lignocellulosic materials [4]

Substrates	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Newspaper (NP)	40 – 50	25 – 40	18 – 30
Wood powder (WP)	40 – 55	24 – 40	18 – 25
Tissue paper (TP)	85 – 99	0	0 – 15
Dry leaves powder (LP)	40 – 50	15 – 25	20 – 25
Cardboard (CD)	60 – 65	19 – 25	14 – 20

Optimization of acid hydrolysis

The optimization of acid hydrolysis was carried out using different concentrations of diluted sulphuric acid (0.5%, 1%, and 2%). 10 gm substrates were added to different concentration of sulphuric acid followed by 30 minutes heating period at 121°C and 15 lb pressure. The concentration of sulphuric acid that led to the most significant concentration of free reduced sugar was further selected for acid hydrolysis.

Substrate hydrolysis

The main constituent of cellulosic waste is cellulose, a complex carbohydrate. Acid hydrolysis shifts cellulose to glucose and other simple sugars that microorganisms can ferment to produce biofuels like bioethanol. 10 gm of substrates were added into the diluted sulfuric acid (1% w/v) followed by a 30 minutes heating period at 121°C for 15 lb pressure. The resulting filtrate was detoxified using NaOH to neutralize the pH and released reducing sugar concentration was estimated with the help of the DNS method. A wash of distilled water was given to leftover substrate residues to neutralize the pH. A 24 h grown inoculum (5%) of isolated cellulose-degrading bacteria was added to the acid-pretreated substrates. Reducing sugar released during enzymatic substrate hydrolysis was analyzed by the DNS method. The sugar obtained through acid and enzymatic hydrolysis was employed to produce bioethanol [7].

Fermentation

To produce bioethanol, the hydrolysates obtained from the acid and enzymatic hydrolysis were utilized in a fermentation medium (yeast extract (10 g/L); KH_2PO_4 (10 g/L); $(NH_4)SO_4$ (2 g/L); $MgSO_4$ (0.5 g/L); distilled water (1000 mL) and pH 4 to 4.5) as a carbon source. Before being supplemented with hydrolysate and added to fermentation media in a shaking incubator at 120 rpm and 30 °C, the strains were first activated in YEM broth. The hydrolysates were incubated for three days at 30 °C and 120 rpm. Before analysis, the sample was taken out of the media during the fermentation process, centrifuged, and the supernatants were filtered. The dichromate method and the DNS method were used to estimate the amounts of bioethanol and residual reducing sugars, respectively [28]. The process of producing bioethanol is depicted in the schematic diagram (Figure 1)**.**

Statistical analysis

All experiments were carried out in triplicates and samples collected from each were analyzed for ethanol production. The means of reducing sugar and ethanol production were calculated. The results obtained in the present study were subjected to SD. Data were analyzed using a one-way variance (ANOVA). Significant differences among means compared using Fisher's LSD test at $p<0.001$.

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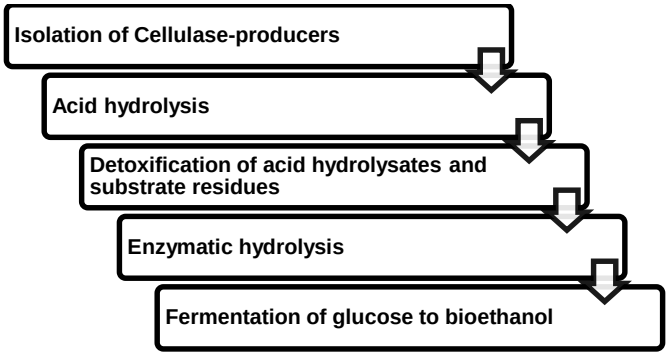

Figure 1. Schematic diagram depicting the method of producing bioethanol from selected substrates

