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A Novel Iraqi Strain of Trichoderma Reesei Shows High Bioethanol Efficiency

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Abstract

Bio-ethanol is considered as one of the most prominent renewable energy sources that affects the environment which are produced from the depleting petroleum deposits. Using agricultural residue, bioethanol might be produced, supplying renewable resources at a low cost. Agricultural residues are currently investigated as feedstocks to manufacture sugar, which is then transformed into ethanol. Rice straw (RS) appears out as a potential raw material caused by its large quantity, lignocellulosic nature, and insufficient practical uses. Some of the strains that belong to the *Trichoderma reesei* genus are used on large scale for the production of cellulase and are essential for different applications such as food and animal feed, textiles, and paper manufacturing, as well as for the production of biofuels. This research focused on producing cellulase using Iraqi strains of *Trichoderma reesei* in a solid state fermentation methods, utilizing alkali-treated rice straw as a substrate. Before ethanol can be produced through fermentation, saccharification must occur to transform cellulose within rice straw residues into fermentable sugars. After that, fermentation of ethanol was accomplished by *Saccharomyces cerevisiae*. The highest fungal strain, number five, exhibited a maximum FPase activity of 10.05 FPU/g. The TRS concentration reached a maximum of 0.668 mg/ml accompanied by saccharification efficiency of 1.92% during 3 days at the fifth strain.. Fermentation by ethanol means performed via *Saccharomyces cerevisiae* at a concentration of approximately 0.146mg/ml in strain5. Assessments of the chemical composition, morphological, structural changes in rice straw that had been exposed to alkaline samples conducted via FTIR, X-ray diffraction and scanning electron microscopy

Introduction

Over the past few years, global energy consumption is continuing to increase, largely due to a reliance on fossil fuels, which account for over 80% of the world's energy production, driven by a steadily growing global population and accelerating economic growth. [1]. Inadequate petroleum supplies, along with intense exploitation of petroleum products and the emission of greenhouse gases (such as CO_2 and CH_4), have resulted in environmental deterioration and social unrest. [2, 3]. Consequently, A growing requirement exists for environmentally friendly and replenishable green energy sources which have minimal environmental and ecological impact, including biofuels such as bioethanol. Bioethanol plays a substantial role in both

manufacturing industries and logistics., also it viewed as a clean, eco-friendly, and perpetual source of renewable energy. [4] representing an reported market share of 10% to 40%, as noted by[5].

Lignocellulosic biomass, particularly rice straw, appears to be the most promising sustainable source of carbon, potentially up to a certain point. Rice straw is a highly prevalent type of lignocellulosic agricultural waste worldwide and offers a viable alternative to produce useful chemicals including biofuel[6]. The composition consists of a cellulose content ranging from 25 to 45%, hemicellulose from 20 to 30%, and lignin from 10 to 15%[7]. Estimated global lignocellulosic waste totals approximately 3.7 to 5 billion metric tons, comprising around 8.9 million tons of grain materials including rice, wheat, and barley[8]. Microorganisms that can produce cellulase are highly valued and of significant interest. The saprophytic filamentous fungus *Trichoderma reesei*, which is one of the many types of microbial organisms, has been studied extensively and is often used as a model to demonstrate the breakdown of lignocellulosic material produced by fungus [9, 10]. *Trichoderma reesei* is a highly regarded organism for its ability to produce a large quantity of the cellulase enzyme, which efficiently breaks down polysaccharides into monosaccharides, enabling their subsequent fermentation into ethanol and other types of alcohol[11]. Cellulases have a lot of effectiveness in the modern world and are currently thought to be the third largest volume of commercial enzymes [12]. It is also utilised in a variety of industrial applications, such as textile manufacturing, brewing, paper production, wastewater treatment, and the production of bioethanol[13]. Using cellulase enzymes, carbohydrates are biotechnologically converted into monomeric sugars. The resulting sugar solution is then fermented to produce biofuels, which can be a powerful substitute for running out of fossil fuels[14]. Our research aims to optimize the bioconversion of rice straw in to biofuel by employing cellulase sourced from a novel Iraqi strain of *Trichoderma reesei* and focusing on the efficiency of biofuel manufacturing pathway.

Experimental procedure

Pretreatment for raw rice straw

Substrates for enzyme production recovered from a field inside the Mishkhab region in Najaf Province, Iraq, specifically from rice straw sources. The rice straws were cut into lengths of approximately 2 to 3 centimeters and then blended. The milled rice straws underwent a 40mesh sieving process and were subsequently submerged in a 5% sodium hydroxide solution at a 1:10 concentration ratio of rice straw to NaOH. The incubating samples located within a boiling water bath during one hour. Following the cooling, the mixture screened using Whatman No.1 filter paper and subsequently wiped twofold to distilled water, maintaining the same ratio to adjust the pH to a neutral level. Conclusively, the mixture desiccated for 12h at 60ºC within an oven. Dehydrated rice straws stockpiled for subsequent enzymatic saccharification processing. This experiments were repeated three times, and the average results are reported.

Isolation of distinct fungal strains

Six novel Iraqi strains were collected from the Penguin city area in Sulaymaniyah Province, Iraq., and were previously identified through examination of their physical characteristics and microscopic examination[15].

Extraction of enzymes from a solid-state fermentation process using six distinct fungal strains

Each 500 ml Erlenmeyer flask held 10 grams of pretreated rice straws and one hundred milliliters of the basal mineral salts medium. The formulation consists of yeast extract at a concentration of 2, peptone at 0.3, $(NH_4)_2SO_4$ at 0.25, KH_2PO_4 at 1.4, $CaCl_2$ at 0.2, $MgSO_4.7H_2O$ at 0.3, $FeSO_4.7H_2O$ at 0.005, $MnSO_4.4H_2O$ at 0.001, $ZnSO_4.7H_2O$ at 0.001, and $CoCl_2.6H_2O$ at 0.0002. The solution included (g/L): Yeast extract 2, peptone 0.3, KH_2PO_4 1.4 , $FeSO_4.7H_2O$ 0.005, $MgSO_4.7H_2O$ 0.3, $ZnSO_4.7H_2O$ 0.001, $MnSO_4$. $4H_2O$ 0.001, $CoCl_2.6H_2O$ 0.0002, $CaCl_2$ 0.2, $(NH_4)_2SO_4$ 0.25, The pH medium is 5.

