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Bioconversion of Rice Straw into Bioethanol by Enzymatic Hydrolysis of Bacillus Subtilis

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Abstract: *Rice straw (RS) is one of the main agriculture residues in Egypt. So, this study was performed on rice straw as a resource for production of bioethanol. Fermenting agricultural biomass, such as rice straw to ethanol is a promising solution to an ongoing waste problem. However, the biomass must first be pretreated to break down lignin, thereby increasing accessibility of the substrate to fermentative organisms. Biological pretreatment by microorganisms represents a potentially economic strategy to prepare the biomass for fermentation. In a series of laboratory experiments, rice straw was pretreated with sodium hydroxide, sulfuric acid and hydrogen peroxide in single and combination treatments followed by biological treatments for bioethanol production by bacteria; Bacillus subtilis were successfully grown on cellulose, hemicellulose of rice straw hydrolysis. Acid hydrogen peroxide treatment was found to be the best chemical treatment. Cellulases from Bacillus subtilis was produced in suitable quantities (2.2 IU ml-1) after 144 h, where Simultaneous Saccharification and Fermentation (SSF) of cellulose by Saccharomyces cerevisiae and cellulases was evaluated in basal media and ethanol was produced at 18.9% v/v (189 mg/g of dry mass) after 72 h at 30°C and pH 5.DNS, FTIR, XRD and GC analysis were done.*

Keywords: *Rice straw, Bacillus subtilis, Saccharomyces cerevisiae, Saccharification, Fermentation, Bioethanol*

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I. Introduction

The creation of ethanol from lignocellulosic waste goes under the second era biofuel generation. It is an option in contrast to the original biofuels which are delivered straightforwardly from the sustenance yields, for example, sugarcane, potatoes, corn, rice straw and so forth and develops into the nourishment and feed concerns [1].

In numerous nations, squander crop buildups are scorched in the open field. This training prompts genuine ecological contamination and results in the departure of an important item that could be utilized to enhance ranch pay. in Egypt as per the Egyptian Environmental Affairs Agency (EEAA, 2016) [2], in excess of 2 million sections of land of rice straw are developed in the nation with a normal generation of ca. 6.12 million tons/year. The preparing of rice yields uncommon amounts of straw as buildup. At the very least 20 % is utilized for paper and composts creation just as grub and the rest of the part is left in the open fields for consuming along a period that may stretch out to > 30 days to dispose of extra flotsam and jetsam. The subsequent emanation clearly adds to the air contamination known as the Black Cloud.

Rice straw is made basically out of sugar (67%) and lignin (28%) [3]. the principle segments of plant cell dividers are cellulose, hemicellulose and lignin, with cellulose being the most plentiful [4]. In any case, the lignin fills in as a boundary to counteract access to the sugar portion. The hydrophobic idea of lignin and the crosslinking of the phenyl propane units make the RS progressively impervious to microbial and enzymatic debasement [5]. Along these lines, this lignin must be evacuated through a pretreatment procedure. The RS can be exposed to a substance pretreatment [6], in any case, the synthetic treatment is might be cost and create elevated amounts of dangerous side-effects that could repress downstream anaerobic assimilation process. Organic pretreatment with parasitic or bacterial strains is additionally detailed [7], however inventive and new pretreatment strategy is required to be created rather than concoction treatment procedure to limit the inhibitors generation and make the pretreatment prudent [8,9]. Organic pretreatments with normally happening microbial strains and chemicals have been investigated [10].

Organic treatment of lignocellulose biomass for lignin debasement particularly utilizing bacterial culture is promising on the grounds that bio-treatment is practical, needs less vitality, and is more affordable

when contrasted with substance treatment. Furthermore, the rate of hydrolysis can be short contrasted with long brooding treatment with contagious strain.

Cellulase compounds can hydrolyze cellulose shaping glucose and other product synthetic concoctions. Scientists have solid interests in cellulases due to their applications in businesses of starch handling, grain liquor maturation, malting and blending, extraction of leafy foods squeezes, mash and paper industry just as material industry [11].

These days, microorganisms are getting to be favored decision for the segregation and creation of various compounds because of their higher development rate, numerous protein edifices and capacity to endure wide assortment of natural pressure [12-13]. Among the various microorganisms financially abused, cellulase creation from major bacterial gathering includes the variety Acidothermus, Bacillus, Clostridium, Pseudomonas and Rhodothermus [14]. Individuals from sort Bacillus produce an incredible assortment of chemicals [12, 15-22]. In addition, they are known to use a wide scope of substrates that incorporate strong natural waste deposits from agribusiness, woodland, plants and so on to furnish simultaneous advantage of waste administration with monetary creation of compounds.

A wide assortment of Gram-positive and Gram-negative bacterial species are accounted for to create cellulose, including Clostridium thermocellum, Streptomyces spp., Ruminococcus spp., Pseudomonas spp., Cellulomonas spp., Bacillus spp., Serratia, Proteus, Staphylococcus spp., and Bacillus subtilis [23].

Certain high-impact bacterial species, for example, Cytophaga, Cellumonas and Cellovibrio have capacity to corrupt cellulose in unadulterated culture [24,25]. The most acknowledged financially appropriate microorganisms are A. niger recombinant, T. reesei, H. insolens, Thermomonasporafusa, Bacillus species and some different life forms

In any case, a few microbes, for example, Bacillus sp., are known to create lignin peroxidase (LiP) and laccase (Lac) [26]. In such bacterial strains, both of these proteins are effectively engaged with corruption of phenols, fragrant amines, diamines, and numerous other xenobiotic particles [27]. Bacterial ligninases are one of a kind in that they can sever certain Cα-oxidation and Cβ–Cβ obligations of the lignin structure which are impervious to the contagious LiPs [28]. A few microscopic organisms can corrupt plant buildup by either burrowing into the inside cell dividers or making stripy disintegrations in the microfibrils of cellulose and have broad communications for lignin debasement [29,30].

Synchronous Saccharification and Fermentation (SSF) is one of the aging innovations embraced as it decreases the expenses and came about into higher ethanol generation as it limits the item hindrance contrasted with Separate Hydrolysis and Fermentation (SHF) [31] and requires shorter home time and low compound stacking and is modest [32]. The significant test for this method is the distinction in the advanced conditions for hydrolyzing and maturation microorganisms [31].

The main concern of the study is for the optimization of various techniques used for the bioethanol production in order to reduce the production cost. The present work is one of the on-going research attempts to improve ethanol production from rice straw by Bacillus subtilis and Saccharomyces cerevisiae. it has been found that Acid Hydrogen Peroxide pretreatment of rice straw resulted into proper delignification of lignocelluloses and then its bacterial treatment with Bacillus subtilis resulted into highest conversion into sugars. Highest ethanol yields (189 mg/g) were obtained after 72h of 6 days of fermentation with S. cerevisiae [33].

II. Materials and Methods

2.1 Materials 2.1.1Rice straw

(Oryza sativa) was collected from the farms of Kafr Sakr / Sharqia, during the harvesting season of the paddy crop, sundried for 7 days to reduce moisture content below 13 %, and then chopped into 2 cm size by using chopper machine and was milled by milling machine to 1-2 mm [34].

2.1.2 Microbiota

Bacillus subtilis (figure 1) obtained from Microbiology department (Faculty of Science- Zagazig university, Egypt) for cellulose-hydrolyzing enzymes production from organic wastes were used. It was cultivated and maintained on nutrient agar medium. Besides, one Saccharomyces cerevisiae strain characterized by its ability for lignocellulosic materials fermentation was obtained from Microbiology department (Faculty of Science- Zagazig university), Egypt. Cultures were monthly subcultured, incubated at 37 °C - 48 hr. and 28°C - 7 days for bacteria and yeast respectively and subsequently stored at 4°C for inocula preparation [35].

