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Bioethanol Production From Hydrolysis And Fermentation Of Rice Straw Using A Combination Of Microbial Isolates

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Abstract

Rice straw (RS) is one of the most prevalent agricultural wastes that can be obtained cheaply and used as a permanent source of biofuel. The saccharification of lignocellulosic wastes is a vital and expensive process in fermentable sugars and bio-ethanol production; therefore, this study focused on maximizing the quantity of fermentable sugars by fungal hydrolysis. In this study, twenty-four fungal isolates were isolated from different lignocellulosic biomass (RS, Water hyacinth, Bagasse, and Saw Dust). RS was subjected to a biological hydrolysis process employing a combination of fungal isolates. Then the resulting sugar is fermented by *Saccharomyces cerevisiae* to bioethanol. The most promising fungal isolate for hydrolysis was the combination of *Trichoderma harzianum* and *Aspergillus terreus* which produced the maximum amount of sugar (13.431 mg/ml). The Response Surface Methodology (RSM) with a Central Composite Design (CCD) was utilized to statistically analyze and optimize the conditions (incubation time, temperature, biomass concentration, and inoculum size) for achieving a maximum total reducing sugars (TRS) production. Under the optimal conditions (12 days of incubation, 20% biomass concentration, 25°C, and 1.14 ml of inoculum size), the study achieved a maximum TRS production of 17.212 mg/ml. This amount of TRS is fermented by *Saccharomyces cerevisiae* to produce 11.05 ml/l of bioethanol. This research emphasizes the significance of rice straw as a renewable waste for bioethanol production. Furthermore, this study demonstrates the relevance of fungal hydrolysis for maximal fermentable sugar production from rice straw. As a result, this study has accomplished two goals: the elimination of rice straw and biofuel production.

Keywords : Bioethanol, Optimization, Response Surface Methodology, Chemical Analysis, Rice Straw, Saccharification, *Saccharomyces cerevisiae*

1. Introduction

Environmental changes, as well as depleting fossil fuel reserves, have raised demand for alternate fuel sources. Energy consumption has expanded considerably in recent decades because to rising economic activity, industrialization, and rapid population growth **[1,2].** The amount of CO_2 generated into our atmosphere duo to the burning of fossil fuels will rise globally. Transportation and the burning of fossil fuels are responsible for 15% of the world's greenhouse gas emissions and 23% of CO2 emissions **[3].** So that, biofuels are generally accepted as a practical substitute for fossil fuels in transportation that can aid in lowering the CO_2 emission. Biofuel is high-value by-products obtained from the conversion of lignocellulosic waste into simple monomeric sugars that can be fermented into useful compounds like bioethanol **[4].** The world consumption of renewable fuels, especially bioethanol, is skyrocketing and will have more than triple by bioethanol production 2035 **[5].**

The feedstocks used in the production of bioethanol are divided into 1st generation feedstocks (such as sugar beets, sugar cane, and cereal grains), 2nd generation (such as lignocellulosic biomass), and 3rd generation (such as algal biomass) **[6].**

The most sustainable carbon source on the planet is lignocellulosic biomass., which produced annually by 200 billion tons **[7].** Because of its renewable nature, lignocellulosic biomass has piqued researchers' interest as well as earned widespread notice **[8]**. Rice ranks third in the world among grains, producing massive biomass residue (straw) each year. In 2017, over 770 million tons of rice were harvested, an

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increase of 1.5% year on year. As a result, the production of by-products such as rice straw grows dramatically. 1 to 1500 gm of rice straw is produced from 1000 gm of milled rice **[9].** Hence, RS is one of the most common types of lignocellulosic waste on the planet, but over the last ten years, burning rice straw in open fields has gained popularity as a post-harvest management technique for paddy fields. This illegal burning technique has major negative effects on the environment and human health. On the other hand, rice straw is a suitable raw material for microbial fermentation and production of energy because rice straw contains carbohydrates in its cell wall and is widely available **[10].**

Cellulose, hemicellulose, and lignin make up the majority of lignocellulosic biomass. Different plant species have different ratios and compositions of these polymers. Furthermore, the age, growth stage, and other factors influence by a single plant's composition **[11]**. The hydrolysis of lignocellulosic biomass for fermentable sugars production is very important step for bioethanol production. There are different hydrolysis methods, such as chemical and biological methods **[12].** Chemical hydrolysis produced furfural, formic and acetic acid which represent the main disadvantage of this process **[13].** Biological hydrolysis has advantages over chemical one, both cellulose and hemicellulose are usually digested to monomeric sugars by microorganisms **[14],** also it demands a low level of energy and mild environmental conditions **[15].**

This work concentrated on the isolation of various fungal isolates from various lignocellulosic biomass (RS, Water hyacinth, Bagasse, and Saw Dust). The rice straw is then fugally hydrolyzed to produce fermentable sugars. This process was optimized using the Response Surface Methodology (RSM) with a Central Composite Design (CCD), which looked at the effects of several parameters and identified the best conditions for obtaining the most sugars. Bioethanol was created by fermenting these sugars by *Saccharomyces cerevisiae*.

2. Material and method

2.1. Collection and Preparation of Rice Straw

Rice Straw (RS) was collected from the field in Aghoor Elkobra, Toukh, Qalyubia, Egypt. RS was air-dried, chipped, ground (Wiley Mill, Philadelphia, USA), and sieved to size (~ 0.5-1 cm). Finally, all amounts of RS were kept at room temperature in plastic bags until used.

