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Bioethanol Production from Economical Agro Waste (Groundnut Shell) in SSF Mode.

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ABSTRACT

Increasing demand of fuel has requisite towards Biofuel production from economical source especially from agro waste. The current work is focussed on Bioethanol production using groundnut shell (GS) waste which is available in plenty in many parts of the world. GS was first pretreated with physical pretreatment using steam explosion followed by various inorganic chemicals (0.25 N HCl, 0.25 N NaOH), and organic chemicals 0.25 N Acetic Acid and 0.25 N Lactic Acid to determine the effective method of pretreatment for saccharification. The effective order of pretreatment was HCl > Lactic Acid >Acetic acid > NaOH. FTIR analysis confirmed the degradation of pretreated GS in ranges of 1100-1400 cm^{-1}. A ethanol yield (16.11%) was obtained with *Bacillus stearothermophilus*, and *Saccharomyces cerevisiae* using 2% w/v pretreated groundnut shell at 50 ^{0}C after 14 days.

Keywords: Yeast, GC, SSF, Lignocellulosic biomass, FTIR, Groundnutshell (GS).

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INTRODUCTION

Due to constant depletion of fossil fuels, demand of global bioethanol is rising constantly. In 2011 the worldwide ethanol production was around 52.0 billion liters by US and Brazil (87.1% of global production by both). India is lagging in ethanol production even enough biomass is available (only 4 % ethanol is produced which is equivalent to around 2 billion liters using molasses as substrate) [1,2].

Presently most of the lignocellulosic agro waste such as groundnut shell, wood chips, and other agricultural wastes including wheat and rice straw is either burnt or wasted. As far as the GS is concerned, India and China are the world's leading groundnut producers, i.e. nearly 60 percent of the total production while, South Africa and Egypt has the maximum productivity and capacity to produce groundnuts (FAOSTAT 1990 to 1998) [1]. According to another report, Forbes, India produces around 125 to 183 million tons of biomass residue which may be sufficient to meet up to 59 % of India's petrol demand up to 2020.

Lignocellulosic has benefit that, 50-80 % higher ethanol can be produced with low emission of Green House Gases (GHG). But the major challenge lies in the release of cellulose from recalcitrant lignocellulosic biomass due to impregnation of lignin. It is necessary to remove the lignin so that cellulose and hemicellulose can be exposed for enzymatic saccharification. Thus, many pretreatment methods prevail till date has been targeted over delignification which changes the recalcitrant nature of lignocellulosic biomass. Hemicellulose conversion has been achieved by using C5 specific enzyme, and thus improves subsequent cellulose hydrolysis. Individual approaches of pretreatment method seems ineffective because of one or other reasons as discussed above, and thus combined approaches has been recommended to improve the delignification which is reported to improve the overall efficiency of hydrolysis. For instance physical treatment improves overall crystallinity, while chemical treatment converts major fraction of hemicellulose, while biological treatment is effective in delignification and depolymerization of cellulose [3]. For microbial based hydrolysis, several cellulolytic microorganisms along with *Saccharomyces cerevisiae*, is beneficial in term of ethanol tolerance [4]. Currently production of cellulosic ethanol is still not cost effective because of the low ethanol yield [5]. Rabah *et al.,* worked on bio-ethanol production by simultaneous saccharification and fermentation, the highest concentration of bioethanol of 0.96 % was produced using a combination of *Saccharomyces cerevisiae* and *Zymomonas mobilis* from groundnut shell. Also, the lowest concentration of 0.11 % of bioethanol was obtained when *Zymomonas mobilis* was used on hydrolysates from groundnut shells [6].

In the present work, Groundnut shell has been used for bioethanol production and both chemical and physical pretreatment has been used for Bioethanol production. The structural modification of groundnut shell was examined by FTIR analysis and saccharification study and ethanol production was done using isolated thermophilic strain.

MATERIALS AND METHODS

Sample Collection

Groundnuts were purchased from the local market of Jalandhar, Punjab and shells were removed manually and were used as raw material in this study. Groundnut shells were mechanically converted into powder form and further air-dried at 50 ^{o}C in hot air oven.