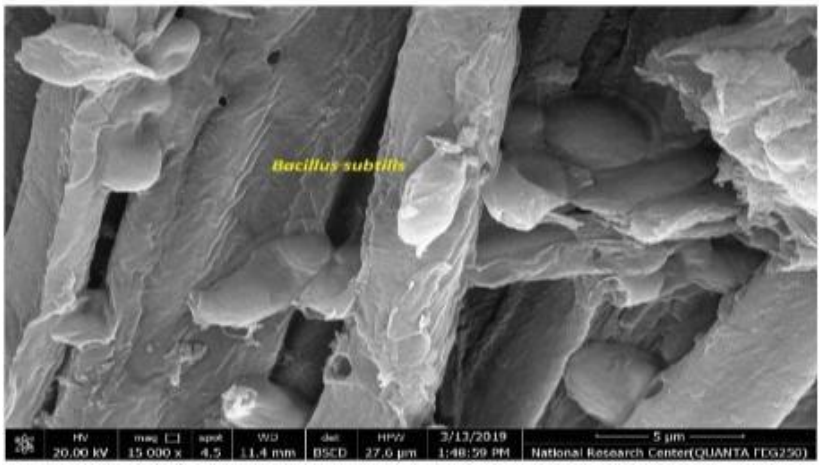


Figure (1). Bacillus subtilis on treated rice straw by SEM at 5 ums

2.1.3 Rice Straw Pretreatment

Rice straw was pretreated chemically then biologically to breakdown cellulose, hemicellulose and reduce lignin percent for high reducing sugars production needed for ethanol production by fermentation of Saccharomyces cerevisiae [36]. For chemical treatment, Sulfuric acid (H2SO4) was used for the acidic pretreatment, Sodium hydroxide (NaOH) was used for the alkaline pretreatment and hydrogen peroxide (H2O2) was used for oxidative pretreatment, in addition to their combinations. For biological treatment B. subtilis was used.

Citric acid, Sodium hydroxide, 3,5 Dinitrosalicylic acid, Potassium tartrate, glucose was used to perform the DNS Analysis to determine the concentration of reducing sugars.

Urea, Sodium Sulphate ((NH4)2 SO4), Sodium Nitrate (NaNO3), Dipotassium phosphate (K2HPO4), Magnesium Sulphate Hepahydrate (MgSO4.7H2O), Calcium Chloride (CaCl2), Manganese Sulphate Hepahydrate (MnSO4.7H2O), Zinc Sulphate Hepahydrate (ZnSO4.7H2O), Peptone, Yeast Extract (Himedia), dextrose was used to prepare the media for SSF. B. subtilis and S. cerevisiae were the strains that has been used. Spectrophotometer readings for DNSA analysis has been taken with the help of Apple PD-303SUV VIS Spectrophotometer at 540 nm.

The samples were sent to Micro-Analytical Center, Cairo University for Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography (GC) analysis. Also, the best results samples were sent to National Research Center, Cairo for X-ray Diffraction (XRD) and Scanning Electron Microscope (SEM) analysis.

2.2 Methods:

2.2.1 Physical (mechanical) Pretreatment

After the hand picking of the raw material to clean the sample, it was used to milled in the grinding machine. The homogenized small size particles were obtained after Milling [37].

2.2.2 Chemical Pretreatment

Chemical pretreatment of rice straw works on breaking the molecular bonds between sugar molecules and building blocks of the fibers. There are many chemical pretreatments available, and the most promising chemicals for pretreatment of rice straw include acidic and oxidative pretreatments. Chemical pretreatment is very important because it was found that later phases of treatment could not effectively convert lignocelluloses to soluble sugars without effective chemical pretreatment. In chemical treatment; 10 g of substrate was dissolved in 100 ml of the chemical solution at ratio of 1:10 (w/v) [37,38]. In this study, two types of chemical treatments were used

2.2.2.1 Simple Chemical Treatment (SCT)

2.2.2.1.1 Dilute Acid Pretreatment(H2SO4)

Acidic treatment is done at three different concentrations of Sulfuric acid were 1%, 2% and 4% [39,40-43,47].

2.2.2.1.2 Alkali Pretreatment (NaOH)

Alkaline treatment was also done at three different concentrations of Sodium hydroxide were 1%, 2% and 4% [42-44,47]

2.2.2.1.3 Hydrogen peroxide "Oxidative Pretreatment" (H2O2)
Oxidative treatment was done at three different concentrations of Hydrogen peroxide were 2%, 4% and 6%. Hydroxyl radicals (•OH) are extremely powerful oxidizing agents that can catalyze highly non-specific reactions leading to the cleavage of covalent bonds in both lignin and cellulose [45-47].

2.2.2.2 Combined Chemical Treatment
2.2.2.2 .1 Acid-Alkali Pretreatment (H2SO4 and NaOH)
Acid Alkali treatment was also done at the same concentrations were 2% and 2% for both Sulfuric acid and Sodium hydroxide respectively [48-50].

2.2.2.2 .2 Acid-Hydrogen Peroxide Pretreatment (H2SO4 and H2O2)
Acid-Hydrogen Peroxide treatment was done at two different concentrations of Sulfuric acid and Hydrogen peroxide were 2% and 4% respectively [51-55].

2.2.2.2 .3 Alkaline- Hydrogen Peroxide Pretreatment (NaOH and H2O2)
Alkaline- Hydrogen Peroxide treatment was also done at two different concentrations of Sodium hydroxide and Hydrogen peroxide were 2% and 4% respectively [56-59].

All the above flasks were the autoclaved at 121˚C, at 15 psi. for 30 ,60 and 90 minutes with control flasks. After treatment, the samples were filtered out with cheese cloth. The samples were then washed out gently, first with the tap water and then with the distilled water [60]. The samples were air dried and then dried at oven at 105˚C for one hour (figure 2), then stored in the refrigerator at 4˚C for further use. The solution which came as the filtrate were also preserved for the DNSA analysis to determine the concentration of reducing sugars present in that samples [61]. All the experimentations had been performed in triplets to get the more accurate results.

Figure (2). Dehydration of treated RS at oven

2.2.3 Biological treatment
2.2.3 .1 Bacterial Hydrolysis
Rice straw which has been treated chemically was then pretreated by microbial enzymes from Bacillus subtilis (figure 1) for sex days in a rotary shaker at 120 rpm and 30˚C.

2.2.3.2. Simultaneous Saccharification and Fermentation (SSF)
The strains Bacillus subtilis and Saccharomyces cerevisiae were grown in Basal Media (figure 3- a&b) which contains: 1.2 g NaNO3, 1.4 g (NH4)2SO4, 3.0 g KH2PO4, 6.0 g K2HPO4, 0.2 g MgSO4.H2O, 0.05 g CaCl2, 0.01 g MnSO4.7H2O, 0.001 g ZnSO4.7H2O, 1.4 g Urea, 1% Yeast extract, 2% peptone were added in 500 ml of distilled water and make up the volume to 1000 ml [62,63,64] and pH of the media was adjusted to 6 by adding 1N HCl [65]. The media was then autoclaved at 121˚C and 15 psi for 15 minutes. 5% dextrose was added after the autoclaving of media [66].

100 ml of this media was then poured in each of 250 ml of flask containing one gram of pretreated rice straw samples. 100 µl of Bacillus subtilis was then inoculated in each of these flasks under sterile conditions and then incubated at 30˚C ± 2˚C on the rotary shaker at 120 rpm for 144h [67]. The sampling from these flasks was done for DNSA analysis for estimation of sugar contents. After 144 hours S. cerevisiae was inoculated in the same flasks for the process of fermentation, at an initial yeast cell concentration of approximately 1×107

cells/mL and incubated on the rotary shaker at 150 rpm at 30◦C± 2˚C for another 144h. Viable yeast cell numbers were determined by the direct counting method using a hemocytometer and the methylene blue staining technique. [68]. Sampling was done after every 48 hours for DNSA analysis for reducing sugar, cellulase and protein content estimation respectively.

Figure (3). (a) SSF of Chemically treated RS with *Bacillus-Bacillus subtilis* and *Saccharomyces cerevisiae*

(b): RS treated with (H_2SO_4 + H_2O_2) and *subtilis*

2.2.4. Filtration and Distillation Process

Samples were then filtered (figure 4-a&b) by using cheese cloth to separate the solid substrate from liquid and then distillation was done at 78.37 ˚C to get the ethanol samples for GC analysis (figure 5-a&b). The solid substrate left was washed with distilled water and then air dried for the FTIR, XRD and SEM analysis.