The flasks underwent autoclaving at a temperature of 121ºC for 15 minutes at a pressure of 15 pounds, with the aim of disinfecting them. Once they had cooled to room temperature, each flask was then inoculated with 3 milliliters of the *T.reesei* cultures strain, containing 106 spores/ml, and the mixture was thoroughly combined. The cultures had grown in 30ºC temperature and agitated on a rotary shaker at 250 rpm during 5 days. 0.1M citrate buffer pH4.8 was then put in flask. After an hour of shackeding at 150 rpm, The flasks were filtered using Whatman filter paper No.1 and then rotated within centrifuge to 10000rpm ,10min , 4ºC to eliminate debris. The filtrate was then putted through a membrane with a pore diameter of 0.2 mm that possessed disinfecting capabilities.

Assay of exoglucanase activity using filter paper.

The filtrate (0.5 ml) was then injected into presoaked paper filters and agitated about one hour and 50ºC. The exoglucanase enzyme assays, specifically FPase, were conducted depending on the experiment by [16], A whatman No.1 filter paper strip, weighing 0.6mg, was immersed in 0.5ml of a 0.05M citrate acid buffer solution with a pH of 4.8, serving as a substrate for filter paperase (FPase) .After that, A filtrate(0.5 ml) injected to presoaked paper filters and agitated around 50ºC about an hour. After incubation, Each tube was terminated with the addition of 3ml of 3,5-dinitrosalicylic acid reagent. The tubes placed whithin a boiling water bath for a duration of 5 minutes. In these assays, reducing sugars expressed through calculating the absorbance at 540nm, as described by [17], with glucose used as the standard. The filter paper assay enzyme was defined as one unit (U) of filter paper, with U of enzyme activity being equivalent to the amount of enzyme that produces 1 µmole of the amount of reducing sugars, as represented by glucose content, is appeared in units per gram of dry substrate U/gds, where each unit regards the amount of glucose present per milliliter per minute.

Enzymatic hydrolysis

The enzymatic hydrolysis process was carried out as described by [18]. Primarily, rice straws treated with alkaline solutions utilized as the substrates in the process of enzymatic saccharification. A 100ml Erlenmeyer flask wa used for the assays, containing 3% alkali rice straw put within a 0.05M citrate buffer of pH 4.8, to which a 10ml cellulase enzyme supplement added. The flasks closed via a cover and the solution were setted within rotary shaker at 150rpm, 50ºC for 72h.The later solution was subsequently supplemented to sodium azide 0.005% for prevent microbial proliferation and to avert the utilisation of sugars resulting from hydrolysis. Additionally, the sampling give up per each flask per cycles 24, 48 and 72hrs , directly placed on ice, centrifuged at 10000rpm/5min, subsequently passed across millipore filter. A filtrate analysed for total reducing sugars via DNSA, followed by calculation of percent saccharification using the equation as previously shown by [19].

$$\text{Saccharification\%} = \frac{\text{formed TRS mg/ml} \times 0.9 \times 100}{\text{Cellulose content of pretreated substrate}}$$

An assays conducted three times. The mean values are presented in the current study.

Analysis of Chemical Composition of rice straw

The levels of hemicellulose, cellulose, and lignin in rice straw were examined both prior to and subsequent to hydrolysis. [20, 21].

Preparation of inoculum yeast

Yeast of the species *Saccharomyces cerevisiae*, commonly used in industrial baking, was obtained from the Agricultural Engineering Sciences College/ University of Baghdad and utilized for ethanol fermentation.

1.0g/100ml of the yeast cells was later transmitted into 250ml flasks constituting 4 g/L yeast extract, 3g/L peptone, 20g/L glucose, pH5.5, and assign within rotary shaker 200rpm, 30˚C during 24 hours.

Ethanol production

The fermentation medium included (g/L): 1g/L yeast extract, 0.25g/L$Cacl_2.7H_2O$, 5 g/L $(NH4)_2\ SO_4$, 1g/L KH_2PO_4, 0.5g/L $MgSO_4.7H_2O$. A cellulosic hydrolyzates obtained from an enzymatic hydrolysis glucose, compressed under the evaporation, after that, these hydrolyzates filtered from filtration through millipore filters 0.45µm after centrifugation at 1000rpm for 10min to get supernatant. In this study 100ml of fermentation medium inoculated to the 250ml anaerobic fermentator bottle consisting hydrolyzates liquid, and later autoclaved at 121℃ and 20min. Saccharomyces cerevisiae is incorporated into the medium of the ethanol fermentation, representing roughly 2% of the totally within fermentation process. The fermentators were then rotated at 150rpm, 35 ˚C, pH4.5 for 72hr . The airlock system ensured a tight seal on each fermenator bottle, thereby preventing oxygen from entering and maintaining an anaerobic environment within the fermenator bottles. Samples were taken in sterilized manner at 24, 48,72 hrs, and centrifuged for 15min , 4˚C at 10000rpm and utilized to determine of the ethanol and total sugars[22]. Each experiment was repeated three times, and the average results along with their standard deviations were recorded.

Analytical methods

Estimation of ethanol by gas chromatography

GC revealed to calculate the amount of ethanol produced within fermentation, using a Porapak Q column in conjunction with a Flame Ionization Detector (FID). The catalyst's gas flow rate recorded 30 ml/min, and the oven at 155 °C, the injector the detector at 250 °C and at 175 °C. The samples were first passed via a 0.2 micron filter prior to undergoing analysis.

Substrate Characterization

1- **FTIR analysis**

Fourier Transform Infrared spectroscopy experiments were performed on untreated, pretreated, and hydrolyzed rice straw samples to discover modifications to the functional groups. FTIR absorption measurements were recorded within the 400-4000 cm^{-1} range via a Fourier transform infrared spectrophotometer.

2- **Analysis by X-ray to assess crystallinity**

X-ray diffraction analysis, employing an XRD-6000 (Shimadzu, Japan) instrument, was used to evaluate the crystallinity of lignocellulosic residues. This analysis utilized a Cu-Ka radiation source, a 30.0 kV voltage setting, and an electric current of 15 mA. A single scan was conducted, consisting of 20 consecutive intervals spanning 4.0-70.0°, with a scanning rate of 2.0° per minute. One scan was conducted with intervals between 4.0° and 70.0°, occurring every 20 minutes, and it progressed at 2.0 degrees/ minute speed.