2.2. Isolation and purification of different fungal isolates

According to Abo-State *et al*. **[16],** five grams of the lignocellulosic waste (rice straw, water hyacinth, bagasse, and sawdust) was added to 45 ml of sterile saline in 100 ml conical flasks. The flasks were shaken for 60 min at 200 rpm. After that, the suspensions were serially diluted to 10^{-1} and grown using a modified dox media. The plates were incubated at 30°C (±2) for 7 days. The well-grown colonies were selected and streaked on a sterile purification medium. After precise purification, sterile slants holding pure separated single colonies were kept in the refrigerator until needed, and these culture slants were monthly sub-culturing.

2.3. Chemical composition of Rice Straw

Hemi-cellulose, cellulose, and lignin concentration in the collected dried RS were determined according to National Renewable Energy Laboratory methods NREL, USA **[17].**

2.4. Fungal Hydrolysis

2.4.1. Spore suspension preparation

According to Abo-State *et al*. **[18]**, the fungal spore suspension was prepared by inoculating the fungal isolates onto test tubes containing Czapek Dox medium. The inoculated tubes were incubated at 30 ˚C (±2) for 7 days, then, the spores were obtained by scratching each tube very well which contain 5 ml sterile saline. Finally, the spore suspension from every tube was gathered and stored in a fresh, sterile flask for inoculation (≈ 4 x 10^7 spores/ml).

2.4.2. *Fungal Hydrolysis by Spore Suspension*

In 100-ml Erlenmeyer flasks add one gram from chipped and grinded RS, this biomass was wetted by 3 ml distilled water before being autoclaved at 121ºC for 20 minutes. Each flask inoculated by 0.5 ml of spore suspension. The inoculated flasks were incubated at 30 ºC for 10 days in a static condition. The soluble total reducing sugars were extracted by vigorously mixing the solid material with 20 ml of distilled water. After that, the solid materials were separated from the flasks using filtration The filtrate was centrifuged for 10 min at 13,000 rpm to measure total reducing sugars (TRS) in the clear supernatant by 3, 5-dinitrosalicylic acid (DNS) method **[19].**

2.5. Combination between selected fungal isolates

The selected fungal isolates which produced the highest TRS concentration from fungal hydrolysis were employed to create dual and triple fungal combinations to investigate the effects of fungal synergy on saccharification and TRS production. the pre-treated RS biomass was inoculated by 0.5 ml spore suspension (as total inoculum) from single, dual, and triple fungal combinations. Then, incubated for 10 days at 30 ◦C under static condition. Then TRS were extracted and measured as mentioned above. Every experiment was run in triplicate.

2.6. Identification of selected isolates

Fugal isolates identified by Sigma Company according to the following method

- ***DNA extraction***

Purified DNA is obtained through processing of a sample that contains fungi in liquid media. There are multiple steps in this process, 0.2 ml of the sample, 0.095 ml of solid tissue buffer (blue), 0.095 ml of water, and 0.01ml of proteinase K are added to a microcentrifuge tube. After the mixture is well combined, it is incubated for two hours at 55ºC. Following incubation, the tube is re-mixed and centrifuged for one minute at 12×10^3x g. Mix 0.6 ml of Genomic Binding Buffer with 0.3 ml of resulting aqueous supernatant. After that, the mixture is moved to a Zymo- Spin™ IIC-XL Column in a Collection Tube and centrifuged for one minute at a minimum speed of 12×10^3x g, the collection tube containing the flow-through is discarded. Following this, 0.4 ml of DNA Pre-Wash Buffer is added to the column in a fresh Collection Tube, and it is centrifuged for one minute at 12×10^3x g. The next step involves adding 0.7 ml of g-DNA Wash Buffer, centrifuging for one minute at 12×10^3x g, and emptying the Collection Tube. the procedure is repeated by 0.2 ml of g-DNA Wash Buffer, the collection tube is then thrown away. Ultimately, the column is filled with0.003 ml of elution buffer, allowed to incubate for 5 minutes, and then centrifuged at 12×10^3x g, for 1 minute in order to extract the purified DNA.

- ***PCR amplification***

A PCR reaction is set up by mixing 8 µl of DNA template, 25 µl of MyTaq Red Mix, 1 µl of forward primers (20 picomoles), 1 µl of reverse primers (20 picomoles), and 15 µl of nuclease-free water. Using a heat cycler, the PCR reaction mixture is subjected to the following thermal cycling procedure: six minutes of first denaturation at 94℃, 35 cycles of second denaturation at 94℃ for 45 seconds, 45 seconds of annealing at 56℃, and one minute of extension at 72 ℃. Finally, an extension step is performed for five minutes at 72℃ to conclude the PCR reaction.

- ***DNA sequencing***

ABI 3730xl DNA sequencer is used to sequence DNA by using forward and reverse primers. the modern 454 technology is combined to the old Sanger technology to sequence DNA in half the time of a typical project with a significant decrease in coatings and gaps. Furthermore, the research community can now use the 454 methods to sequence genomes due to significant cost advantages.

2.7. Optimization of biological hydrolysis parameters

The optimization process for maximum TRS production was performed through two optimization steps.