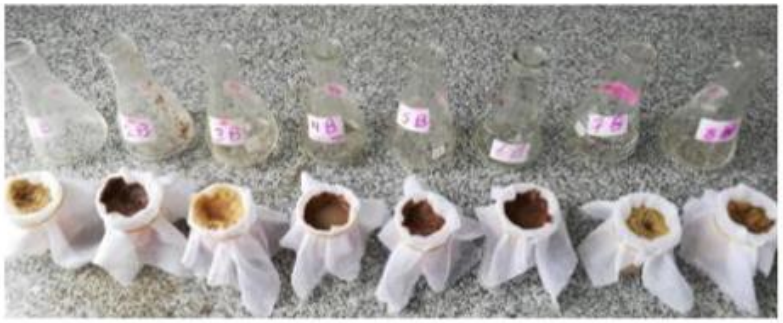


Figure (4). (a) Samples filtration by cheese cloth to separate the solid substrate from liquid.

(b)Filtrate and pretreated RS

Figure (5). (a) Distillation of samples to get the ethanol

(b)Ethanol evaporated at 78 ˚C

2.2.5. Analytical Methods:

Different analytical methods were used to determine whether the degradation of sample has done and further for the qualitative changes in the sample (DNS, FTIR, XRD). The estimation of ethanol yield was done by gas chromatography.

2.2.6. Standard Preparation

A 0.009 g/ml standard stock solution of glucose was prepared by dissolving 0.9g of glucose in 100.0ml distill water. Working standards were prepared by pipetting 25, 50, 75, and 100µg aliquots of the standard stock solution into separate 100 ml volumetric flasks and diluting to volume with 50 ml distill water. Five tubes were prepared for standard preparation; one tub e for blank, the four tubes for glucose standard. 2ml of sample

containing glucose standard was pipette. 2ml of 5% phenol was add to all tubes and mixed. Then 10ml of 96% concentrated sulfuric acid was added, simultaneously the tubes were shaking to effect fast and complete mixing. To develop color, the tubes are allowed to stand 10 min for shake, and the placed in water bath (25℃ - 30℃) to cool and display color. Blank solutions were prepared in the same way as above, except that the 2 mL of the standard solution was replaced by distilled water. The same procedure was used to prepare standard of xylose the only difference D- xylose used as a standard. The absorbance was measured at 490nm from hexoses group glucose and 480nm from pentose groups" xylose [69]. The amount of total of total carbohydrate present in the sample solution was calculating using the standard graph.

$y = mx + b$

Where: y is absorbance x is concentration m is the slop and b are the intercept

Con. of unknown sample = (absorbance of unknown sample) − (y−intercept) x Df /Slope Where: Df is dilution factor

Sugar yield (%) = gram of sugar/ raw material used x100%

Ethanol yield (%) = (gram ethanol produced/gram glucose used) x (100)

2.2.6.1. Chemical composition of RS 2.2.2.5.1.1. Cellulose content

Rice straw was refluxed with 10 ml of 80% acetic acid and 1.5 ml HNO3 for 20 min. The mixture was oven dried at 105°C. The residue heated at 650°C for 6h. [70-72].

2.2.2.5.1.2. Hemicellulose content

Hemicellulose was determined by subtracting the sum of weights of cellulose, lignin, protein, fats, waxes and ash from that of rice straw [72,73].

2.2.2.5.1.3. Lignin content

Rice straw was treated with 72% sulfuric acid at 15°C for 2 hours. The mixture was diluted with water to 3% acid concentration and refluxed for 4 h. the residue was washed thoroughly with water [72-74].

2.2.6.2. Protein content

Soluble protein was determined according to the methods described by Lowry et al. (1951) [75]. The protein content was calculated from a standard curve of bovine serum albumin.

2.2.6.3. Determination of reducing sugar

Reducing sugar was determined according the methods described by Miller et al. (1960) [76]. Three ml of 3,5-dinitrosalicylic acid (DNS) reagent were added to 2 ml aliquot sample in a test tube. The mixture was heated in a boiling water bath for 5 minutes, and then cooled to ambient temperature under running tap water. The absorbance was determined using a spectrophotometer at 640 nm against a reagent blank.

2.2.6.4. Determination of ethanol

Determination of ethanol was measured according to the methods of Caputi et al. (1968) [77]. The standard curve was prepared under similar set of conditions by using standard solution of ethanol containing 2 to 25 % (v/v) ethanol in distilled water and ethanol content of each sample was estimated and graph was made which obtained the equation y= 3.6727X to applied in all treatments. The ethanol absorbance was measured using a spectrophotometer Apple 303S -Vis Spectrophotometer at 600 nm., Ethanol quantification was done based on a standard curve generated with pure of standard ethanol then the calculation with the following equation:

O.D = 3.7526x C

Where O.D is optical density of Ethanol solution at different concentrations and C is the concentration.

2.2.6.5. Filter paper-Ase

Filter paper-Ase activity was measured according to Ghose (1987). the methods of Reducing sugars were estimated by (DNS) reagent [76,78].

2.2.6.6. FTIR Analysis

The samples in which maximum reducing sugar were obtained and maximum lignin content removal was found, FTIR analysis of those samples were done to determine the effect of pretreatment on various bonding present in the sample compared to the untreated samples. FTIR was also done for the samples obtained after distillation in which maximum bioethanol production was expected according to the DNS test analysis in order to check if peaks representing ethanol bonding were present or not [79].

XRD Analysis
XRD was used on finely ground material to investigate changes in crystallinity and hence spacing before and after treatment [80].

2.2.6.7. GC Analysis:
The samples for which peaks showing the presence of ethanol were good in FTIR analysis were used to perform gas chromatography. GC analysis has done for volatile samples in order to determine the concentration of ethanol in the sample obtained after distillation. Ethanol production was analyzed by gas chromatography as described by Paulina et al.,2015 [81]

2.2.6.8. SEM Analysis
In order to investigate the effectiveness of pretreatment SEM was used and the results are shown in this study.

III. Results and Discussion

3.1 Physical Pretreatment
Super fine grinding of rice straw decrease particle size and improve reactive surface to the largest content, and it had been no more energy consuming than traditional mechanical grinding with respect to the increase of surface area.

3.2 Chemical composition of RS before and after pretreatment:
3.2.1 Chemical composition of RS before pretreatment
Chemical composition of untreated rice straw (Native RS) was done under standard exploratory conditions. Results appeared as in figure 6, cellulose, hemicellulose and lignin of rice straw were 60.65%, 7.01% and 4.32% separately.

3.2.2. Chemical composition of RS after chemical pretreatment

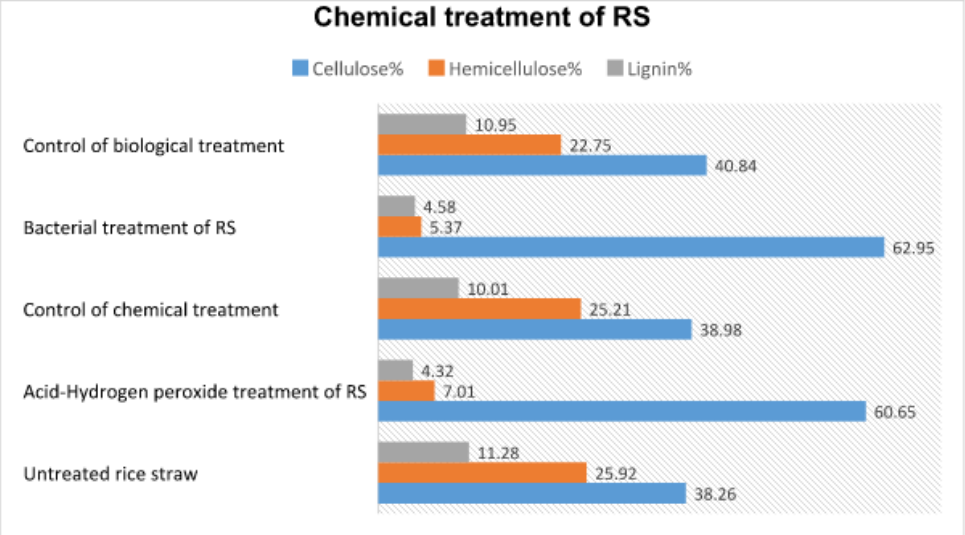


Figure (6). Chemical composition of Rice straw

The result of Acid Hydrogen Peroxide pretreatment, shown in figure 6, where hemicellulos decline from 25.92 to 5.37%, with an expansion in cellulose (from 38.26 to 62.95%).