3. RESULTS AND DISCUSSION

Identification of cellulase producers

CMC agar (1%) as a selective media was used to isolate cellulase-producing microorganisms. Out of 6 different cellulosic wastes, total 46 isolates have showed growth on CMC agar. After flooding each plate with Congo red, a zone of cellulose hydrolysis was detected to validate the cellulase producers (Figure S1). Further, the cellulolytic index (CI) was calculated for each cellulase-positive isolate (Table 2). CI suggests the efficiency of cellulase producers depicted in Figure 2. Four (KW3, BY7, CD5, and SF4) out of 27 isolates were found to be efficient cellulase producers subjected to further morphological characterization and enzyme activity estimation (Table S1). All four selected isolates (BY7, CD5, SF4, and KW3) were screened for their enzyme activity in the submerged fermentation process. The maximum cellulase activity was found to be 2.004±0.03 U/mL (BY7) inferred from Figure 3. Cellulase activity was calculated using the standard curve of glucose (Figure S2). The enzyme activity determined from isolate BY7 was higher than reported in the study of Malik and Javed [19]. In their study, they discovered that the maximum cellulase activity from the bacterial isolate *Bacillus tequilensis* (ON754229) was approximately 0.59 U/mL. This is comparatively less than the maximum cellulase activity found in our study and previous work of Islam and Roy where they found 0.9 U/mL for *Paenibacillus* sp [16]. Further reports of cellulase activities for *Arthrobacter woluwensis* 0.253 U/mL) and *Bacillus pumilus* (0.079 U/mL) have been reported by Das and Islam respectively [11][17]. These literature reviews suggested that BY7 was a good candidate for further investigation.

Table 2. Different bacterial isolates along with their hydrolytic zones and hydrolysis capacity

Source of the organism	Sample code	Hydrolytic zone diameter (mm)	Colony diameter (mm)	CI (Cellulose hydrolysis capacity)
Sawmill soil (SM)	SM3	11	7	1.57
	SM4	8	5	1.2
	SM5	4	3	1.3
	SM6	12	8	1.5
	SM7	13	9	1.4
Kitchen waste soil (KW)	KW1	9	6	1.5
	KW3	16	4	4
	KW6	6	4	1.5
	KW7	10	6	1.67
Dumping site soil (DS)	DS1	12	8	1.5
	DS2	10	7	1.4
	DS3	13	8	1.62
	DS5	9	8	1.1
	DS6	6	5	1.2

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	DS8	10	8	1.25
	DS9	5	3	1.7
Cow dung soil (CD)	CD1	4	3	1.33
	CD3	5	4	1.25
	CD5	14	5	2.8
	CD6	4	4	1
Backyard soil (BY)	BY1	6	5	1.2
	BY2	4	3	1.33
	BY3	13	5	2.6
	BY7	18	3	6
	BY8	11	4	2.75
Sugarcane farm soil (SF)	SF1	7	5	1.4
	SF3	6	5	1.2
	SF4	14	4	3.5