The crystallinity index (C.I) obtained using this pattern [23]:

$$C.I\% = \frac{I200-Iam}{I_{200}} \times 100 \qquad (1)$$

Crystalline substance is represented by I_{200}, the peak intensity from the 200 lattice plane (2θ=22.4º), wherease a amorphous substance of rice straw is shown by I_{am}, the diffracted intensity peak at 2θ=18º. The following formula was used to determine the percentage of crystallinity in the raw, pretreatment, and treated rice straw[24]:

$$\text{Crystalline } \% = \frac{I22}{I_{22+}I18} \times 100 \qquad (2)$$

Where I_{22} and I_{18} are the crystalline and amorphous intensities, respectively, represented by the 2θ scale of 22º and 18º.

The fiber samples' crystallite sizes were assessed via the Scherrer form [25]:

$$L = K\lambda / \beta \cos \theta \quad (3)$$

In this context, L represents the crystallite size, the form factor is denoted by K (0.94), λ signifies the X-ray wavelength, B indicating the maximum half-width of the equatorial reflections, and θ refferring the Bragg angle in relation to the 200 plane.

Using the Z-discriminant (Z-value) evaluation, it is possible to determine if the crystalline cellulose structure represents I_α (monoclinic) or I_β (triclinic) (Wada and Okano, 2001). The Formula below determines this evaluation [26].

$$Z = 1693d_1 - 902d_2 - 594 \quad (4)$$

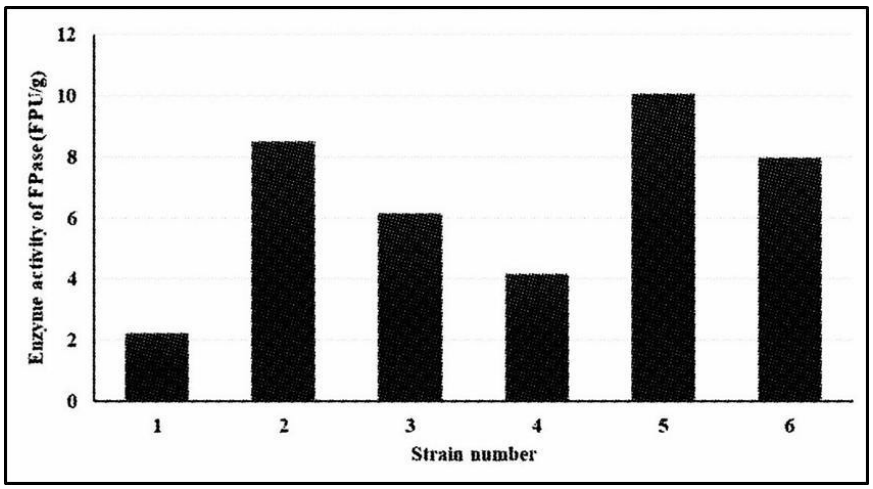
where $Z > 0$ indicates I_α, and $Z < 0$ indicates the I_β dominating cellulose type, in addition d_1 represents the d-spacing of the peak (1–10) and d_2 appears the d-spacing of the peak (110) [27].

3- Scanning electron microscopy (SEM)

The structural morphological alterations in the untreated, pretreated, and treated samples were examined using SEM analysis. After being glued on appropriate stubs, the dry samples were treated with Au using a Doerlikar-Balers, Batzers, Liechtenstein SCD 050 sputter coater. After fixation, a Zeiss, SIGMA model of electron microscope used to take pictures at various magnifications.

Results and discussion:

Table 1 Estimation FPase enzyme via six novel fungus strains



A greatest activity of enzyme levels were attained with pre-treated RS at a level of approximately 10.05FPU/g in fungus strain (5), which showed lower levels of hemi-cellulose and lignin compared to other strains.

Pretreatments may decrease the repulsion for lignocellulosic waste, hence enhancing digestibility and microorganism availability of cellulose [28]. As a result, the elimination of both lignin and hemicelluloses may have facilitated fungal access to the cellulosic structure, resulting in increased secretion. In addition to higher secretion, another crucial element to consider is the time spent producing enzymes. Studies conducted with various strains of *T. reesei* exposed to different residues indicate that the most suitable time periods fall between four to eight days, which is equivalent to 96-192 hours[29]. Research has shown that the strain *Trichoderma reesei* Rp698 exhibits higher FPase activity than *T. reseei* QM9414 [30]. The study with accordance of those discovered by[31], who showed that Fpase in RS was more pronounced within *Aspergillus terreus* compared to *Trichoderma viride*.

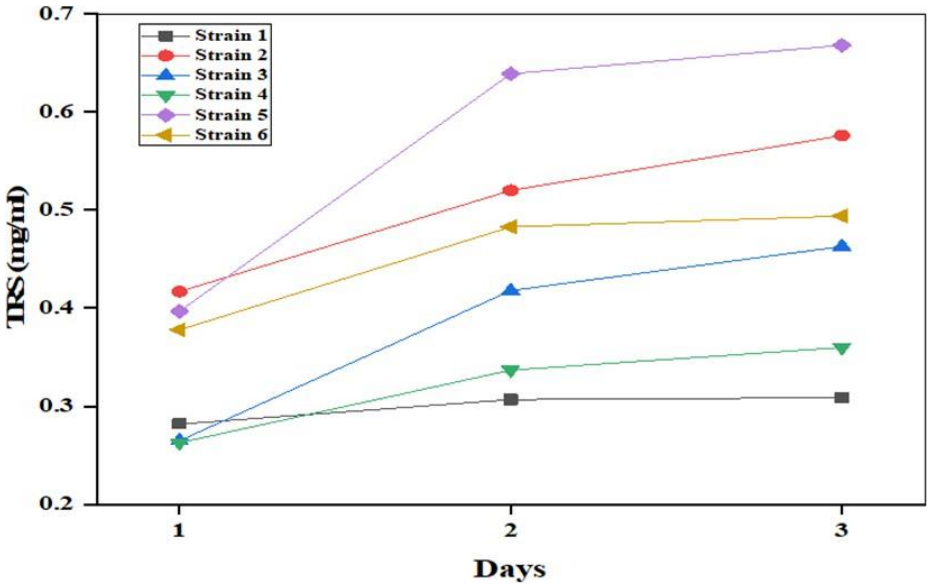


Figure 1 Determination of total reducing sugar yield(TRS)(mg/ml) via a UV- visible spectrophotometer