Isolation and characterization of microbes

Cellulolytic microbes were isolated from the soil of Rice field at approx. 10 cm deep from Lovely Professional University Campus at Jalandhar. Soil sample was diluted from 10^{-1} to 10^{-7} and cultured over screening media 2% carboxymethyl-cellulose (CMC) agar. Isolated strains were screened on the basis of hydrolytic zone formed. Screened strains were further characterized on the basis of saccharification studies and 16-S rRNA sequencing.

Media Composition

2% Carboxy-methylcellulose was used as the screening media for isolation of cellulolytic bacteria from soil. SSF, was performed using 20% pretreated GS; CMC, 20 g; $NH_4H_4PO_4$, 0.5 g; KCl, 0.1 g; MgSO4.7H2O, 0.5 g, Yeast Extract, 0.5 g (Each constituent is per 1000 ml) pH was set at 5.6.

Composition analysis of Groundnut shell (GS)

The composition analysis of GS was done to determine the cellulose and lignin content. Cellulose content was estimated by Anthrone Assay according to Updegraff [7] .Lignin content was determined experimentally by TAPPI Test Method according to Aldaeus *et al.,* [8]. Nitrogen was estimated by Jackson Method [9]. Organic carbon in groundnut shell was estimated according to Walkley and Black [10].

Pretreatment Studies

Physical and chemical methods were used for pretreatment studies. Powdered GS was treated with inorganic chemicals such as 0.25 N HCl and 0.25 N NaOH and organic chemicals such as 0.25 N Acetic Acid and 0.25 N Lactic Acid. Physical pretreatment was done using steam explosion method. The sample was washed with hot water for several times to remove acidic or basic content and treated with isolated bacterial strain for saccharification studies.

FTIR Analysis of GS

Physical and structural changes in GS after and before pretreatment was confirmed by FTIR analysis as described by Lojewska *et al.,* [11]. Groundnut shell sample was washed with water and then hot air dried to remove the moisture content to eliminate the peak overlapping by water molecules.

Simultaneous saccharification and fermentation (SSF)

SSF was performed using (20 % w/v) pre-treated groundnut shell (GS) using 500 ml flask, along with other media components as mentioned above. Media was autoclaved and inoculated with 2% (v/v) overnight grown culture of *Saccharomyces cerevisiae* and *Bacillus stearothermophilus*. Flask was incubated in rotating shaker at temperature 37 ^{0}C at 100 rpm, while pH was maintained by adding 2 % of 0.05 M citrate buffer for 14 days. Samples were taken from the flask at regular time intervals for the analysis of glucose and ethanol concentration. Sugar content was determined by DNS Assay by Miller et al., [12]. Ethanol content was determined by method using Acidified Potassium Dichromate Method by Bennett [13]. After 16 days, fermented broth was processed for distillation process and ethanol content in distilled sample was determined by Gas Chromatography.

Ethanol Assay

Ethanol production was estimated by potassium dichromate method by Bennett [13] and Gas chromatography. The acid dichromate solution was prepared by dissolving 33.76 gm of $K_2Cr_2O_7$ in 400 ml distilled water and 325 ml concentrated sulphuric acid, cooled and then volume was made up to 1 liter with distilled water. Calibration curve was prepared using 5% ethanol in different dilution, making final volume 1 ml to which 2 ml of $K_2Cr_2O_7$ (1N) was added and mixed. This solution was boiled for 10 minutes in a water bath. 2 ml of 2 N NaOH was added and mixed after cooling and absorbance was taken at 600 nm in spectrophotometer.

GC analysis

GC was done maintaining following conditions: Oven temperature: 155°C, Inlet temperature, 175°C; Detector temperature, 250°C, Carrier gas flow rate, 30 ml/min, Injection volume, was 2 µl.

Statistical analysis

Results obtained in triplicate were used for mean and SD, while one way ANOVA analysis was done using excel software.