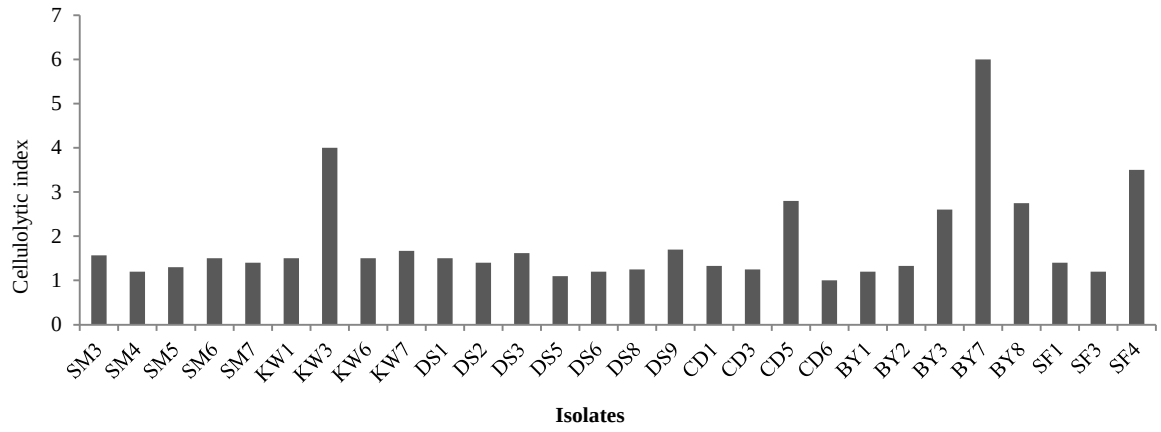

Figure 2. Cellulolytic index of cellulose-degrading isolates

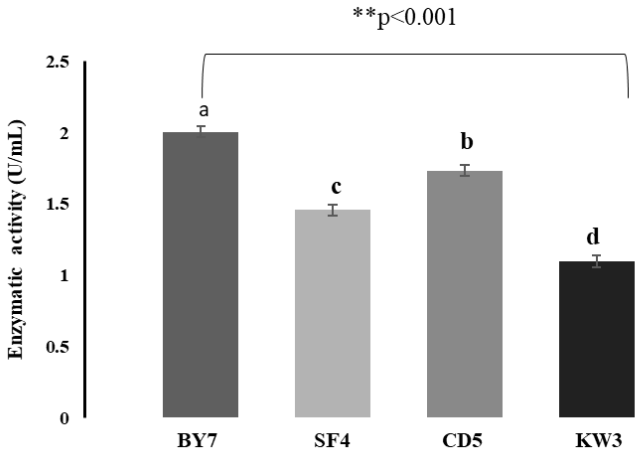

Figure 3. Graphical representation of cellulase enzyme activity of selected isolates. Each value in the figure is represented as mean ± SD (n = 3). Different letters indicate significant differences (p < 0.001) between groups (Fisher's LSD)**

Pretreatments

Pretreatment involves the Chemical and enzymatic hydrolysis of the substrates. The three most agricultural raw materials composed of sugar, starch, and cellulose can be easily applied to the fermentation process to produce ethanol. Moreover, waste sulfite liquor from pulp and paper mills, agricultural residues, and wood cellulose must all be converted to sugars, usually through mineral acids [5]. Thus, a variety of waste materials, such as cardboard, newspaper, tissue paper, wood powder, and dried leaves, were used in our experiment as the cellulose source for ethanol production. Pretreatment is the initial step in the production of ethanol from lignocellulosic materials. It entails breaking open the lignocellulose bundles to expose cellulose and hemicellulose polymer chains. Consequently, the pretreatment step could be the most crucial in ethanol production due to its impact on cellulose conversion rates, sugar hydrolysis, and fermentation.

During the sulphuric acid pre-treatment, the structure of the cellulosic biomass will change, making cellulose easily accessible to the acid that more quickly and effectively converts carbohydrate polymers into fermentable sugars [21]. In the present study, 20 gm of substrate was used to produce the bioethanol by acid hydrolysis using different concentrations of acid (0.5%, 1%, and 2%). It can be apparent from Figure 4, the quantity of total reducing sugar in the 0.5% acid treatment was lower than in the other two concentrations, but the amount of sugar released from the 1% and 2% treatments was comparable (Table S2). Moreover, avoiding the use of higher acid concentrations (2%) discourages the conversion of sugar into toxic and inhibitory compounds like furans and phenols [9]. Inhibitors affect fermentative metabolism and hence its final product. The released reducing sugar from NP, TP, WP, CB, and LP were estimated by the DNS method and the amount of released sugars were about 6.36 ± 0.06 g/L, 5.91± 0.04, 6.09 ± 0.02 g/L, 10.82 ±0.01 and 11.8 ± 0.01 g/L, respectively. The maximum amount of sugar released from dry leaf powder can be inferred from Figure 5a. Increasing the effectiveness of sugar conversion from cellulose waste is a common tactic used in enzymatic hydrolysis. With this method, the cellulose structure is partially broken down by acid, increasing its accessibility to enzymatic action. This parameter involved 72 h of enzymatic hydrolysis, performed after acid hydrolysis. Following the pre-treatment procedure, the cellulose-degrading organism is used in the enzymatic hydrolysis to convert the cellulose present in the substrate into ethanol. In the present study, the BY7 isolate was used for the enzymatic hydrolysis process at 7 pH. The process of hydrolysis involves the transformation of cellulose into sugar. The DNS approach was used to estimate the released sugar content. The reducing sugar amounts from NP, TP, WP, CB, and LP after enzymatic treatment (72 hours) were 7.44 ± 0.02 g/L, 7.41 ± 0.04 g/L, 9.61 ± 0.01 g/L, 14.41 ± 0.07, and 15.44 ± 0.04 g/L respectively. According to these findings, the maximum quantity of sugar released from dry leaf powder after 72 hours of incubation can be inferred from Figure 5b.