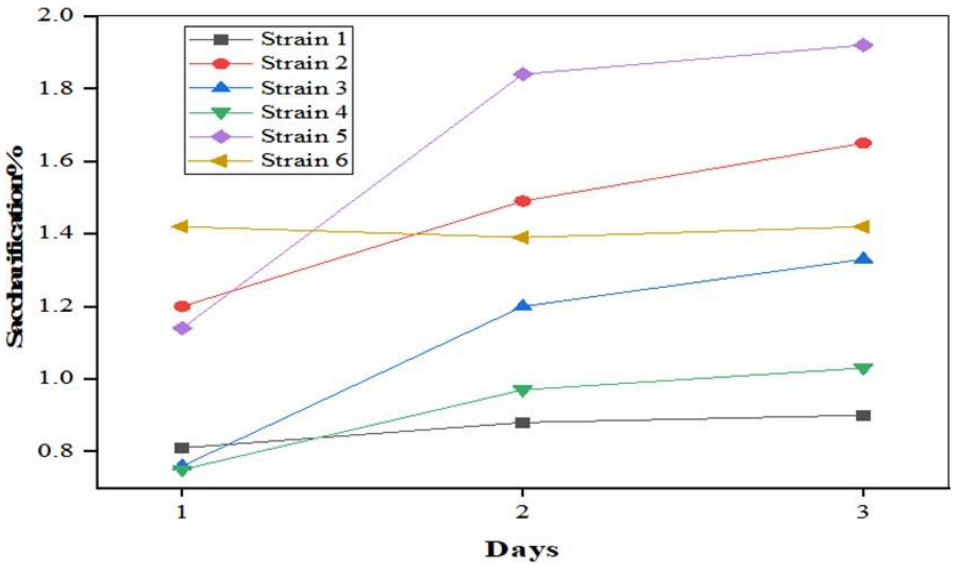


Figure 2 Saccharification percent during 24, 48 and 72h of treated RS.

Figures 1 and 2 depict the total reducing sugars (TRS) yields, which serve as an estimate of the amount of sugars that can be released through hydrolysis.

The highest concentration of TRS was achieved as 0.668 mg/ml, resulting from 1.92% saccharification at strain five after three days, with lower concentrations observed in strains 2, 6, 3, and 4, and then in strain 1. Specifically, the TRS concentrations were (0.576, 0.463, 0.309) mg/ml, corresponding to (1.65, 1.42, 1.33, 1.03, 0.9)% saccharification, respectively, following the same three-day timeframe.It is evident that enzymatic

hydrolysis yielded the highest concentration of fermentable sugars, particularly in strain 5. Several researchers have used microbial enzymes to break down lignocellulosic materials. The saccharification rates achieved using *T. viride* culture supernatant were 9.9% for cotton, 59.4% for filter paper, and 41.8% for newspaper[32]. Using *Sporotrichum thermophile* culture filtrate to hydrolyze saw dust, filter paper, and newspaper, the rate of saccharification was 3.5, 1.5, and 3.0%, respectively. [33]studied the hydrolysis of paddy straw that had been treated with an alkali using an industrial cellulase, yielding 65% total reducing sugar. *T. reesei* cellulases were utilised by [34] to enzymatically break down alkali-treated rice straw, resulting in a maximum glucose yield of 1.07% after a 16 hour period. Research by[35], indicated that fungal treatment to the rice straw using *T. reesei* and *Aspergillus awamori* yielded 73.7 and 62.7 mg/g of TRS, respectively. [36]study found that the decrease in sugars yield and saccharification level after treated willow residue reported 28.3% and 24%, respectively. Furthermore, TRS levels were measured at 43.38, 64, and 51 mg/g after undergoing solid-state fermentation with the strains *Aspergillus niger*, *A. awamori*, *T. reesei*, and *T. viride*, as reported by[37]. [38]who found the hydrolysates of RS straw contained a concentration of 12% (w/v) reducing sugars.

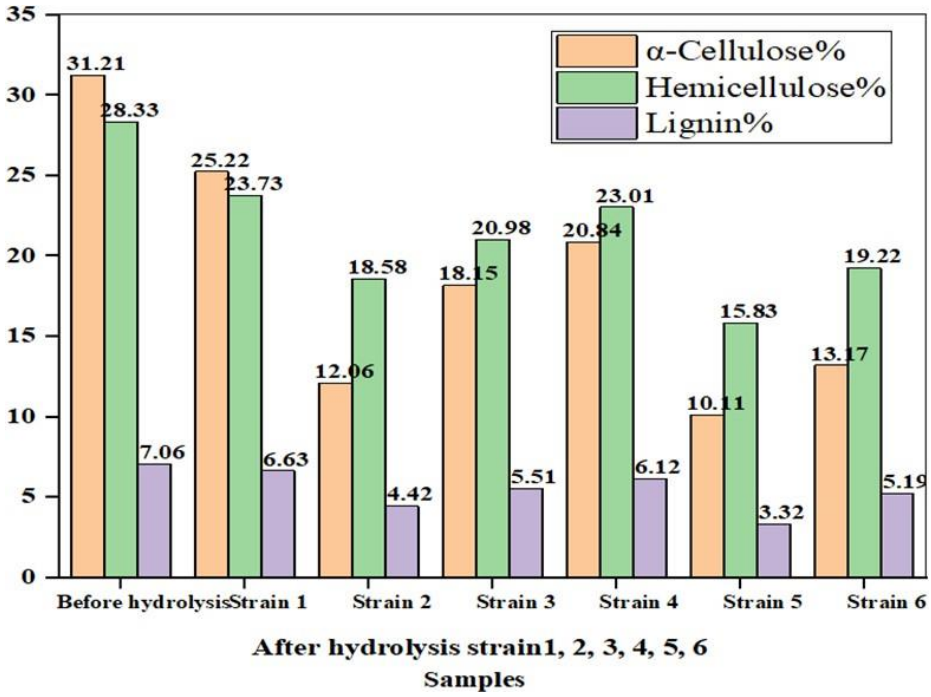


Figure 3 Chemical constitution of rice straw both prior to and subsequent to hydrolysis.

For hydrolysis to release high sugar, it is essential to pre-treat lignocellulosic biomass so that saccharifying enzymes have access to cellulose, as lignin interferes with the enzyme's ability to attack cellulose. Prior to saccharification, a pretreatment step is necessary to decrease lignin levels and enhance the surface area in order to facilitate optimal enzyme activity. Throughout this investigation, it was discovered that weight loss induced by treatment promotes the degradation of ligno-cellulosic residue.