2.7.1. *One-factor-at-time (OFAT)*

The traditional strategy of optimization by one-factor-at-time (OFAT) (i.e., all factors are kept constant except one factor was varied) was utilized to optimize the hydrolysis process and TRS production by the most potent fungal isolates. Different parameters such as incubation time (6, 8, 10, 12, and 14 days), temperature (15, 20, 25, 30, 35, and 40℃), pH (5, 5.5, 6, 6.5, 7, and 7.5), biomass concentration (2.5- 25%), and inoculum size (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ml) were investigated. At the end of each experiment, the TRS was extracted and measured as mentioned above.

2.7.2. *Statistical analysis*

The impact of four factors on the production of TRS from fungal hydrolysis of RS **(Table 1)** was investigated using a central composite design (CCD). These factors are: incubation time (A), temperature (B), biomass concentration (C), and inoculum size (D). The model used in this study to estimate the response surface is the quadratic model. The impacts and interactions between these factors were examined using five levels [-1, 0, 1, and Axial point (low and high)] for each factor. Design-Expert software (ver. 7.0.0; Stat-Ease Inc., Minneapolis, MN, USA) was used to create the experimental designs. The resultant model's experimental significance was examined using the F test (derived P value. The statistical parameters for maximal TRS production were estimated using analysis of variance test (ANOVA).

Table (1): Variables and their levels used in the experimental design.

Variable	Symbol coded	Range and level				
					Axial point	
		(-1)	(0)	(+1)	Low	High
Incubation time (day)	A	8	10	12	6	14
Temperature (℃)	B	25	30	35	20	40
Biomass concentration (%)	C	10	15	20	5	25
Inoculum size (ml/g)	D	0.75	1.00	1.25	0.50	1.50

2.8. Fermentation for bioethanol production

According to **Abo-State *et al*. [16]** with little modification the fermentation process carried out in medium contain ($MgSO_4 \cdot 7H_2O$ (1.0 g/l), KH_2PO_4 (2.0 g/l), peptone (10.0 g/l) and RS hydrolysate, the medium pH adjusts to 5.5. This medium was distributed at test tubes each contain 10 ml of medium. These tubes were autoclaved at 121℃,1.5 atm for 20 min. Each tube inoculated by 1 ml of yeast suspension (*Saccharomyces cerevisiae)*. Then incubated at 30℃ under 50 rpm for 4 days. Finally, bioethanol resulting from this fermentation was calculated using the dichromate oxidation and solvent extraction methods.

2.9. Assay of bioethanol

According to Miah *et al and* El-Sheekh *et al* **[20, 21]**, bioethanol was quantitatively estimated using Tri-n-butyl phosphate (TBP) and $K_2Cr_2O_7$ reagent as following. 1ml of supernatant after fermentation process was mixed with 1 ml of Tri-n-butyl phosphate solvent in small tube, vortexed for 1 minute, then centrifuged for 5 minutes until separate into the upper (clear) and lower (turbid) layers. add 0.5 ml of the upper layer to 0.5 ml of $K_2Cr_2O_7$ reagent in other tube, shaking for 1 min. Then, the mixture was allowed to oxidize and form blue-green color in the lower layer for 10 min at room temperature 1800 µl of deionized water was mixed with 200 µl of the blue-green layer, and the absorbance was measured at 595 nm using a spectrophotometer. The standard curve was plotted using different absolute ethanol concentrations starting at 1:5% according to the method described above.

3. Result and Discussion

3.1. Isolation and purification of different fungal isolates.

Twenty-four fungal isolates were isolated from different lignocellulosic biomass such as (RS, Water hyacinth, Bagasse, and Saw Dust) as shown in **(Table 2)**. Nine fungal isolates were isolated from RS, so it was the best source for isolation, followed by WH where seven fungal isolates were isolated. Five isolates were isolated from bagasse The least source of fungal isolation was Saw Dust, where three fungal isolates were isolated.

Abo-State *et al*. **[18]** stated that Sugar-cane bagasse was used to isolate twelve different microorganisms, including four yeasts, five bacteria, and three fungi. Also, rice straw was used to isolate eight microorganisms (three fungi and five yeast) were isolated from by Abo-State *et al.* **[16]**. Also, according to Zhang *et al.* **[22],** 36 microbial isolates (7 basidiomycete fungi, 7 filamentous fungi, and 22 bacteria) were isolated from lignocellulosic wastes. This indicated that lignocellulosic biomass is a suitable source for microbial isolation.

Table (2): Isolation of different fungal isolates from different sources.

Substrate	No. of fungal isolates	Fungal isolate Code
Rice Straw	9	F3, F6, F14, F15, F20, F21, F22, F23, F24
Water Hyacinth	7	F4, F5, F8, F9, F12, F13, F16
Bagasse	5	F1, F2, F7, F10, F11
Saw Dust	3	F17, F18, F19

3.2. Chemical composition of rice straw

The data recorded in **Table (3)** showed the proportion of cellulose, hemicellulose, and lignin in RS. The percentage of hemicellulose (43.8%) was higher than the percentage of cellulose (38.4%) and lignin (17.8%). These results agreed with Syaftika and Matsumura **[23]** which reported that RS is composed of hemicellulose (55%), cellulose (28%), and lignin (11%). However, cellulose content (34.80%) was the highest percentage in RS than hemicellulose (31.22%) and lignin content (10.18%) as shown by Abo-State *et al*. **[16]**. Also, Imman *et al* and Dai *et al* **[24,25]** stated that the RS is composed of high concentration of cellulose (35.8 and 38.3%) than hemicellulose (21.5 and 21.3%) and lignin (24.4 and 12.5%), respectively. Differences in the composition of RS might originate from its source, the growth state of the RS, the time of harvesting, and the nutritional conditions in the plant habitat.