In the investigation of Annamalai, they obtained about 12.6 g/L and 6.12 g/L from the office paper and newspaper respectively [3]. The hydrolysis was done using 0.1% diluted sulphuric acid followed by 121°C for 30 minutes which was similar to the present work. In Another investigation of Saleem reported the increase in acid concentration contributed to the release of more sugars (g/L) (i.e., 52.3 ± 0.01 at 3% and 56 ± 0.04 at 5%) at 100 °C for 30 minutes of sulfuric acid hydrolysis of pomegranate peels [25]. Additionally, they discovered that higher acid concentrations lead to the release of more sugar along with inhibitors such levulinic acid, furfural, and hydroxymethyl furfural (HMF). In the investigation of Gomes sugarcane bagasse (SB) was used for enzymatic hydrolysis after pretreatment [13]. The changes in the structure and chemical composition of the pretreated SB resulted in a high TRS (Total Reducing Sugar) value (28.2 g/L) after enzymatic hydrolysis, for the pretreatment condition employing 6% citric acid, compared to the raw SB (3.06 g/L). Similarly, Annamalai pretreated office paper and newspaper with 0.5% v/v of H_2O_2 at 121 °C for 30 minutes for a considerable increase in available cellulose and sugar yield additionally to efficient delignification [3]. Enzymatic hydrolysis of pretreated office paper and newspaper produced a sugar yield of 24.5 and 13.26 g/L under ideal conditions. Furthermore, Yadav used free and functionalized immobilized enzymes (CellicCTec2) for the breakdown of sugarcane bagasse [31]. They found the maximum reducing sugar was about 33 g/L and 34.6 g/L from the 70.71 ± 1.43 and 74.2 ± 0.27 hydrolysis yields at 72 h, respectively. All of these literature works advise carrying out alternate experiments with various pretreatment methods in order to enhance the results of enzymatic hydrolysis.

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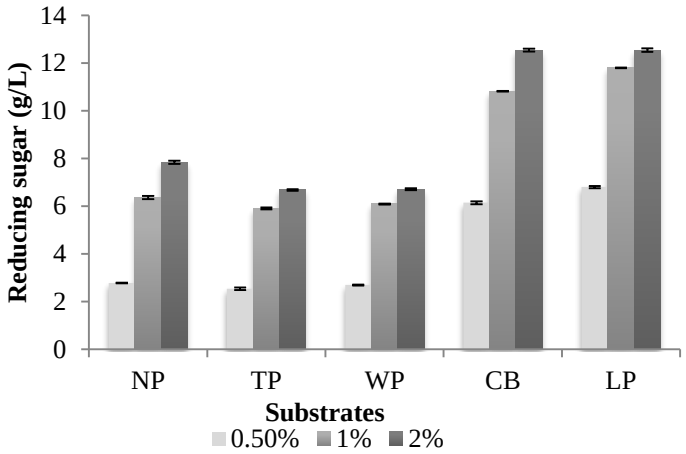

Figure 4. Optimization of different concentration of diluted H_2SO_4 for Acid hydrolysis. Each value in the figure is represented as Mean ± SD (n=3)