3.2.3. Chemical composition of RS after Bacterial treatment
Bacillus subtilis have been broken the lignin. The lignin is decreased from 11.28 % to 7.2%, while cellulose where increased from 38.26% up to 48.75%, also hemicellulose was utilized good (figure 6).

3.3. DNSA Results
3.3.1. Chemical treatment results
The results indicated that as the concentration of chemical treatment and the time of treatment increased as the reducing sugars discharged from rice straw increased gradually.

3.3.2. DNS Results for chemical hydrolysis of RS

3.3.2.1. Simple Chemical Treatments of RS (SCT)

The results of simple chemical treatments (SCT) show that, sulfuric acid (4%H2SO4) treatment of rice straw for 90 min was more successful than alkaline and Hydrogen peroxide treatments

sulfuric acid (H2SO4) of rice straw at 4% concentration, results in 190.33 mg/ml of reducing sugars after 90 min at 121 c as compared to 6% Hydrogen peroxide treatment or 4% sodium hydroxide treatments at the same conditions where they produce 181.85 and 175.15 respectively (figure 7)

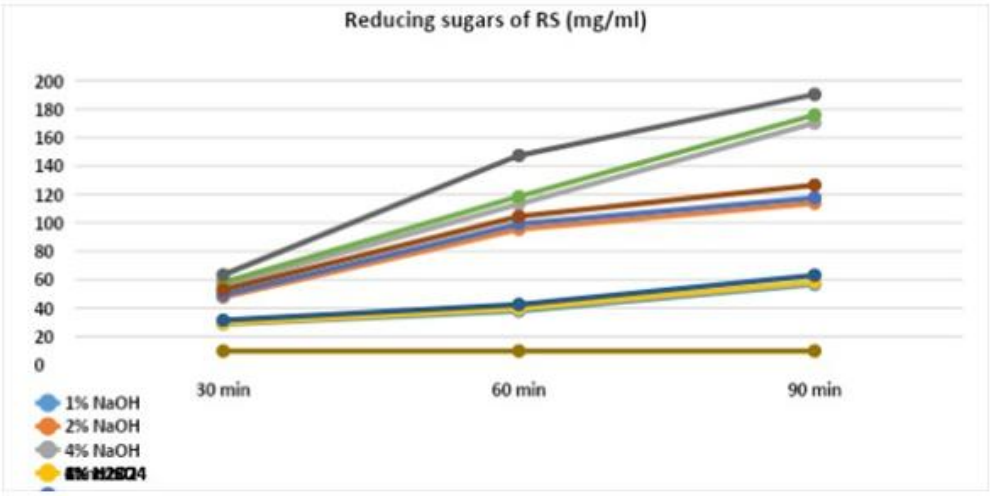


Figure (7). Reducing sugars from Simple chemical treatments of Rice straw

3.3.2.2. Combined Chemical Treatments of RS (CCT)

Consequences of Combined Chemical Treatments of RS (CCT), demonstrates that Acidic hydrogen peroxide (2%H2SO4 + 4%H2O2) treatment of rice straw for 90 min discharged higher amounts of reducing sugars than Acid-soluble base or Alkaline-hydrogen peroxide medications. Where it has been discovered that Acidic hydrogen peroxide is a lot more grounded oxidizing specialist than Acid-salt or basic hydrogen peroxide. Under acidic conditions, the lignin macromolecule is widely debased and broken up by hydrogen peroxide [94.95]. It appeared to be likely, subsequently, that acidic arrangements of hydrogen peroxide should

More promptly delignify rice straw than basic arrangements.

Adding concentrated sulfuric acid to solutions of hydrogen peroxide results in the production of peroxymonosulfuric acid (6). This acid is a much stronger oxidizing agent than hydrogen peroxide. It can also be produced by adding peroxydisulfuric acid salts to concentrated sulfuric acid. It has been found that as the concentration of peroxymonosulfuric acid increase, as the delignification of rice straw increased, and this is agreed with Edward (1990) [96]. So, rice straw treated with Acidic hydrogen peroxide was used for further experiments for bioethanol production (figure 8).

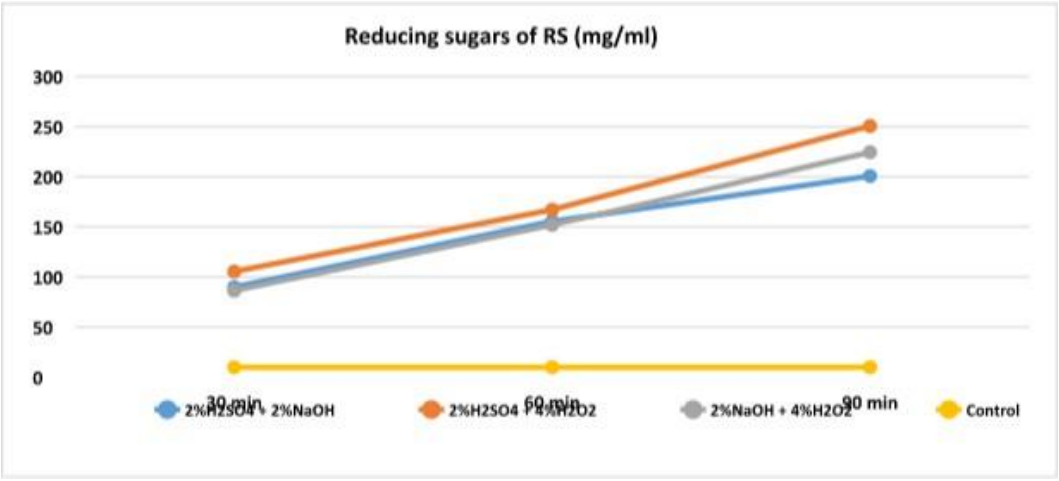


Figure (8). Reducing sugars from Combined chemical treatments of Rice straw

As shown from chemical treatments results of rice straw; the reducing sugars increased with increasing in time of treatment as well as increasing in the concentration percent of chemicals.it is clear that the hydrogen peroxide treatment of RS is very effective as compared to acid or alkali treatments (single treatment), but acid-hydrogen peroxide treatment is the best treatment in this study neither compared to single or combined chemical treatments. Likewise, this pretreatment technique is probably not going to achieve mechanical size of biomass pretreatment due to the staggering expense of the hydrogen peroxide and the ignitable idea of the unadulterated oxygen [97]. So, Acid-Hydrogen Peroxide treatment was preferred.

3.3.3. DNS Results of Bacterial hydrolysis

In contrast with regular chemical and physical pretreatment techniques, biological pretreatment is considered as a proficient, naturally protected and low-vitality process. Nature has plenteous cellulolytic and hemicellulolytic organisms which can be explicitly focused for successful biomass pretreatment [98].

Bacillus subtilis ready to debase rice straw pretreated with Acid-Hydrogen Peroxide progressively through Saccharification and Fermentation procedure giving most elevated reducing sugars (388.51 mg/ml) after 144h at 30°C and pH 5, at that point the reducing sugars diminished steadily because of fermentation into ethanol (figure 9). what's more, this is drawing closer to Saha et al. (2016) [99] where About 394 mg of reducing sugars of treated corn stover were accomplished by enzymatic saccharification of microorganisms

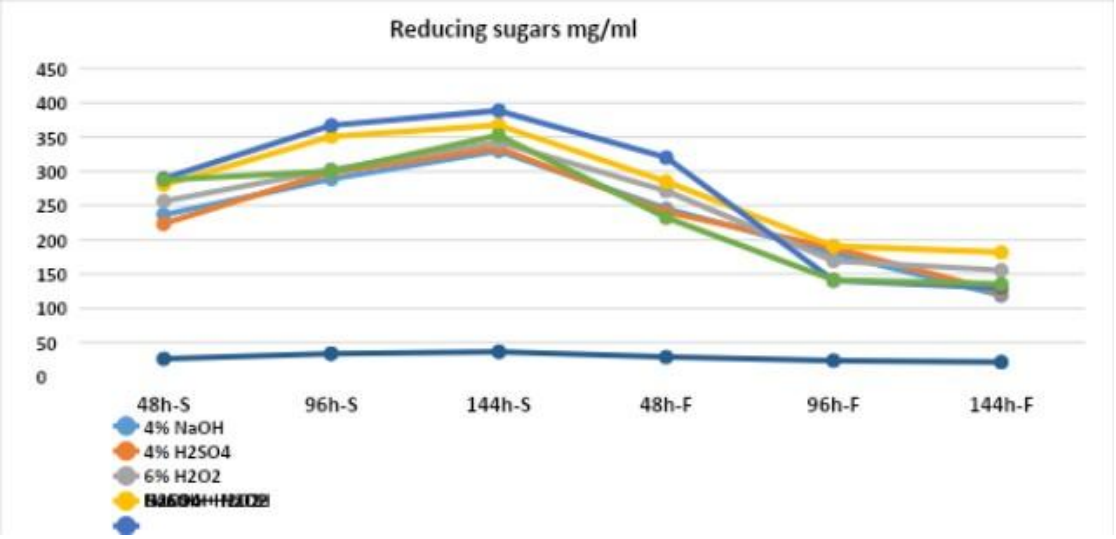


Figure (9). Total reducing sugars of pretreated rice straw during SSF with Bacillus subtilis and Saccharomyces cerevisiae