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RESULTS AND DISCUSSION

Composition analysis

Result of groundnut shell has been shown in Table 1. Biochemical analysis shows that groundnut shell contains 34% cellulose while lignin was around 28%. Similarly Osman *et al.*, estimated 33 % of cellulose in groundnut shell [14]. Rivilli *et al.,* reported lignin content in peanut shells was 26.4 % [15]. The most abundant lignocellulose agricultural residues are corncobs, corn stover, wheat, rice, barley straw, sorghum stalks, coconut husks, sugarcane bagasse, switchgrass, pineapple and banana leaves that are rich in C5 & C6 sugars [16,17].

Biomass Pretreatment

Results of pretreatment and saccharification of groundnut shell has been shown in **Table 2.**

Therefore pretreatment studies was done using physical and chemical method which is essential to remove recalcitrant lignin. After steam explosion, chemical pretreatment was done with HCl, NaOH, lactic acid, and acetic acid. Chemical pretreatment, done using 0.25N HCl followed by physical treatment and SSF using *B. stearothermophilus*, resulted in 16.84 % of glucose (w/w) while with NaOH glucose yield was 8.1 % (w/w) on 6^{th} day of saccharification, while pretreatment with organic acid, glucose yield was comparatively low for instance with lactic acid glucose yield was 15.7% and with acetic acid glucose yield was 11.35%. So order of glucose yield after 6 days was better with 0.25N HCl. Therefore, order of chemicals in delignification observed was Hydrochloric Acid> Lactic Acid>Acetic acid > Sodium Hydroxide. Thus delignification efficiency of 0.25 N HCl was maximum.

Similarly Rosgaard *et al.,*reported that acidic pretreatment was better for various lignocellulosic materials [18]. If alkali or acid treated biomass is given steam under high pressure and temp (160-260 ^{0}C and 0.69 to 4.83 Mpa for 2-30 min it results in high expansion of lignocellulosic biomass and facilitates better hydrolysis of cellulose because of rapid de-polymerization[19, 20].

Most commonly studied chemicals for pretreatment is HCl or H_2SO_4 which has been used at very high temperature (120-160 ^{o}C) and at very high concentration (10-30%) which is essentially needed to liberate oligomer from the compactly associated chains, because most cellulose is present in crystalline form, impregnated with lignin and hemicellulose. Thus, for better pretreatment, acid concentration, temperature and time are crucial factors, which must be controlled to avoid the sugars and lignin degradation to by-products.The harsh condition is not recommended for the hemicellulose hydrolysis, though mechanism of liberation of glucose and other molecule is same. Harsh conditions promote the degradation of hemicellulose against cellulose, owing to its amorphous nature and also become corrosive against the reactor. Thus, recommended working acid concentration for hemicellulose hydrolysis is (1-4%) while temperature is (120-160 ^{o}C) [21].

FTIR Analysis

To confirm the degradation FTIR study was done which was in the range of 1100-1400 cm^{-1}, as comparison to non pretreated groundnut shell, (1100-1300 cm^{-1} wavenumber i.e. it depicts the lignin, cellulose and hemicellulose degradation as shown in Fig 2 and 3.

Thus, showing the more degradation of cellulose and hemicellulose content in pretreated hydrolyzed groundnut shell as compared to non-pretreated hydrolyzed groundnut shell. The peaks in GS shows C-H deformation in cellulose and hemi-celluloses at 1,369 cm^{-1} ; C-H vibration in cellulose and C-O vibration in Syringyl derivatives at 1,319 cm^{-1} ; syringyl ring and C-O stretching in lignin and xylan at wave number 1,226 cm^{-1}; C-O-C vibration in cellulose and hemicellulose at 1,151 cm^{-1} ; aromatic skeletal and C-O stretch at 1,116 cm^{-1}; C-O stretch in cellulose & hemicelluloses at wave number 1,024 cm^{-1} and C-H deformation in cellulose at 892 cm^{-1} [11]. Gelbrich *et al.*, had characterized the bacterial degradation of waterlogged softwood using FTIR spectroscopy [22]. The lignin degradation lies in spectrum peaks at wave number of 1,599, 1,511,1,467, 1,429, 1,157 and 1,054 cm^{-1} as reported by Adapa, *et al.,* [23]. Further, wave number 1300-1250 has been assigned for acylated hemicellulose degradation (Himmelsbach, 1998) [24]. thus we can see different wave number is

related to degradation of different components.