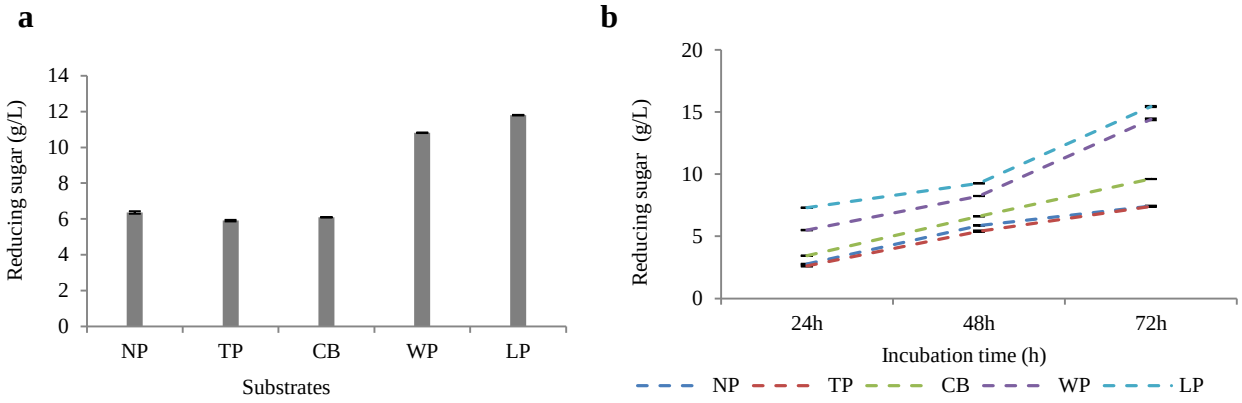

Figure 5. Total reducing sugar concentration obtained under (a) Acid hydrolysis of substrate and (b) Enzymatic hydrolysis of pretreated substrates. Each value in the figure is represented as mean ± SD (n = 3)

Fermentation of cellulosic hydrolysate for bioethanol production

Saccharomyces cerevisiae can be used as a fermenting agent to convert the sugar released by hydrolysis into bioethanol. In the initial stage of fermentation inoculating *Saccharomyces cerevisiae* at a low cell concentration will inhibit bioethanol production because yeast increases biomass by reproduction instead of producing bioethanol in the presence of a high reducing sugar concentration [18]. Therefore, a high inoculum of yeast cells is required for ethanol production, thus a 10% inoculum was used during the fermentation process. The dichromate method was used to analyze the production of bioethanol during the three days that the fermentation process was conducted. Fermented samples were taken every 24 h to estimate the alcohol production. At first, the culture produced ethanol at a where ethanol reached a maximum concentration of 3.4 ± 0.026 g/L, 3.80 ± 0.035 g/L, 3.90 ± 0.050 g/L, 2.33 ± 0.03 g/L and 5.31 ± 0.051 g/L of alcohol produced (Figure 6a) from the acid hydrolyzed substrate and 5.47 ± 0.03 g/L, 5.72 ± 0.02 g/L, 6.60 ± 0.06 g/L, 5.47 ± 0.04 g/L, 9.09 ± 0.06 g/L (Figure 6c) from enzymatic hydrolyzed substrate at 72 h of incubation from substrates NP, TP, WP, CB and LP respectively with approximately complete depletion of glucose (Figure 6b and 6d). The finding reveled that, among other wastes utilized for producing the maximum ethanol, dried leaf powder is the great source of cellulosic waste. Guerfali used *Saccharomyces cerevisiae* CTM-30101 to hydrolyze office paper pretreated with diluted phosphoric acid [14]. Another study by Yildirim used cotton stalks for enzymatic hydrolysis. They obtained 9.5 g/L ethanol from the 0.36 g sugar/ g biomass [32]. The suggested the optimization study should be carried out in consideration of the ideal physiological condition for *S. cerevisiae.* Additionally, some genetically modified strains that use hexose and pentose sugars are being developed, which increase the ethanol yield even more.