Figure(3) shows that untreated rice straw had 31.21% cellulose, 28.33% hemicellulose, and 7.06% lignin. The saccharification of treated rice straw using six different strains showed the lowest chemical content in strain 5, which yielded 10.11%, 15.83%, and 3.32% respectively. This attributed to a maximum degree in strain 5 for deliginification of rice straw compared to other strains. According to [36], an analysis of a treated willow

content revealed the presence of cellulose at 0.5%, hemicellulose at 21.3%, and lignin at 25.5%. The primary effect of alkaline pretreatment is to enhance delignification efficiency by breaking down the lignin side chain, thereby releasing low molecular weight components. Findings corroborate those of [31], who found untreated rice straw to consist of 34.80% cellulose, 31.22% hemicellulose, and 10.18% lignin. In contrast, chemical analysis of rice straw treated with *Trichoderma viride* revealed compositions of 25.20%, 19.22%, and 7.52% respectively, while *Aspergillus terreus* hydrolysis yielded 20.70%, 15.03%, and 6.73% respectively. In relation to our results, [39] discovered that chemical treatment led to a larger destruction of lignin and hemicellulose, which in turn released more sugars and phenolic compounds. According to [40], the hydrolysis of carbohydrates by alkali caused a decrease in the cellulose amounts of tobacco residue following a 10% NaOH pretreatment, ranging from 44% to 27.6%.

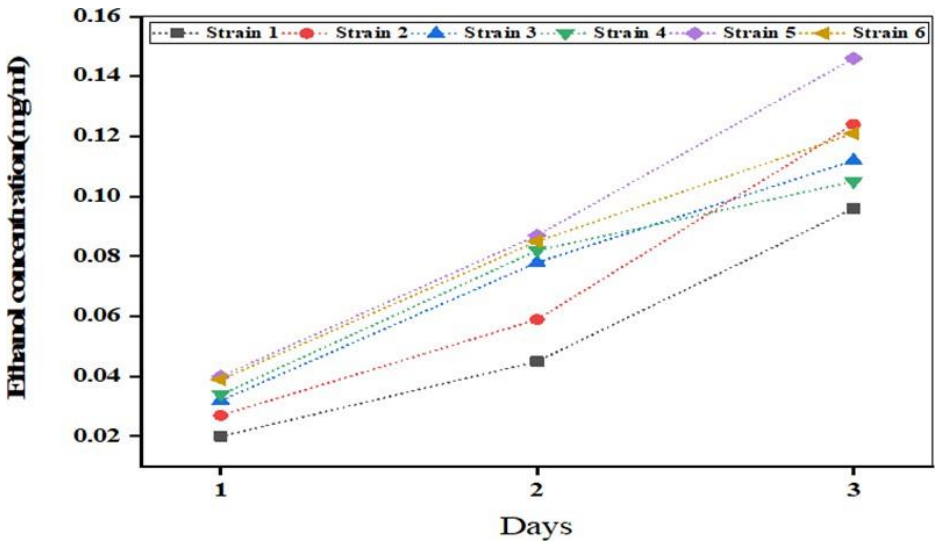
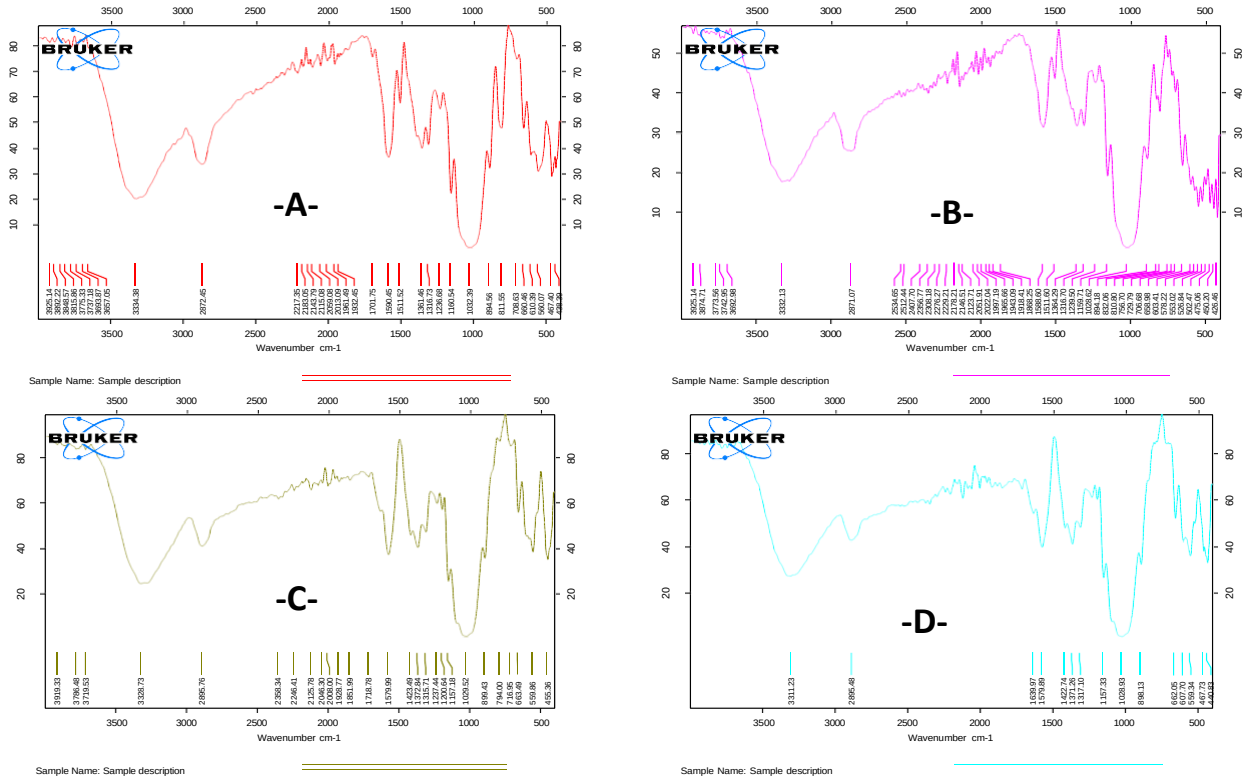