Table (3): Chemical composition of rice straw.

Compounds	(% w/w)
Lignin	17.8
Hemicellulose	43.8
Cellulose	38.4

3.3. Fungal hydrolysis of RS biomass

3.3.1. Fungal hydrolysis by fungal isolates

RS was subjected to fungal hydrolysis by 24 fungal isolates **(Table 4).** The results indicated that the fungal isolate F5, F8 and F23 showed the highest amount of TRS. So, these three isolates were selected for studying the effect of a combination between selected fungal isolates on TRS production.

3.3.2. Combination between selected fungal isolates
The data recorded in **Table (5)** showed the effect of combination between selected fungal isolates on hydrolysis and TRS production. Some combinations decrease TRS amounts, i.e., there is an inhibition relationship between the fungal isolates. while other combinations increase them, i.e., there is a synergism between the fungal isolates. The data recorded in **Table (5)** demonstrated that all the combination between the selected isolates increase the efficient of hydrolysis and TRS production. The best combination for hydrolysis was F5&F8 which produced the maximum TRS concentration (6.587 mg/ml).

Ramarajan and Manohar **[26]** demonstrated that the combination between the ligninolytic isolates and the cellulolytic isolates showed increasing in enzyme activity. According to Taha *et al*. **[27]** five bacterial and four fungal isolates were **selected to form dual** and triple microbial combinations to examine synergistic effects of microbes on saccharification, and founded that the dual combinations between selected isolates were the best in all condition and increased saccharification compared with their single cultures.

Table (4): Total reducing sugar (TRS) from fungal hydrolysis of rice straw by different fungal isolates

Fungal isolates	TRS (mg/ml)
F1	3.098
F2	2.684
F3	1.706
F4	1.748
F5	4.096
F6	2.386
F7	2.801
F8	3.629
F9	2.099
F10	2.734
F11	2.466
F12	2.056
F13	2.156
F14	1.096
F15	2.106
F16	1.184
F17	3.188
F18	2.629
F19	2.008
F20	2.474
F21	2.837
F22	2.368
F23	3.542
F24	2.243

Table (5): Combination between selected fungal isolate for hydrolysis of rice straw without any pretreatment

Fungal isolate	TRS (mg/ml)
F5	4.351
F8	3.668
F23	3.187
F5&F8	6.587
F5&F23	6.226
F8& 23	4.994
F5&F8&F23	5.644

3.4. Identification of selected fungal isolates
Based on the 18S rDNA sequence fungal isolate F5 was 100 % similar to *Aspergillus terreus* and F8 isolate was 99% similar to Trichoderma harzianum. *Aspergillus terreus* is a member of 344 recognized species in *Aspergillus* genus. *Aspergillus terreus* belongs is a common soil saprophyte fungus **[28].** *Aspergillus terreus* was used to degrade different lignocellulosic waste such as sugarcane bagasse **[29]** and RS **[30].** *Trichoderma harzianum* is one of the nine aggregate species recognized by Rifai **[31]** and found on a wide variety of substrates. *Trichoderma harzianum* was used to degrade lignocellulosic biomass including sugarcane bagasse, wheat bran, corncob, corn stover miscanthus, switchgrass, and sunchoke stalks **[32].**

3.5. Optimization of hydrolysis parameters
TRS which produced from hydrolysis is very important for bioethanol production. So, it is necessary to optimize all factors affecting on hydrolysis process and TRS production. The optimization process for maximum TRS production was performed through two optimization steps. OFAT experiments were applied to determine the low and high levels that further used in the factorial design experiment.

3.5.1. *Incubation time*
Fig. (1) showed the effect of the different incubation times on hydrolysis and TRS production. TRS increased gradually with time until reached to maximum concentration (5.580 mg/ml) on the 10th day. Then, the TRS concentration decreases gradually with time. The same result occurred by Dias *et al*. **[33]** who stated that the optimal hydrolysis of wheat straw that had been biologically pre-treated by fungi was achieved after 10 days. Also, Accossato *et al* **[34]** stated that TRS from RS inoculated by *Trichoderma asperellum* increase with time until the 10 th day and become stable between 10 and 15 days then decrease with time.

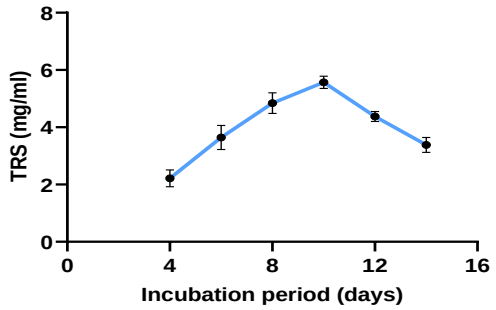

Figure (1): Effect of different incubation time on hydrolysis of rice straw at temperature 30℃, pH. 7, concentration of substrate 5% and inoculum size 0.5 ml

3.5.2. *Temperature.*

Temperature is among the most crucial factors. affecting on fungal hydrolysis and hence controlling the amount of TRS. At lower temperature, transport of nutrients was blocked and at higher temperature, the organism had to expend a lot of energy for survival **[35].** The optimum temperature for hydrolysis was 30℃ **(Fig. 2)**, which produced the maximum TRS (5.890 mg/ml).