3.4 Enzyme activity- filter paper assay for saccharifying Cellulase (FPU)

Cellulase activity was examined by estimating the measure of reducing sugars freed from FP-Ase (Filter paper test) as an outcome of catalyst substrate response utilizing 3, 5-dinitrosalicylic corrosive (DNS) reagent [76].
Among most broadly examined life forms having outstandingly high cellulolytic movement, include various fungal species like Humicola, Trichoderma, Penicillium and Aspergillus. Some bacterial species include; Pseudomonas, Bacilli, Actinomycetes, Streptomycetes, Cellumonas, Streptomyces and Actinomucor [100,101].

The most astounding Cellulase FP-Ase created from Bacillus subtilis was 2.2 IU/mL after 144h at 30°C and pH 5 in Acid Hydrogen Peroxide pretreated RS medium (figure 10).

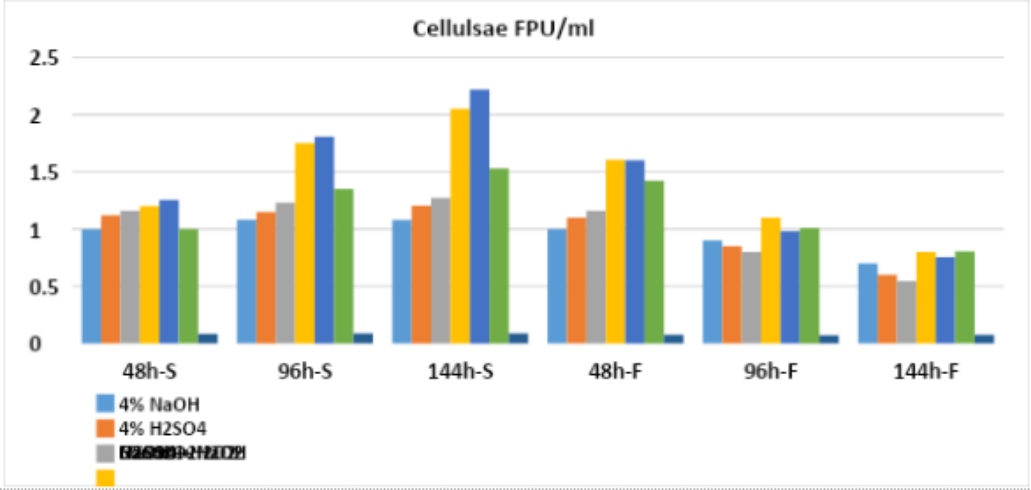


Figure (10). Cellulase (FPU) from Bacillus subtilis during SSF.

3.5 Protein analysis

Contrasted with purged ligninolytic enzymes, rough ligninolytic catalysts utilized for delignification offer a few advantages in the presence of components, for example, protein and broker in the medium that improves the activities of the compound [102].

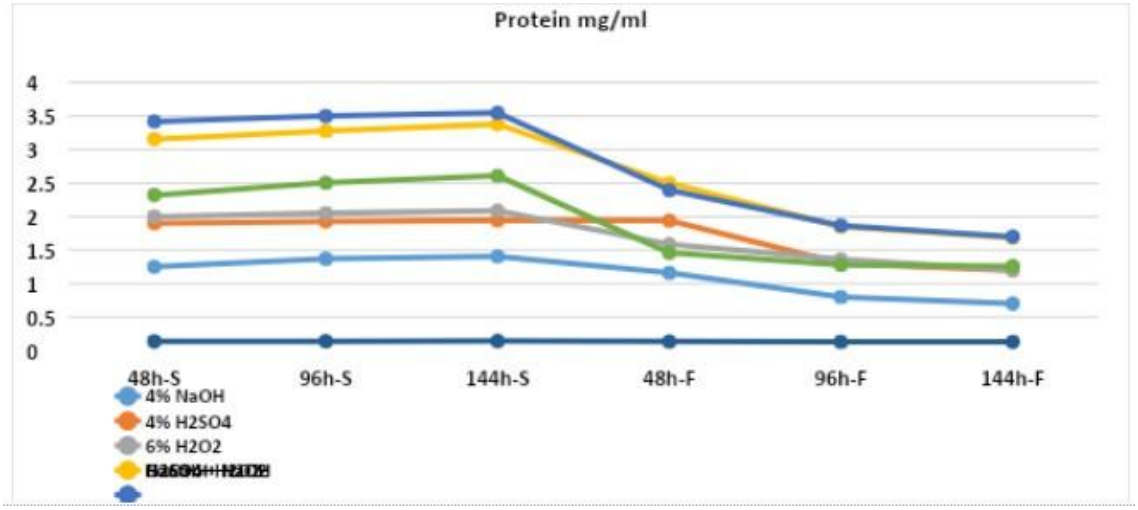


Figure (11). Total protein mg/ml during SSF

As indicated by Fig. (11); The highest results of total protein content delivered during rice straw saccharification by Bacillus subtilis were 3.5 mg/ml in in Acid Hydrogen Peroxide pretreated RS medium after 144h, at 30°C and pH 5.

3.6 FTIR results before and after pre-treatment

FTIR investigation affirmed changes in microstructure and utilitarian gatherings after biotreatment. FTIR spectra with a goal of 4 cm-1 were utilized to decide changes in the useful gatherings and structure of cellulose, hemicellulose, and lignin of rice straw after the synthetic (H2SO4+H2O2) and natural (Bacillus subtilis) pretreatments. The spectra of untreated and treated rice straw examples are given in Fig. (12).

The band at 899.95 cm-1 is the normal for the glycosidic bond β-(1→4) cellulose [103,104]. The range between 1200 cm-1 and 1100 cm-1 is district of hemicellulose and cellulose, which achieved a greatest incentive around 1059 cm-1 because of C-O extending [49]. A band around 1429 cm-1 is mishappening of lignin CH2 and CH3 and 1647 cm-1 is accounted for to stretch of the C=C and C=O lignin sweet-smelling ring [105]. In addition, a band around 1735 cm-1 is the normal for C=O extending of unconjugated hemicellulose while the crest at 2919 cm-1 is because of hitter kilter extending goodness CH2 and CH which indicated the attributes of cellulose. The area between 3800 cm-1 and 3000 cm-1 shows the crystalline structure of cellulose.

This range covers the whole of the vibration of valence groups of the hydrogen obligation of the O-H gathering and the groups of infra atomic and intermolecular hydrogen bonds [106].

The region between 1324 cm-1 and 1159 cm-1 showing the expulsion of hemicellulose. It very well may be seen that the area at 1717 cm-1 is influenced after compound and natural pretreatments which means the abatement in hemicellulose content. Be that as it may, the region 1642 cm-1 which appeared to be lignin macromolecule additionally demonstrates a few changes after delignification process [107].

Moreover, area between 2917 cm-1 and 2134 cm-1 is influenced during concoction and organic pretreatments. The band between these two pinnacles demonstrate that cellulose chain is being influenced though the expansion in line width and asymmetry of bends inside 3863 cm-1 to 3000 cm-1 demonstrates that aggravation in the crystalline structure of cellulose. These progressions allude to the interruption of intramolecular hydrogen holding in cellulose [108].

The peak assignments shown in table (1) below were made to corresponding infrared absorption at the given frequencies [109, 110].