Figure 4 Ethanol concentration (mg/ml) estimation

The analysis shows a concentration of around 0.146 mg/ml was achieved after 3 days in the fermented mixture at strain 5 .The variations in performance are observed among different fungus strains is attributed to their preferred utilisation of pentose and hexose sugars found in the hydrolysate. The variation could also depend on the specific strain. The disparity in ethanol production can be attributed for the accessibility of fermentable sugars from cellulose contained in biomass, which justified for removing lignin and hemicellulose, thereby making cellulose more amenable to enzymatic treatment in SSF. Research on the formation of bioethanol from rice husks confirmed our findings that SSF produced a higher quality ethanol output [41, 42]. Using different yeast strains to produce ethanol, [43] discovered that *Kluveromyces marxianus* produced a great deal of ethanol, yielding 11.8g/L in the wheat straw hydrolysate. Studies by [44] revealed that maximum ethanol output in solid-state fermentation occurred after 72 hours of fermentation. The direct conversion of resulting sugars to ethanol, without any feedback inhibition, is thought to be the reason behind the high ethanol output in SSF. *Trichoderma harzanium*-treated rice straw yielded an excess of bioethanol at approximately 5.4g/L, whereas *Aspergillus terreus* produced 4.7g/L. [45]. Between 24 and 72 hours, the maximum ethanol production, which ranged from about 6g/L to 10g/L, was achieved. This rise in ethanol production may be explained by the nutritional supplementation, which increased *S. cerevisiae* capacity to produce ethanol[46]. According to [47] , in research findings has consistent with earlier research indicating the ethanol production from rice hydrolysate using

Zymomonas mobilis yields more than *Candida shehatae*, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *Pichia stipites*.

Data from Fourier transform infrared (FTIR) spectroscopy collected both untreated and treated RS samples, with an aim of identifying of non-cellulosic components of the substrate at two stages: before and after the reaction of cellulose. Peaks for samples of untreated and alkali-treated at 3334 cm^{-1} and 3332 cm^{-1} were indicative of the –OH stretching to the hydrogen bonds. Additionally, peaks have relatively insignificant compared to hydrolysed treated samples. The disruption of hydrogen bonds between the O-H groups of hemicellulose is likely the cause [48]. In untreated and alkali-pretreated samples, peaks at 1701 cm^{-1} and 1719 cm^{-1} were associated with the ester linkage of carboxylic groups from ferulic and coumaric acids present in hemicellulose. However, in hydrolyzed samples, these bands completely vanished. The results suggested enzymatic activity was present. [49]. Peaks are observed a 1236 cm^{-1} and 1239cm^{-1} within untreated and alkali-pretreated tests, corresponding to the presence of phenolic hydroxyl groups in lignin, whereas these peaks are diminished in hydrolysed treated samples, indicating a removal of lignin. [50]. A peak at 898 cm^{-1} identified for cellulose and it rises after samples have undergone hydrolysis with an enzyme[51]. Absorption bands within the 3309-3328 cm^{-1} range within the hydrolysed samples exhibit the characteristic of cellulose, that is in contrast to untreated and alkali-pretreated samples. It can be inferred that the levels of chemical contents pectins and hemicellulose have lowered. [52] Figures (5-C-H). In Figure 5-G,. The treated samples exhibit a heightened intensity of the cellulose peaks at 900cm^{-1}, 1023cm^{-1}, 1156cm^{-1}, and 1640cm^{-1} as a result of the increased cellulose content after the elimination of feculences within the fiber system [53].



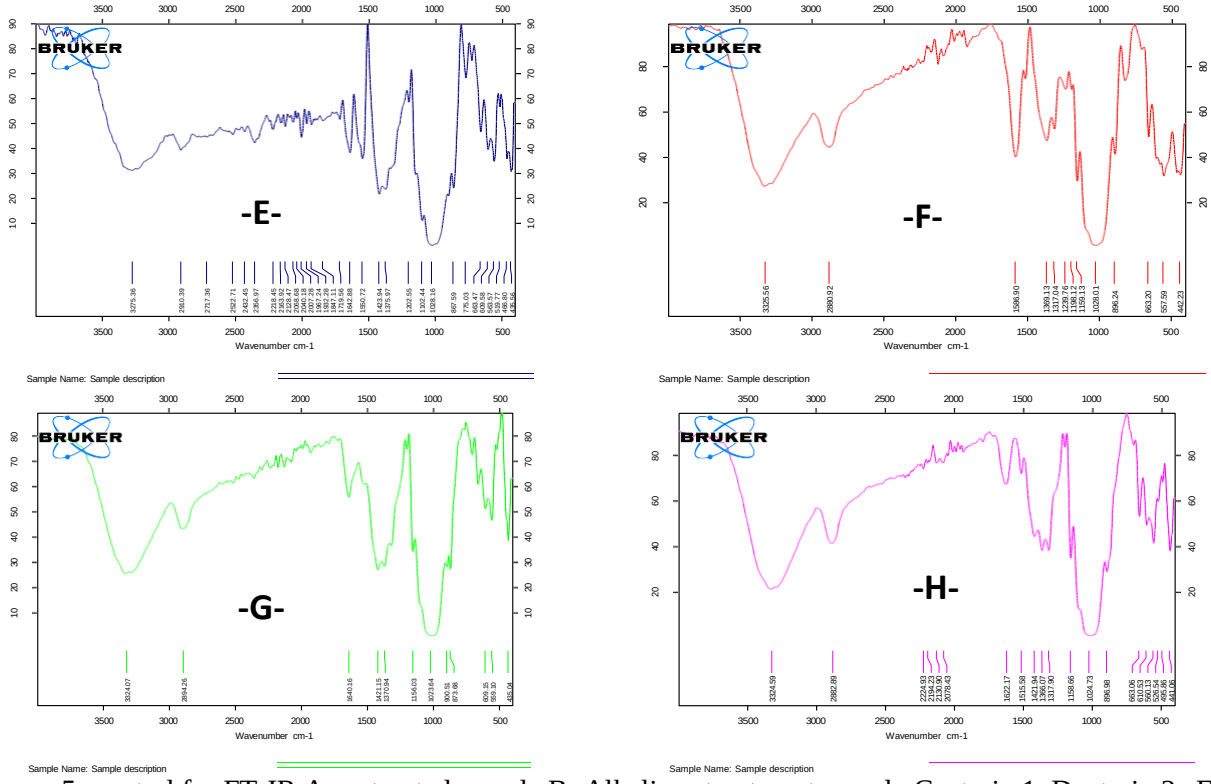


Figure 5 spectral for FT-IR A- untreated sample B- Alkali-pretreatment sample C- strain 1, D- strain 2, E- strain 3, F- strain 4, G- strain 5, H- strain 6. Note: From C to H represents hydrolysed samples

The untreated, alkali-pretreated, and treated RS fibers are illustrated within Figure 6 by their patterns of X-ray diffraction. The diffraction of all samples revealed the existence of reflections at 2θ 18 and 22.4, indicating both crystalline and amorphous patterns. In figure G, the diffractogram pattern of the hydrolysed sample (5) exhibited a prominent sharpened peak at 2θ 22.4 and a lower, expanded peak at 2θ 18 in comparison to the other samples. The shift in cellulose structure's relative crystallinity from treatment may attributable primarily to hydrolysis and the elimination of non-cellulosic components areas occurring during the process. Our findings are consistent with earlier documentation by different docmentations [54, 55, 56], it demonstrate the crystallinity consistuents deformation within the lignocellulosic system.