According to Singh *et al* **[36],** the level of TRS from hydrolysis of RS by two indigenous fungal strains was maximum at 30℃. Furthermore, Deswal *et al.* **[37]**, showed that the best temperature for cellulase production by *Fomitopsis* sp. was 30℃. On the other hand, Belal **[38]** reported that the maximum TRS obtained from RS residue at 25℃. Furthermore, Gilna and Khaleel [39] reported that the cellulase activity of *Aspergillus fumigatus* on lignocellulosic substrate was maximum at 32℃.

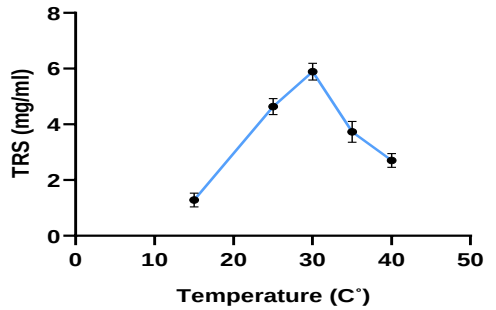

Figure (2): Effect of different temperatures on hydrolysis of rice straw at incubation time 10 days, pH 7, concentration of substrate 5% and inoculum size 0.5 ml

3.5.3. *Initial pH*

The medium's pH affected on the morphology of microorganisms and enzyme secretion. The pH change which occurs during microbial growth has an impact on product stability in the medium **[40]**. Additionally, the pH directly affected the charge of the cell membrane, which in turn affected the permeability of the membrane and the release of cellulases into the extracellular environment **[41].** The results recorded in **Fig. (3)** showed that the best pH for hydrolysis was 6 which corresponded to the amount of TRS 6.955 mg/ml.

Belal **[38]** had the same result which produced maximum TRS from hydrolysis of RS residue at pH.6. likewise, Xu *et al* **[42],** founded that the synthesis of cellulolytic enzyme by *Inonotus obliquus* on wheat bran was highest at pH 6. However, in contrast, Singh *et al* **[36]**, reported that the best TRS from hydrolysis of RS by *A. niger* and *A. heteromophous* was obtained at pH.5. Furthermore, Deswal *et al.* **[37],** shown that the maximum amount of cellulase was produced at pH 5.5. Also, Ong *et al*. **[43]** and Das *et al.* **[44]**, founded that mild acidic pH 5.5 was optimal for saccharification of the pretreated RS enzymatically.

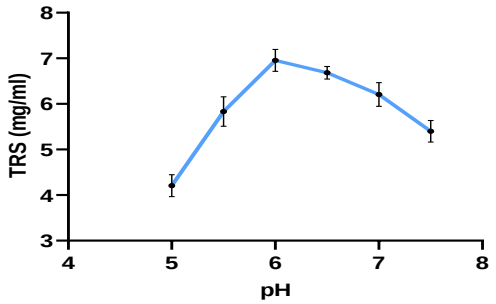

Figure (3): Effect of different pH on hydrolysis of rice straw at 30℃, 10 days, concentration of substrate 5% and inoculum size 0.5ml

3.5.4. *Substrate concentrations*

Fig. (4) showed that the amount of TRS increased gradually until reached the maximum (13.525 mg/ml) at 15% substrate concentration, then decreased gradually by increasing the substrate concentration, this could result from the synthesis of inhibitors [**45**] such as furfural, hydroxymethylfurfural, and lignin **[46,47].** Das *et al.* **[44]**, founded that increasing the RS concentration (over 10 %) decreased cellulase activity. While, Ong *et al.* **[43]**, reported that TRS produced by *A. niger* from hydrolysis of RS had a maximum value at 12% substrate concentration.

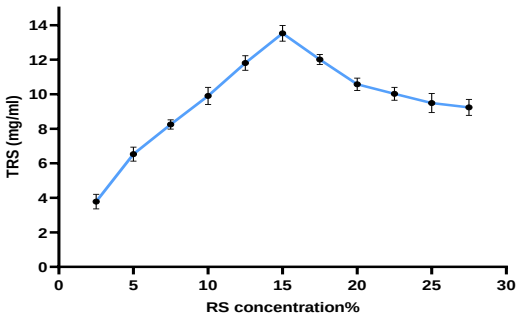

Figure (4): Effect of different concentrations of rice straw on hydrolysis at 30℃, 10 days, inoculum size 0.5 ml; and pH.6

3.5.5. *Inoculum size*

The importance of inoculum size in promoting cellulase synthesis was particularly clear. Due to poor mycelia biomass, a small amount of inoculum had a negative impact on the synthesis of cellulases enzymes. On the other hand, larger amounts of inoculum resulted in an immense and rapid nutrients consumption for cell growth, causing malnutrition in the fungus, which impacted enzyme synthesis and saccharification process **[35].**

As shown in **Fig. (5)**, the optimum inoculum size from fungal spore suspension was 0.5 ml for maximum