Isolation of cellulolytic microbes

Cellulolytic bacteria were isolated from the soil of rice field on agar media supplemented with 2 % CMC and two bacteria RFS1 and RFS2 (Rice field Soil) were screened on basis of zone of hydrolysis and pure colonies were obtained by subculturing. Gram staining showed both the isolates as Gram +ve bacilli.16S rRNA followed by phylogenetic analysis identified bacteria RFS1 as *Bacillus coagulans* and RFS2 as *Bacillus stearothermophilus* as shown in Fig.4. Based on hydrolytic zone formed,we have used *Bacillus stearothermophilus* for further study.

Bacillus stearothermophilus is a rod-shaped, Gram-positive bacterium and a member of the division Firmicutes. The bacteria is a thus thermophile is more suitable for cost-effective simultaneous saccharification and fermentation of lignocellulosic biomass.

Hatami *et al.,* isolated the cellulolytic bacteria from farming soil and forest soil by plating method on CMC media, and screened by zone of hydrolysis using Hexa Decyl Trimethyl Ammonium [25]. Ibrahim *et al.,* isolated thermophilic cellulolytic bacteria from hot spring and isolated bacteria were identified by sequencing followed by phylogenetic analysis as *G.thermodenitrificans* and *G.stearothermophilus.* [26]. 0.96 % conc. of bioethanol was produced form groundnut shells using a combination of *Saccharomyces cerevisiae* and *Zymomonas mobilis.* Also, the lowest concentration of 0.11 % of bioethanol was obtained when *Zymomonas mobilis* was used alone on hydrolysate from groundnut shells [6].

Fermentation and Saccharification in SSF mode

In our case we had used very low concentration of acid around 9% which is essential to check the release of pentoses during pretreatment. Though, pentoses had not been converted and also Low glucose was formed during hydrolysis which may be due to high pentoses in groundnut shell which remains unutilized by yeast which may be the reason of low ethanol yield. Other reason for low bioethanol may be due to inhibitory effect on the hydrolytic ability of some organic component over the cellulase enzyme complex. Tewari *et al.,*suggested that saccharification can be increased by increase in enzyme concentration (1–4 IU ml^{-1}) and treatment duration (12–72 h) along with maintaining substrate-acid ratios of 1:8 [27]. An ethanol yield of 13.6g/l was obtained when Tapioca stem var. 226 white rose (50 % substrate concentration) was used in SSF mode using cellulase enzyme and *Saccharomyces cerevisiae* yield at pH 5, temperature 35°C [28].

The total reducing sugars in most grasses have been reported ranging from 500–600 mg/g grasses (70–80% yield). For the ethanol production, simultaneous saccharification and co-fermentation (SSCF) technique have been used with cellulase and xylanase, while for pentose utilization, the yeasts, *Saccharomyces cerevisiae and Pichia stipitis,* were used for co-fermentation at 35°C for 7 days. From the results, the highest yield of ethanol, 1.14 g/L or 0.14 g/g substrate equivalent to 32.72% of the theoretical values was obtained from Sri Lanka ecotype vetiver grass (Jinaporn Wongwatanapaiboon,2012) [29].

A combined effect of simultaneous saccharification and fermentation and separate hydrolysis and fermentation (SHF) for ethanol production by *Kluyveromyces marxianus* 6556 was studied using two lignocellulosic feedstocks viz., corncob and soybean cake. The ethanologenic efficiency of *K. marxianus* 6556 was observed as 28% (theoretical yield) in a fermentation medium containing glucose, but, there was no ethanol production by cells grown on xylose. A maximum sugar release of 888 mg/g corncob and 552 mg/g soybean cake was achieved through acid hydrolysis pretreatment. Furthermore, corncob and soybean cake treated with commercial cellulase (100 IU for 48 h) from *Trichoderma reesei* yielded reducing sugars of 205 and 100 mg/g, respectively. Simultaneous saccharification and fermentation resulted in highest ethanol production of 5.68 g/l on corncob and 2.14 g/l on soybean cake after 48 h of incubation [30].