The hydrolysed sample, as shown in table(2), exhibited a marked increase in crystallinity index, crystalline content, and crystallite size, with values rising by approximately 39%, 62%, and 4.59nm respectively, surpassing those of all other tested samples, and this was confirmed by the FTIR spectra. The process involves the totally amorphous regions breakdown, specifically wax, hemicellulose, pectin and lignin within the primary cell wall. Previous studies, including those by [57, 58, 59, 60], found that our findings align with observations of enhanced crystalline cellulose structure following pretreatment with various agricultural wastes. [61]resulted the crystallinity forms of Bombax ceiba have shown modifications following pretreatment with sodium hydroxide in recent X-ray diffraction analysis. The Z-values of RS fibers showed that I_β was the dominant type of cellulose structure in higher plants because I_β was found exclusively in these plants, as reported by [62]The data obtained were in agreement with several previously published studies[52, 63, 64].

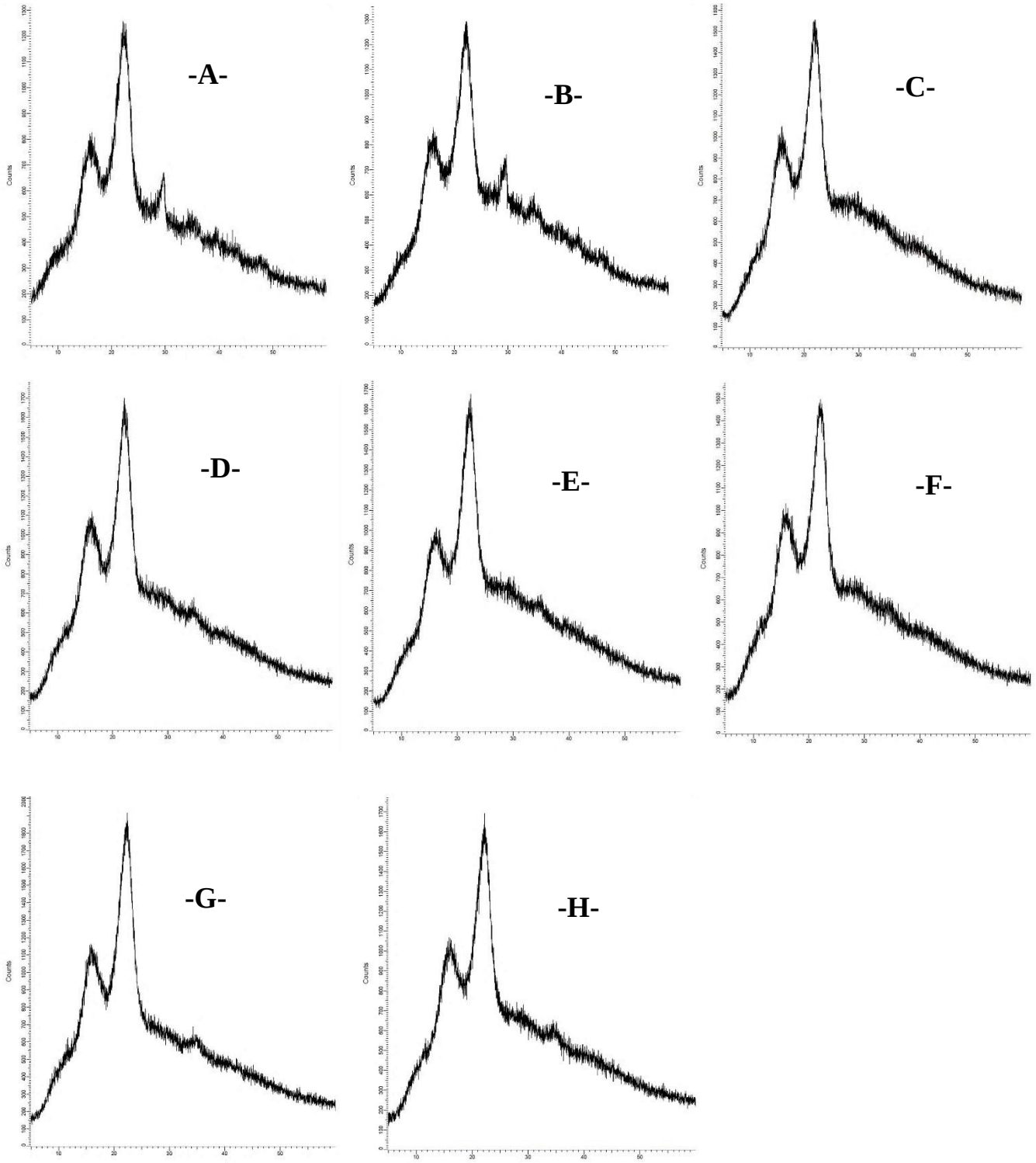


Figure 6 Patterns of X-ray diffractograms A- untreated sample B- Alkali-pretreatment sample C- strain 1, D- strain 2, E- strain 3, F- strain 4, G- strain 5, H- strain 6.