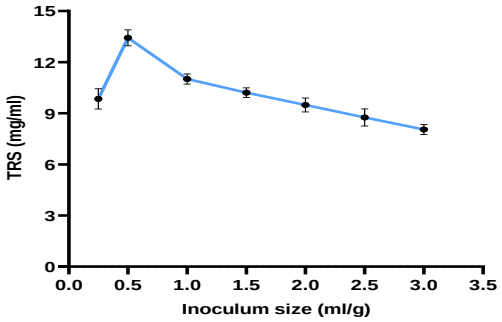

Figure (5): Effect of different inoculum size on hydrolysis of rice straw at 30℃, 10 days, 15% substrate concentration, and pH 6

hydrolysis. Azzaz *et al.* **[48]** demonstrated that the cellulase production from *A. niger* using wheat straw as a sole carbon source had maximum activity at 4% inoculum size.

3.6. Statistical analysis of biological hydrolysis of RS

The statistical analysis of the investigated variables and experimental response values are shown in **Table (6).** Furthermore, the optimum conditions solutions for TRS production from hydrolysis of RS are demonstrated at **Table (7)**. It was founded that the best circumstances were 12 days, 25℃, 1.14 ml spore suspension, and 20% biomass concentration, which give the amount of TRS about 17.212 mg/ml.

The validity of the response surface full quadratic model was assessed using the ANOVA, and the statistical significance was assessed using the F-test. The substantial Model F-value of 28.37 from the analysis of variance (ANOVA) in **Table (8)** demonstrated that the response surface complete quadratic model is statistically acceptable. Only 0.01% of times would noise result in such a high F-value. The P rob > F" values were used to determine the importance of the model terms. B, C, A^2, B^2, and D^2 are significant model terms since they have values smaller than 0.0500. The model terms are not significant if the value is bigger than 0.1000. The model effectively fits the data, as shown by the R-Squared value of 0.9455 and Adj R-Squared value of 0.9121. Furthermore, the Pred R-Squared value of 0.7695 is in fair agreement with the Adj R-Squared.

According to the obtained result and its evaluation, this is the final equation represents the effect of the different factors and interacting factors on TRS (in terms of coded factors):

$$TRS = +11.02 + 0.29 *A - 0.92 *B + 2.56 *C - 0.16 *D - 0.27 *A *B + 0.26 *B*C - 0.22 *B *D + 0.37 *C *D + 0.46 *A^2 + 0.64 * B^2 - 0.39 *D^2$$

Where A is the incubation time (day), B is the temperature (℃), C is the biomass concentration (%), and D is the inoculum size (ml/g).

As a result, using this model to guide the design process and make predictions is possible. The variables of the quadratic model were displayed as plots in three-dimensional charts (as depicted in **Fig. 6)** to analyze their interaction and identify the optimal conditions required for each factor to obtain the highest possible TRs yield.

Fig. (6 a) showed the effect of inoculum size and incubation time on TRS yield, where the TRS amount is small at low and high inoculum size and reached optimum at inoculum size 1.14 ml. On the other hand, the TRS amount increased with the prolongation of incubation time until it reached a peak level at 12 days. **Fig. (6 b)** Illustrated how temperature and biomass concentration interact to effect on TRS yield, where the amount of TRS increased by the increasing of biomass concentration and decreasing of temperature, maximum amount achieved at 20% biomass concentration and 25℃.

Figure (6 c) represented the effect of inoculum size and temperature on the production of TRS, where TRS yield increased by the increasing of inoculum size until reached to optimum at 1.14 ml then decrease again above this value and also decreased above 25℃.

Figure (6 d) showed the impact of both inoculum size and biomass concentration on TRS yield. The results indicated that the TRS amount was relatively low when the inoculum size was either too low or too high, and it reached an optimum level at an inoculum size of 1.14ml. However, the TRS amount increased with the increase in biomass concentration until optimum at 20% concentration.

The saccharification of rice straw was improved by Gupta and Parkhey **[49]** using CCD. The optimum conditions were determined to be 1.84% (w/v) for rice straw concentration, 40 U (µmol/min) for enzyme load, 57.4 hours for incubation time, and 0.76 mM for Tween-80 concentrations. Under these optimal conditions, a saccharification rate of 69.5% was another study, CCD was used to optimize hydrolysis of waste-broken rice and founded that the maximum TRS amount was 0.689 g/g at optimum conditions **[50].**

Das *et al*. **[44],** employed the CCD to maximized the production of reducing sugars from NaOH-pretreated

rice straw. They determined that the maximum yield of reducing sugars (24.9 g/l) was achieved under the following conditions: 10% (w/v) substrate concentration, pH 5.5, 24 hours of reaction time, and an enzyme concentration of 40 U/g.