Ethanol Production Kinetics

Theoretical yield (Y_{PS}) is based on mass of glucose actually used for synthesis of ethanol.

Y_{PS} , 0.51 gg^{-1} i.e. 0.51 g ethanol/g glucose [9].

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While observed yield (Y'_{PS}) is experimentally determined
Y'PS,0.23 gg^{-1} (for *B. stearothermophilus , S. cerevisiae*)
Cellulose conversion efficiency 29% of GS
Ethanol conversion efficiency 51.9% of GS

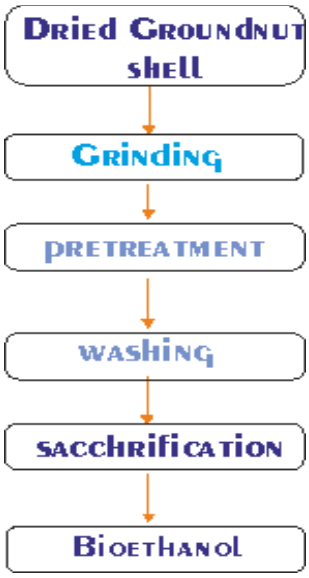

Figure 1: Pathway in Bioethanol production via groundnut shell

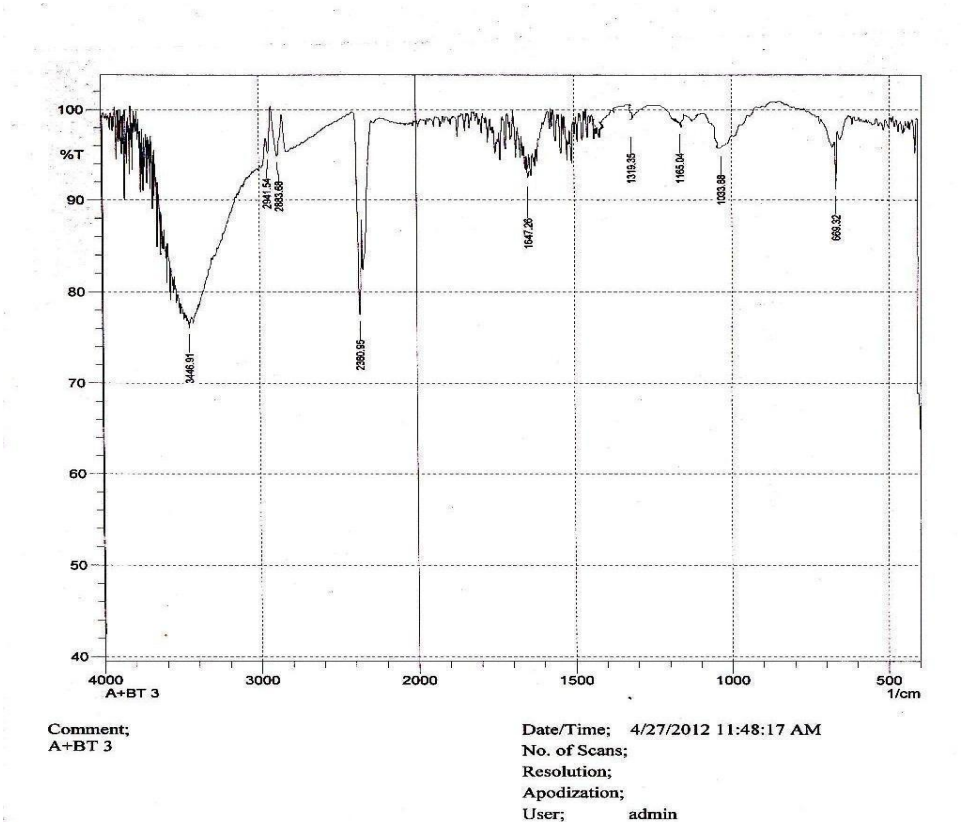

Figure 2(a): FTIR of non treated GS

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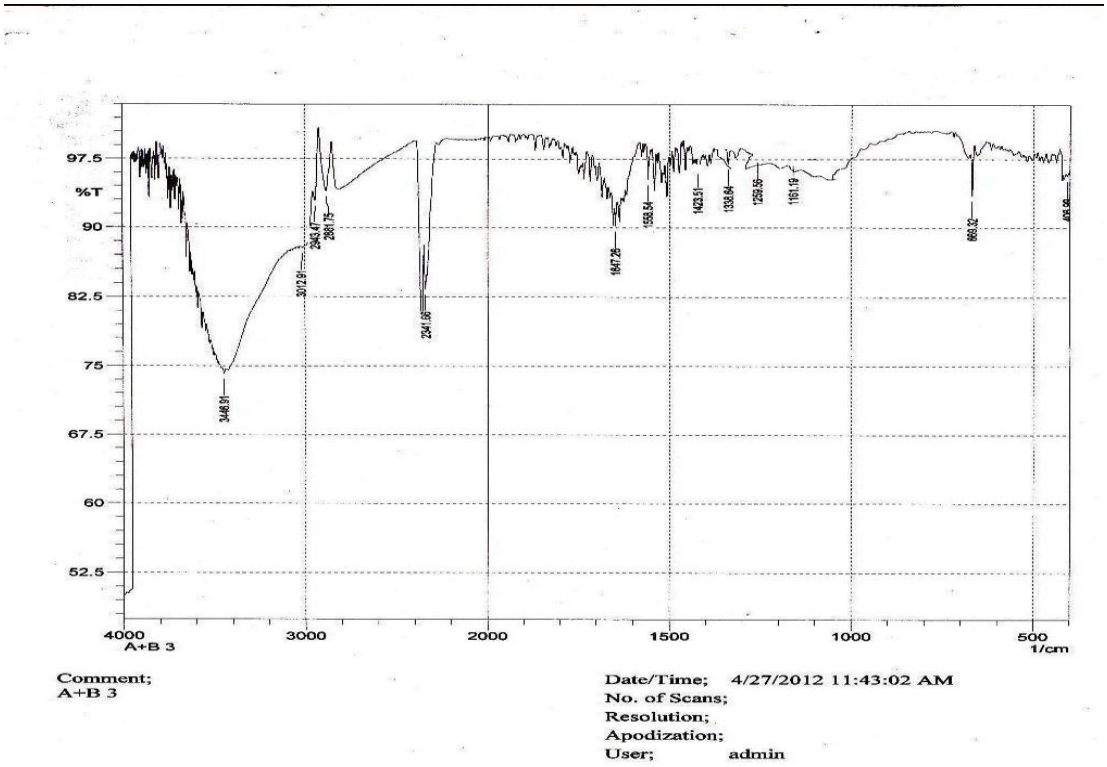