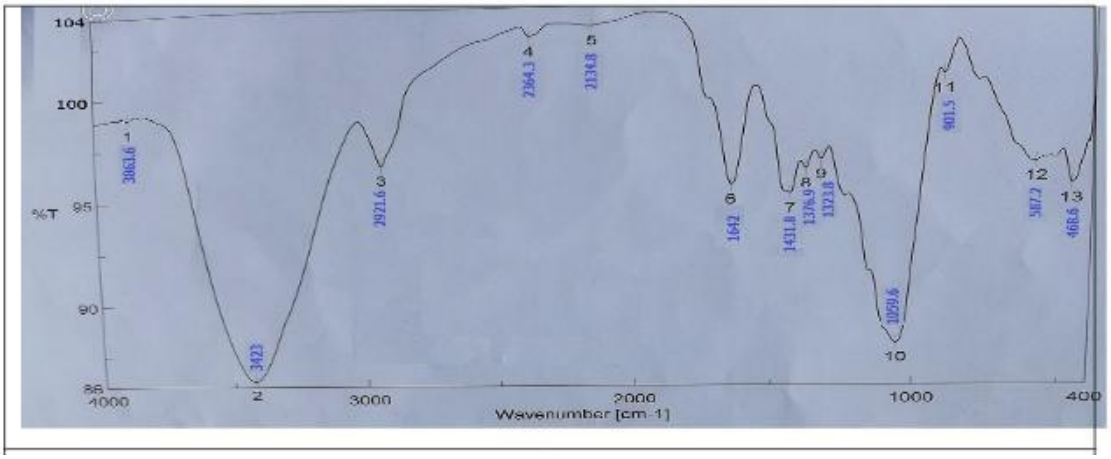
3.7 XRD results before and after pre-treatment

Little removal of lignin during biotreatment added to the increasement in the estimations of crystallinity record [111,112]. X-ray diffraction was utilized to investigations the examples' crystallinity. The diffraction force was estimated in the scope of 2ɵ = 10 – 45 degrees with a stage size of 0.02 degrees every second. The technique for Segal et al. (1959) [113]. was utilized to figure the crystallinity records (CrI). Nickel-sifted Cu Kα radiation (ʎ =0.1542 nm) was utilized at 40kV and 40mA. The diffraction power was estimated in the scope of 2ɵ =10 60°, with a stage size of 0.02°at a rate of 2°/min. The CrI was determined dependent on the Segal et al. (1959) [113] strategy.

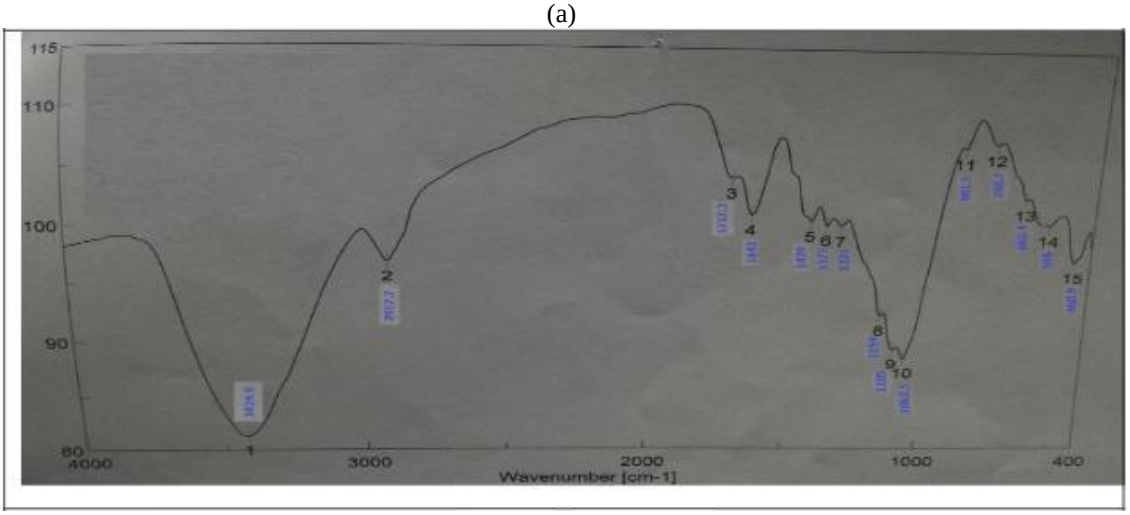
CrI (%) = [(I002–Iam)/I002] x 100 Equation for crystallinity index calculation

Where I002 is the intensity of the 002 crystalline peak at 2Ɵ = 22.4 °and Iam corresponds to the amorphous cellulose region for cellulose, hemicellulose and lignin at 2Ɵ = 18.0° [114].

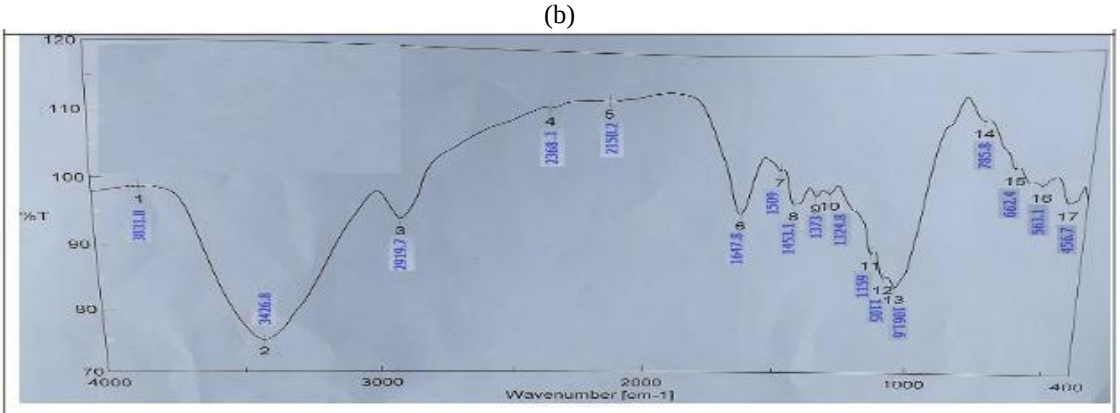
X-ray diffraction results demonstrate that there was a noteworthy increment in the crystallinity of the rice straw after pre-treatment. The expansion in crystallinity was deciphered as due to hemicellulose solubilization and fractional lignin debasement as revealed by Sun and Cheng (2002) [115]. This implies cellulose was presently progressively uncovered in anticipation of compound hydrolysis. Figure 13-b on the following page indicates X-beam diffraction diagrams of these outcomes. The CI esteems demonstrate a sharp increment upon pre-treatment from CI estimations of 29% for the crude untreated material to 45.8%. As a result of the organic pretreatment, morphological changes were seen in cellulose structure. The CrI of cellulose is considered as a standout amongst the most significant elements for assurance of enzymatic hydrolysis proficiency. In this way, CrI of cellulose has been watched for over fifty years for translation of progress in cellulose structure because of the execution of different pretreatment strategies and enzymatic hydrolysis [116] (figure 8-c). Corrosive Hydrogen Peroxide is the most supported pre-treatment techniques in view of the minimal effort of sulfuric corrosive and capacity to perform well on various feed stocks. X-beam diffraction (XRD) examination demonstrated that the crystallinity Index (CrI) of rice straw expanded by about 10% after Acid Hydrogen Peroxide treatment (Table 2) and the conceivable purpose behind this obvious increment is the expulsion of shapeless hemicellulose and lignin and the consequent enhancement in the grouping of crystalline cellulose [117,118,119] (figure 8-b).