In a study by Pattraa and Sittijunda **[51],** CCD was utilized to optimize the chemical hydrolysis of water hyacinth. Also, Mihajlovski *et al*. **[52]** applied a statistical design to optimize the hydrolysis of waste bread. Under the optimal conditions of 100.73 hours of hydrolysis, 20.36% waste bread concentration, and 200 rpm agitation speed, the resulting waste bread hydrolysate contained 19.89 g/l of reducing sugars. Furthermore, the enzymatic hydrolysis of non-treated corn stover produced a maximum reducing sugar yield (3.85 g/l) under the following optimal conditions: corn stover concentration of 6.6% and a hydrolysis time of 78.8 hours (approximately 3 days and 7 hours) **[53].**

3.7. Fermentation process

Hydrolysate obtained from saccharifying rice straw using a combination of *Trichoderma harzianum* and *Aspergillus terreus* were fermented by *Saccharomyces cerevisiae* to produce bioethanol. The fermentation process indicate that *Saccharomyces cerevisiae* produced bioethanol at a yield of 11.05 ml/l. This value of ethanol yield (11.05 ml/l, equivalent to 8.84 g/l) is small in comparison with the other studies. A concentration of 9.45 g/l of ethanol was obtained from the fermentation of RS sugar with *Saccharomyces tanninophilus* **[54].** Also, ethanol productivity from rice straw saccharified with the laccase-supplemented immobilized enzyme cocktail was 0.478 g/l/h **[55].** Ethanol amount produced from hyper-thermal acid hydrolyzed and enzymatic saccharified water hyacinth was 15.3, 19.5, and 22.7 g/l of ethanol by *Saccharomyces cerevisiae*, *Pichia stipites*, and *Candida lusitaniae*, respectively **[56].** So that in the next study, the fermentation process will be optimized to increase the bioethanol yield.

Table (6): Experimental design matrix prepared using central composite design with the actual and predicted values of total reducing sugars (TRS) from hydrolysis of rice straw (RS).

Run	Incubation time (Day)	Temperature (°C)	Biomass concentration (%)	Inoculum size (ml/g)	Experimental Value of TRS (mg/ml)	Predicted Value of TRS (mg/ml)
1	12.00	35.00	10.00	1.25	7.813	7.263
2	8.00	35.00	20.00	0.75	13.041	13.623
3	12.00	25.00	10.00	0.75	11.666	11.239
4	12.00	35.00	20.00	0.75	13.003	13.666
5	8.00	35.00	20.00	1.25	13.153	13.598
6	10.00	30.00	15.00	1.50	8.536	9.136
7	10.00	30.00	15.00	1.00	11.001	11.019
8	10.00	30.00	5.00	1.00	5.563	5.903
9	12.00	35.00	10.00	0.75	8.023	8.768
10	12.00	35.00	20.00	1.25	12.823	13.641
11	10.00	30.00	15.00	1.00	10.953	11.019
12	8.00	25.00	20.00	0.75	13.573	13.956
13	8.00	25.00	20.00	1.25	15.068	14.809
14	10.00	30.00	15.00	1.00	10.996	11.019
15	8.00	35.00	10.00	0.75	8.153	8.726
16	8.00	25.00	10.00	0.75	10.643	10.101

17	10.00	30.00	15.00	1.00	11.081	11.019
18	8.00	25.00	10.00	1.25	10.143	9.474
19	10.00	30.00	25.00	1.00	17.181	16.135
20	12.00	25.00	20.00	0.75	14.988	15.094
21	10.00	30.00	15.00	1.00	10.666	11.019
22	10.00	30.00	15.00	1.00	11.008	11.019
23	12.00	25.00	10.00	1.25	10.893	10.611
24	10.00	20.00	15.00	1.00	14.048	15.423
25	8.00	35.00	10.00	1.25	6.866	7.221
26	6.00	30.00	15.00	1.00	12.568	12.281
27	12.00	25.00	20.00	1.25	16.711	15.946
28	10.00	40.00	15.00	1.00	13.411	11.742
29	10.00	30.00	15.00	0.50	10.683	9.788
30	14.00	30.00	15.00	1.00	13.468	13.461

Table (7): Optimum conditions solutions for TRS production from hydrolysis of RS.

Run	Incubation time (Day)	Temperature (°C)	Biomass concentration (%)	Inoculum size (ml/g)	Experimental Value of TRS (mg/ml)	Predicted Value of TRS (mg/ml)
1	**12.00**	**25.00**	**20.00**	**1.14**	**17.212**	**16.025**
2	12.00	25.00	19.93	1.03	16.394	15.925
3	8.00	25.00	20.00	1.14	15.020	14.889
4	8.83	25.00	20.00	1.13	14.913	14.816
5	12.00	25.77	20.00	0.75	15.086	14.809
6	12.00	34.99	20.00	0.99	15.281	14.041
7	8.00	35.00	20.00	1.01	13.990	13.999
8	8.00	33.66	20.00	0.94	13.222	13.774
9	12.00	32.05	20.00	0.77	12.518	13.612
10	9.87	35.00	19.99	1.00	12.274	13.556

Table (8): Analysis of variance (ANOVA) of the quadratic regression model for total reducing sugars production from hydrolysis of RS.

Source	SS*	df*	MS*	F Value	p-value Prob > F
Model	208.563	11	18.960	28.369	< 0.0001
A*	2.088	1	2.088	3.125	0.0940
B*	20.323	1	20.323	30.408	< 0.0001
C*	157.056	1	157.056	234.995	< 0.0001
D*	0.639	1	0.639	0.956	0.341
AB	1.199	1	1.199	1.794	0.1971
BC	1.087	1	1.087	1.626	0.2185
BD	0.770	1	0.770	1.152	0.2973
CD	2.190	1	2.190	3.277	0.0870
A^2	6.001	1	6.001	8.979	0.0077
B^2	11.496	1	11.496	17.201	0.0006
D^2	4.242	1	4.242	6.347	0.0214
Residual	12.030	18	0.668		
Lack of Fit	11.924	13	0.917	43.281	0.0003
Pure Error	0.106	5	0.021		
Cor Total	220.594	29			

*A:Incubation time (Day); B:Temperatures (°C); C:Biomass concentration (%); D:Inoculum size(ml/g); SS: sum of squares; df: degree of freedom; MS: mean square.