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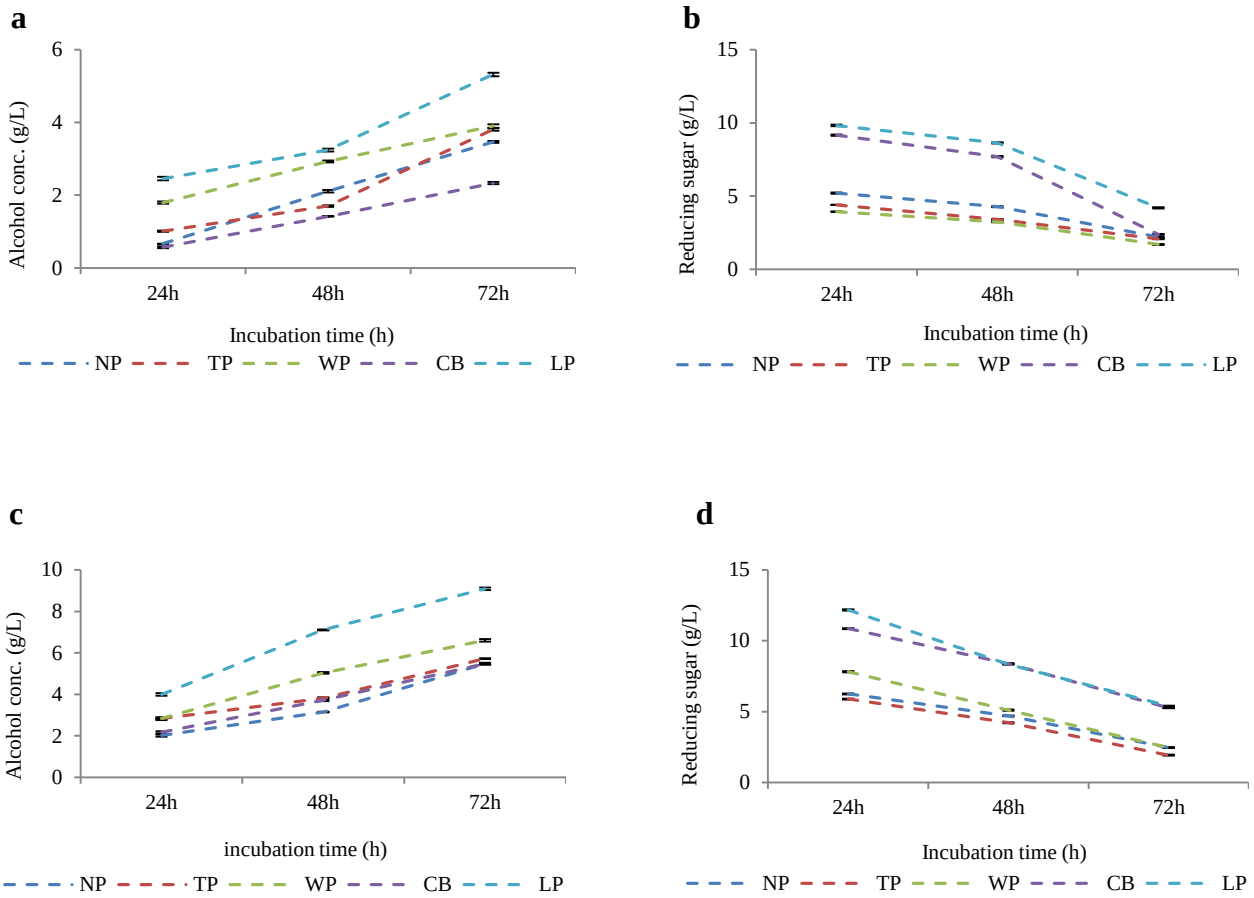

Figure 6. Graphical representation of (a) The alcohol production from acid hydrolyzed substrate (b) Utilization of acid hydrolyzed reducing sugar in fermentation (c) The alcohol production from enzymatic hydrolyzed substrate (d) Utilization of enzymatic hydrolyzed reducing sugar in fermentation. Each value in the figure is represented as mean ± SD (n = 3)

4. CONCLUSION

In the current study, we employ environmentally friendly technologies to address the issues of conventional energy scarcity and generate renewable energy. The present work deals with studies on the production of bioethanol from different cellulosic waste. Based on all findings it can be concluded that the selection of soil isolates was done depending on their potential of cellulose degradation on the CMC plate. We isolated BY7 as a promising cellulase producer and demonstrated that this isolate can efficiently use cellulosic wastes as a sole source of carbon to secrete cellulase. Acid and Enzyme hydrolysis were employed for substrate hydrolysis. In the present study, 1% of diluted sulfuric acid was used for the acid hydrolysis whereas the potential bacterial isolate was applied to the hydrolysis of pretreated substrates to obtain reducing sugar. Thus, fermentable sugar obtained through hydrolysis were subsequently bioconverted into ethanol using *Saccharomyces cerevisiae* suggested that the selected cellulosic waste have the potential to serve as a substitute feedstock in the development of a practical and cost-effective process for the eventual expansion of bioethanol production.

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Competing interest: The authors have no relevant financial or non-financial interests to disclose.