Note: From C to H represents hydrolysed samples

Table 2 Analysis of the XRD parameters for prior to and subsequent to enzyme treatment on RS fiber

RS Samples	CI (%)	Crystalline (%)	Crystallite size (nm)	Z- value
Untreated	28	47	1.9	-21.4
Alkali-pretreated	30	48	2.2	-22.8
Hydrolysed 1	31	50	2.6	-24.5
Hydrolysed 2	38	61	4.1	-34.9
Hydrolysed 3	35	54	3.54	-29.1
Hydrolysed 4	32	51	3.03	-26.7
Hydrolysed 5	39	62	4.59	-36.2
Hydrolysed 6	37	58	3.82	-33.4

Fig.7 Scanning Electron Microscope images of the alkali and enzymatic treatment for morphological and structural surfaces of the RS samples

Samples of untreated RS possess a specific fibre system. A closed structure exhibited a high degree of resistance, as evident in Fig. (7A), resulting in a limited number of pores. The entire plant cell wall, including vascular bundles, has an extremely fibrillar composition and is easily visible. Earlier studies have documented the characteristics of untreated sorghum biomass, which were found to be highly conserved, compact, and nonporous in structure[41]. Pretreatment with alkali caused modifications to the fabrillary structure. As a result of the pretreatment's structural disruption, the substrate had an uneven, unsmooth, hard, and tough surface, with some exterior surface components lacking. The findings indicate that several adjacent fibers have undergone a decrease in crystallinity of cellulose, which has also led to an increase in porosity, ultimately allowing the cellulosic component to facilitate bioconversion, as illustrated in Figure 7B. This outcome was also corroborated by [61], who discovered that lignin and hemicellulose can be substantially reduced through alkali pretreatment , [65] employed a scanning electron microscope to identify irregular surfaces of pores in pretreated palm tree stem debris stem debris.

The morphological change to the fiber after enzymatic treatment is indicative of a smoothed surface, which could be the result of the enzyme building up in the fiber surface, the form of pores on wall system takes place simultaneously with the removal of unwanted microfibrils. The effect was more pronounced in saccharification processes with elevated efficiency rates , particularly in strain five, as shown in Figures C, D, E, F, G, and H. An analogous evaluation was conducted with treated jute residues, as reported by[66, 67].

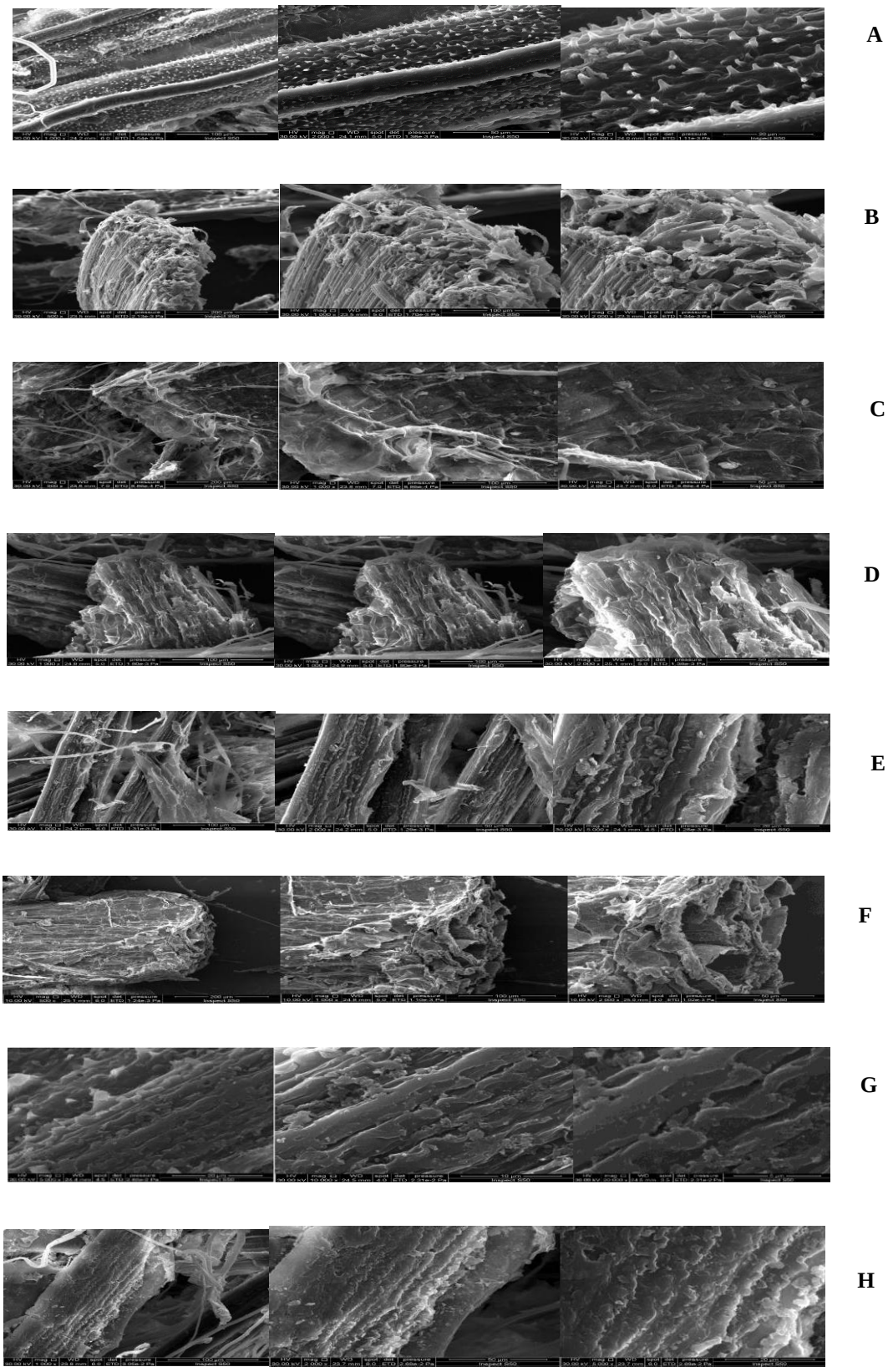


Figure 7 A-Untreated R.S, B- alkali-pretreated R.S, C- Strain1, D- Strain2, E- Strain 3, F- Strain4, G- strain5, H- strain6.

Conclusion

Ongoing research is focused on developing biofuels derived from microorganisms, with the goal of reducing reliance on finite fossil fuels, generating employment opportunities, and promoting a healthier environment planet. Significant progress has been made in the production of biofuels from *Trichoderma reesei* Iraqi strains, particularly strain five. Furthermore, this study found that *T. reesei* strain five was a hyperproducer of cellulase than other *T. reesei* strains, resulting in yielded bioethanol production. This global momentum towards renewable energy provides a significant opportunity for countries like Iraq to diversify their energy portfolios and align with international sustainability objectives.

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