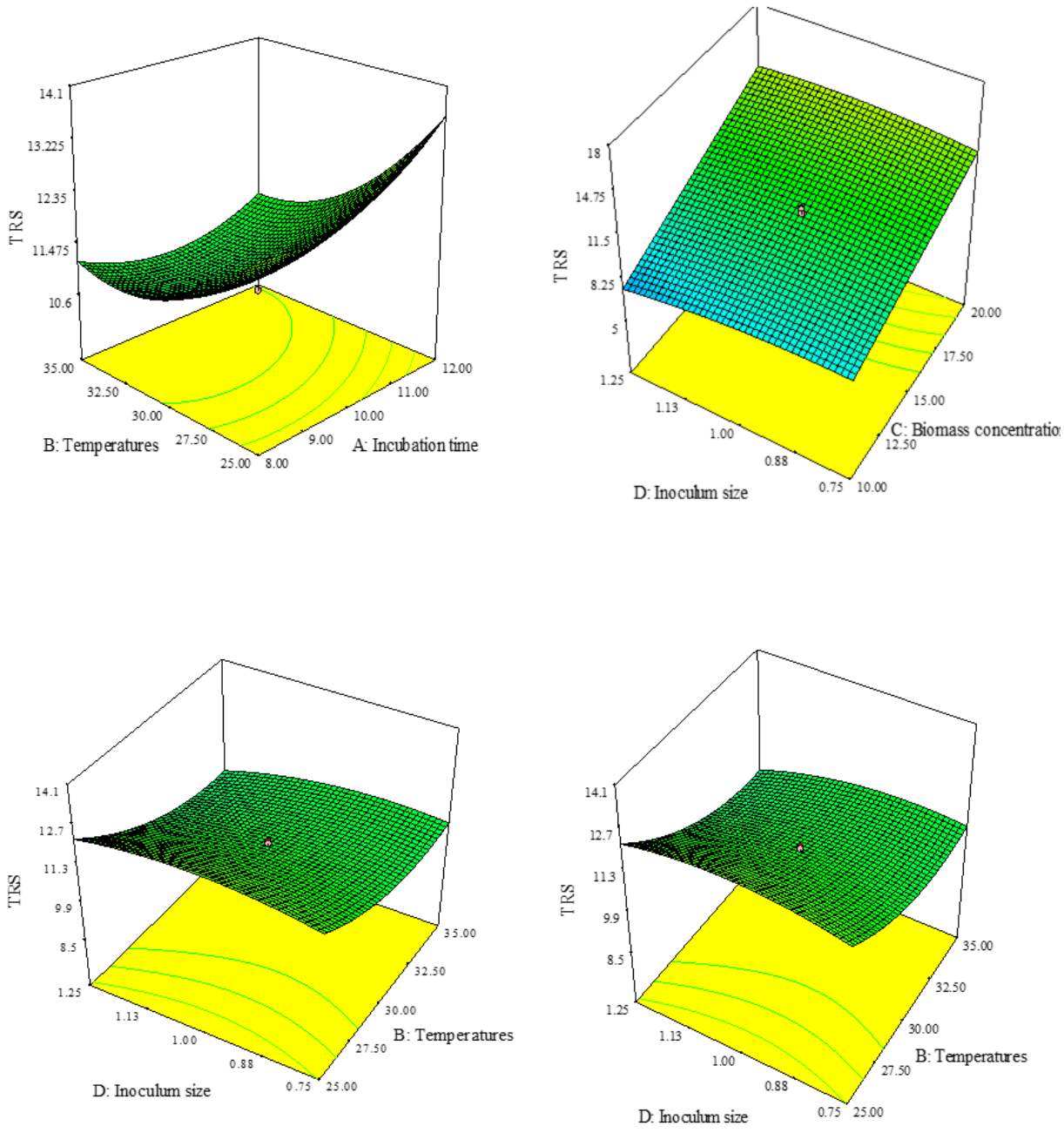

Fig. (6): Response surface plots showing the effect of incubation time (Day), temperatures (°C), biomass concentration (%), inoculum size (ml/g), and their mutual interactions on the production of total reducing sugars (TRS) from RS hydrolysis

4. Conclusions

In present study, the potential of rice straw as affordable and sustainable source for bioethanol production was explored. *Trichoderma harzianum* and *Aspergillus terreus* were used to saccharify RS. The process was optimized by statistical optimization using a central composite design to obtain the highest total reducing sugar (17.212 mg/ml) under optimal conditions (12 days of incubation time, a temperature of 25°C, a biomass concentration of 20% w/v, and an inoculum size of 1.14 ml). The sugar obtained from the previous step was fermented by *Saccharomyces cerevisiae* to produce 11.05 ml/l of bioethanol. In the futures, the next step of this study aims to optimize the fermentation process to achieve the highest ethanol yield.

5. Conflicts of interest

The authors confirm that there are no conflicts of interest

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