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Author's contribution:

Conceptualization, Methodology, Writing-review and editing, Supervision: **Smita Parekh**; Writing-original darft, Formal analysis and investigation: **Jainish Panchal**; Review and Editing: **Tulasa Khatik**

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Supplementary Materials:

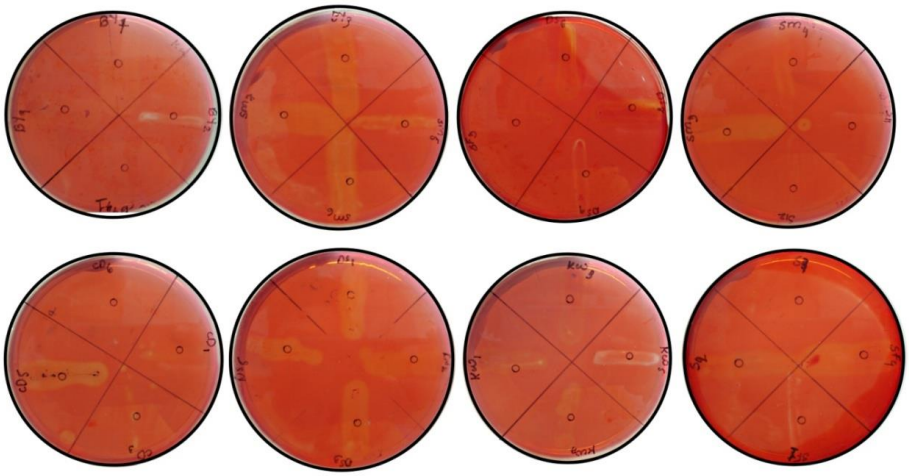

Fig. S1: Zone of cellulose hydrolysis on CMC agar plate after flooding Congo red staining

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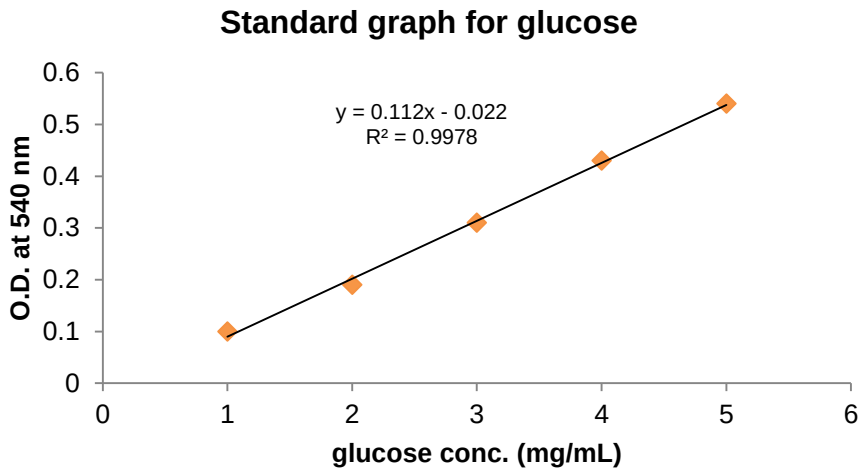

Fig. S2 Standard curve of Glucose

Table S1 Enzyme activity of selected isolates at different time intervals

Isolate	Enzyme activity (U/mL)		
	24h	48h	72h
BY7	0.79 ± 0.003	1.47 ± 0.009	2.00 ±0.039
SF4	0.74 ± 0.003	1.10 ± 0.009	1.14 ± 0.083
KW3	0.89 ± 0.003	1.47 ± 0.014	1.74 ± 0.061
CD5	0.64 ± 0.003	0.80 ± 0.014	1.09 ± 0.045

Each value in the table is represented as Mean±SD (n=3)

Table S2 Optimization of H_2SO_4 concentration for acid hydrolysis

Sample	Reducing sugar conc. (g/L)		
	0.5%	1%	2%
NP	2.78 ± 0.014	6.36 ± 0.068	7.84 ± 0.063
TP	2.54 ± 0.055	5.91 ± 0.040	6.68 ± 0.030
WP	2.69 ± 0.017	6.09 ± 0.020	6.71 ± 0.045
CB	6.14 ± 0.058	10.82 ± 0.015	12.54 ± 0.058
LP	6.80 ± 0.043	11.80 ± 0.015	12.54 ± 0.070

Each value in the table is represented as Mean±SD (n=3)