Figure 2 (b): FTIR Treated GS

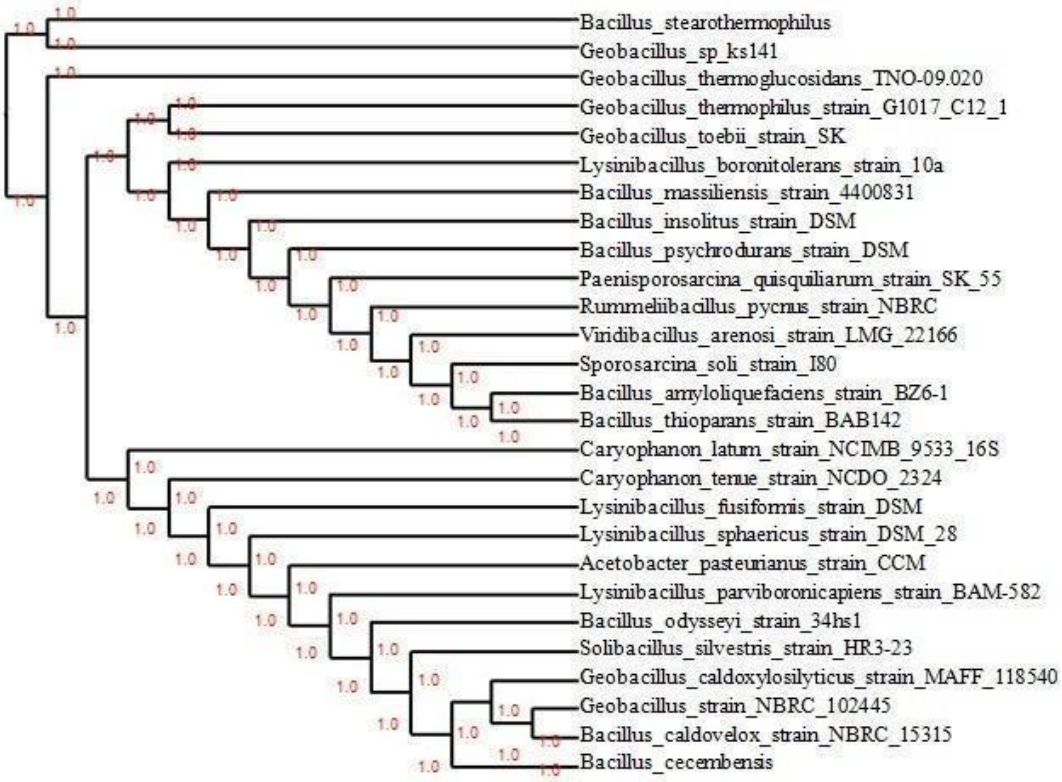

Figure 3: *Bacillus stearothermophilus* Phylogeny

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Table1. Composition analysis of Ground nut Shell

Carbon	27.7%
Nitrogen	23.4%
Cellulose	34.33%
Lignin	28.6%

Table 2 Glucose Yield % (w/w) after steam explosion and Chemical pretreatment with inorganic and organic chemicals and saccharification with *B. stearothermophilus*

Time (Days)	HCl (0.25 N)	NaOH (0.25 N)	Lactic Acid (0.25 N)	Acetic Acid (0.25 N)
1	9.19	3.4	4.9	3.39
2	10.31	4.2	6.5	4.71
3	12.09	5.9	9.9	6.6
4	14.33	7.2	12.8	7.33
5	17.4	7.9	14	9.4
6	19.04	8.1	15.7	11.35

Table 3: Time course of Ethanol production from 20 gm GS. Results are shown in triplicate at p<0.002.

Time (Days)	Glucose (g/L)	Ethanol (g/L)
6	16.8366 ± 0.11547	3±0.58387
7	14.72667±0.02309	6±0.38974
8	13.56 ±0.07291	6.5±0.64291
9	10.45 ±0.15275	7.5±0.50332
10	9.00333 ±0.57735	8±0.39954
11	7.14333 ±0.57735	10.4±0.49652
12	4.75 ±0.17321	12.3±0.49652
14	2.62798 ±0.19	16.11±0.49652

CONCLUSIONS

Current study was focused on bioethanol production from groundnut shell via simultaneous saccharification and fermentation(SSF) using thermophilic cellulolytic bacteria and yeast. Cellulolytic bacteria used was B*acillus stearothermophilus*, while yeast was *Saccharomyces cerevisiae*. Biochemical analysis of groundnut shell showed 35 % Cellulose, 32.10 % Lignin, 4.3% other Substances, 27.7 % Organic Carbon content and 23.4 % Nitrogen content. Groundnut shell was pretreated using 0.25 N HCl for lignin removal and then used for simultaneous saccharification and fermentation followed by distillation process.

Groundnut shell was found to be an effective lignocellulosic material for bioethanol production if used with mixed microbes. In this study, combination of *B. stearothermophilus* and *S. cerevisiae* was found to be better in terms of bioethanol production from pretreated groundnut shell overall % cellulose conversion was low because of yeast was not able to utilize the pentoses form as has been confirmed by FTIR. Further, for enhancements in bioethanol production, was possible with microbes specialized in pentose metabolic pathway such as *Z. mobilis* along with *S. cerevisiae* for the hydrolysis of both pentose and hexose sugars as *S. cerevisiae* can only utilize hexose sugars but *Z. mobilis* can use pentose sugars as well. Thus by adding mixture of microbes, the present methodology can be improved which can be helpful in providing a sustainable process for cellulosic ethanol production from GS, which would be a better alternative of petroleum fuels.

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