(a)



(b)



(c)

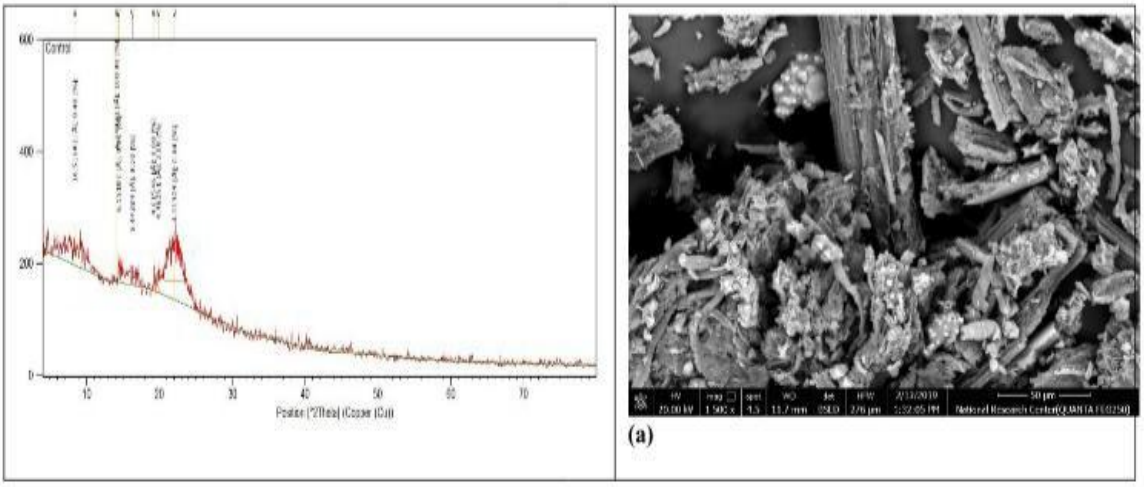
Figure (12). FTIR of (a) Native rice straw and pretreated rice straw with(b) chemicals (H_2SO_4+H_2O_2) and with (c) *Bacillus subtilis.*

Table (1). FTIR peak assignments

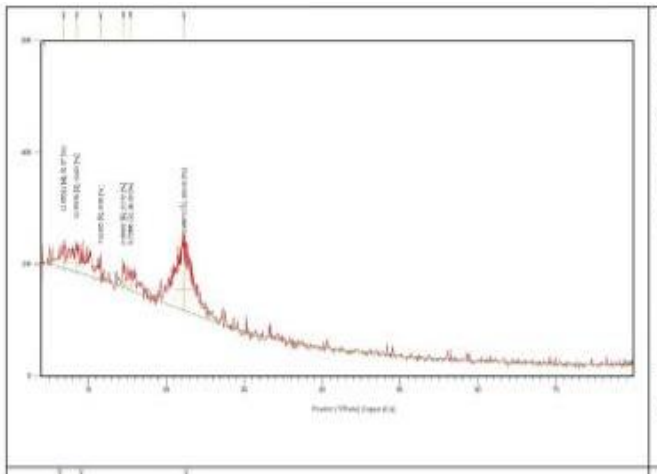
Wavenumbers (cm-1)	Peak assignment
3800 - 2800	A.3414 cm-1 O – H stretch, intermolecular hydrogen bonding. B. 2854 and 2925 - C – H stretch
1900 – 1500	C.1737 aromatic ring stretch (typical lignin) B. 1619 very strong aromatic ring stretch, aromatic –C – O stretch. C = C, C = O, C = N , aromatic skeletal vibration
1500 – 1300	1459 – C – H deformation, 1376 – weak C – O stretching, acetylated hemicelluloses, 1320 –CH2
1300 – 1100	1161 – glycosidic linkage, C – O – C – Hemicellulose ring vibrational stretching, 1717 – cellulose C – O stretch
1050 – 1200	E. 1060 – β- polysaccharide, 1045- C – OH bending, 1035 – C – O , C= C and C – C – O vibrational stretching

Table (2). Crystallinity Indices

Material/treatment	Crystallinity Index (%)
Native straw	29.0
Acidic-Hydrogen Peroxide treatment	45.8*
Bacillus subtilis hydrolysis	56.4*



(a)



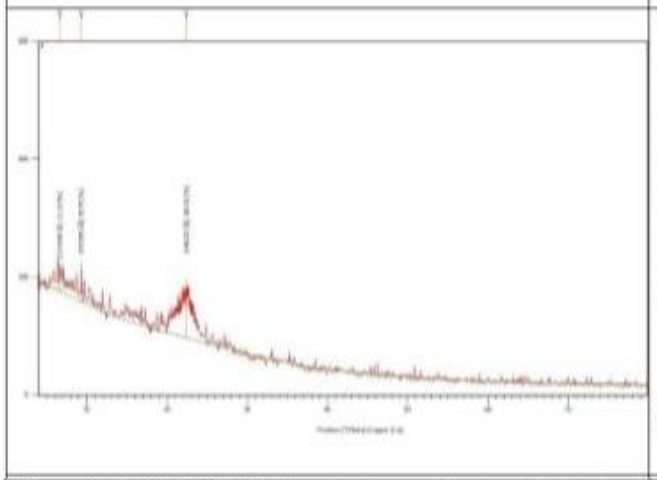
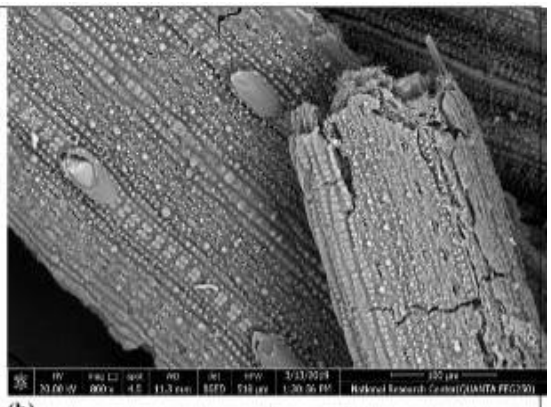


Figure (13). XRD of (a) Native rice straw and pretreated rice straw with(b) chemicals (H2SO4+H2O2) and with (c) *Bacillus subtilis.*



(b)

(c)

Figure (14). SEM of (a) Native rice straw and pretreated rice straw with(b) chemicals (H2SO4+H2O2) and with (c) *Bacillus subtilis*

3.8 SEM results before and after pre-treatment

The surface structures of the untreated and pretreated RS were analyzed utilizing SEM (figure 14). The untreated RS had an exceedingly conservative, smooth, and homogeneous structure that was corrupted after bacterial culture pretreatments. The profoundly thick structure was lost, and the surface was plainly corrupted and contorted. The pictures show broken fibrils and upset packages in the cell divider complex of each pretreated test. Our outcomes are like SEM investigation of RS treated with ligninolytic microorganisms and ligninolytic compounds in which comparable corruption and mutilations were watched [120,121]. In another investigation of RS debasement by a microbial consortium, the SEM micrographs indicated entrance of the external structure and disturbance of the substrate [122].

The free of microstructure and increments in surface territory brought about by Bacillus subtilis encourages the consequent hydrolysis process by enabling the microorganisms and proteins to enter all the more effectively [123]. In this way, biotreatment with Bacillus subtilis improve the hydrolysis procedure and expands the yield of reducing sugars.

Meng et al.,2015 [124] found that smooth unblemished physical structure in SEM (filtering electron microscopy) examination of local rice straw was changed to mole/papillae type structure shaped by deconstruction of fingernail skin wax and silica layers after Acid hydrogen peroxide treatment. Sun et al. [125] likewise, revealed that breaks and isolated sinewy structure were seen after Acid hydrogen peroxide treatment. The proposed purpose behind this impact was the evacuation of hemicellulose and the opening of macropores which were about twofold in size as for the untreated rice straw [126]. Corrosive hydrogen peroxide treatment has been accounted for to keep a great part of the silica with pre-treated solids [127] (figure 14)

SEM pictures of untreated RS (Fig.14-a) demonstrated a total, smaller, and continuous surface though the topology of organic treatment was mutilated (Fig. 14-c). These adjustments in the surface topology could be because of the movement of ligninolytic proteins created by Bacillus subtilis during natural pretreatment. The SEM pictures of substance treatment indicated particular and unmistakable unpleasantness with more profound

forests and looser topology (Fig. 14-b) when contrasted with the untreated RS. During the natural pretreatment, the outside surface of the lignocellulosic biomass was harmed and the surface region for cellulose of RS wound up open to cellulases present in the rough chemical. [128].

3.9. Ethanol Determination

3.9.1 Ethanol Yield (Ethanol productivity)

High temperatures and high ethanol fixations are real pressure factors for yeast during the maturation procedure, restraining cell division and the particular development rate. [129]. Likewise, these elements influence different vehicle frameworks, for example, the general amino corrosive permease and glucose take-up procedures [130,131]. The yeast disconnects acquired in our examination, which have high temperature and ethanol resistance levels, possibly create both present moment and long haul pressure reaction instruments to adapt to the harmful impacts of these burdens, including the amalgamation of heat shock proteins (HSPs) and trehalose just as adjustments to the lipid arrangement of the plasma film [132.133].

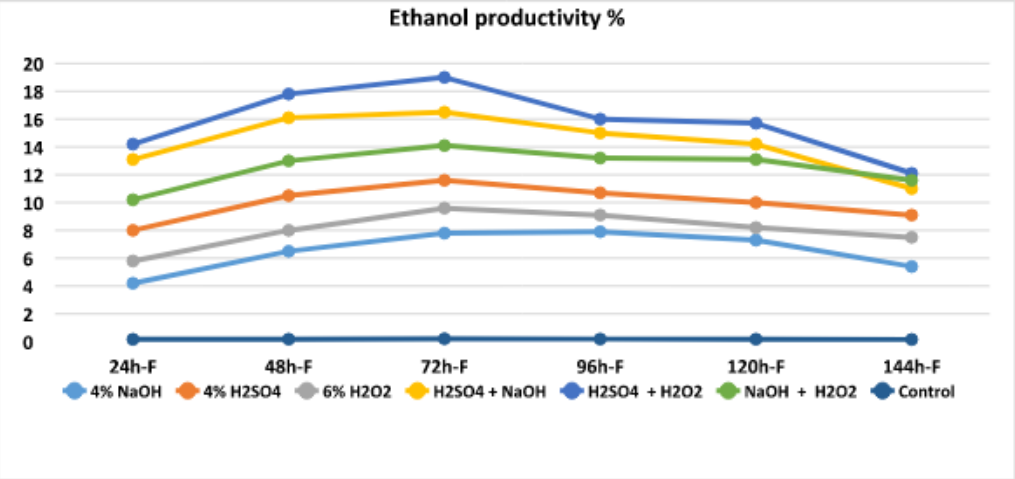


Figure (15). Ethanol productivity of pretreated rice straw during fermentation with Saccharomyces cerevisiae

Figure (15) likewise, demonstrates the impact of brooding time and yeast strain on ethanol generation. As per impact of brooding time on ethanol generation there was expanding pattern in ethanol sum and was seen from 24 hours to 72 hours while, the most reduced ethanol sum was found at 24 hours. Information demonstrated that following 72 hours of hatching was the most elevated estimation of ethanol pursued by 48 hours with huge contrast between them which is as per arrival of all out sugars and this showing the effectiveness of saccharifying and fermenting enzymes. These outcomes affirmed with Farag et al.,2015 and Ali et al., 2011 [34,134].

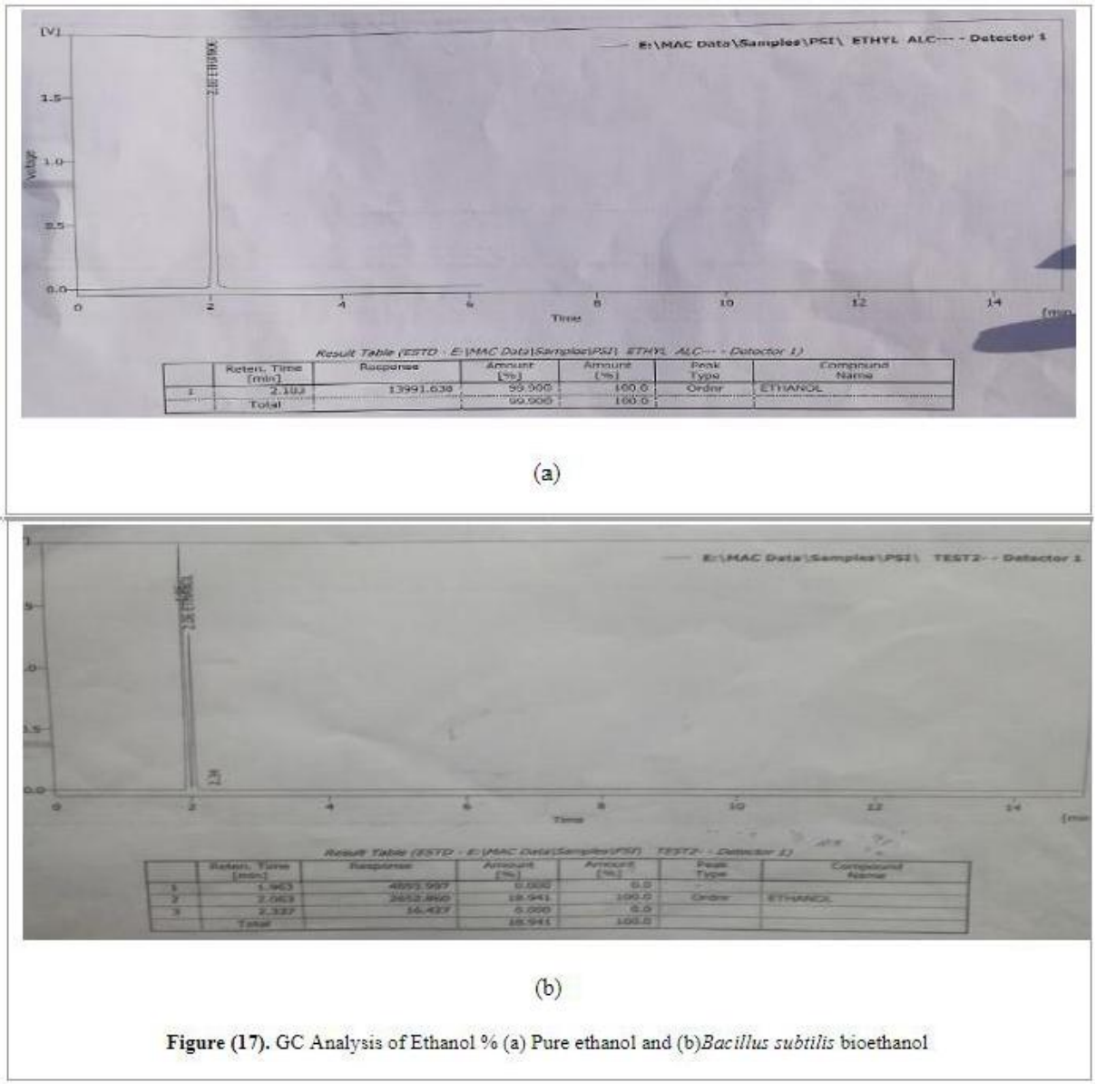
3.9.2. GC Analysis:

The examination of the fermentation medium (Figure.16) by GC-MS demonstrated an ethanol grouping of ≈189 mg/g (Fig. 17). There was a steady increment in the measure of ethanol created with increment in aging time from one day to fourth day. The aftereffects of maturation are appeared in figure (17-b) where the ethanol percent created after RS treatment with Bacillus subtilis is 18.9% (≈189 mg/g dry mass).

Figure (16). Ethanol yield after SSF of chemically treated rice straw with Bacillus subtilis and Saccharomyces cerevisiae

These results showed that there was a higher concentration of ethanol when B. subtilis was used to degrade the rice straw after Acid-Hydrogen Peroxide treatment, which suggested there was a higher conversion of the substrate to reducing sugars. Banerjee et al. (2010) [135] explained that enzymatic hydrolysis is done by cellulase enzymes that are highly substrate specific. The obtained yield can be compared with the yield obtained from other wild-type bacteria. Svetlitchnyi et al. (2013) [136] reported a maximum ethanol yield of 3.5 g/L from the wild-type bacterium Caldicellulosiruptor DIB 004C. Sato et al. (1993[137]) reported a bioethanol production of 4 g/L from the wild-type Clostridium thermocellum strain I-1-B, and an improved 23.6-g/L ethanol yield by the same strain when it was grown on an optimized medium.

The bioethanol yield obtained in this study was lower than the yield from banana pseudo stem (17 g/L) reported by Ingale et al. (2014) [138]. However, the yield from the co-culture was moderate to the yield (7.5 g/L) obtained from the fermentation of sugarcane bagasse hydrolysate using Pichia stipitis DSM 3651, as reported by Ira et al. (2016) [139].



(a)

(b)

Figure (17). GC Analysis of Ethanol % (a) Pure ethanol and (b)*Bacillus subtilis* bioethanol

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