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Enhance the bioethanol production from the extracted fermentable sugar by sonication

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Abstract

The petroleum fuel price hiking is in alarming stage. To combat this situation, bio ethanol is one of the alternatives for petrol transportation fuel. To synthesize bioethanol from waste rice straw lignocellulose, different chemical synthesis procedure has been applied. Dilute acid hydrolysis of waste rice straw lignocellulose causes a break down a rigid structure to comparative less rigid structure such as lignin, hemicellulose and cellulose. For better saccharification from rice straw a new ultrasonic treatment process was implemented as a post hydrolysis treatment, which was proofed highly effective. The sonication of hydrolyzed rice straw samples under 0.5% acid concentration, 100% sonication amplitude, 0.8 sonication cycle, and 10-minute sonication time was given optimum results. It was observed that the improvement of TRS extraction from dilute acid hydrolyzed sonicated rice straw was about 90% better than that dilute acid hydrolyzed without sonicated sample. The capabilities of the ethanol production from dilute acid hydrolyzed sonicated rice straw by Saccharomyces cerevisiae, and Candida shehatae was 78% and 65% respectively better than the dilute acid hydrolyzed rice straw without sonication. This process is one of the phase change processes, where solid waste has been transformed to liquid bioethanol as a renewable resource.

Keywords: lignocellulose, bioethanol, rice straw, acid hydrolysis, sonication, renewable, Phase Change

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Public Interest Statement

While many of the advances in materials science have been driven by breakthroughs in material design and fabrication, understanding the changes that occur in a material during its utilization or operation in devices is of immense importance for its successful integration. Considering these aspects and the continued growth in materials research, there is a clear need for new topical journals which can serve researchers to understand the phase transitions and exploit the phenomena to current, state-of-the-art research in the field of materials science, moreover with full accessibility.

1. Introduction

In the twenty-first century, fossil fuels are becoming scanty and more expensive. Efforts are now being given to manufacturing biofuels from various biomass resources, which are nothing but sources of organic carbon, and can be transformed into fuel. Cellulose is the most plentiful substance on the earth, and cellulosic ethanol has been considered as a favorable secondary generation of biofuel for partial alternatives of petrol oils [1]. However, the natural recalcitrance of lignocellulose could not avoid a high-cost process of impending unexpected waste deliverance into the environment [2][3]. Rice straw is considered one of the largest biomass feedstock resources of second-generation biofuels in the world (i.e., 7.31 x 1011 kg of dry rice straw per year) and 90% of it is produced in Asia [4]. According to FAOSTAT [5], annual rice production was around 650 million tons in 2019 and, China and India were at the top of the list. Therefore, it is understandable that it has a tremendous potential to be considered as feedstock for fermentable sugar production, which can be converted to bioethanol by the fermentation process. Lignocellulosic feedstock such as rice straw has become attractive raw materials to produce ethanol in comparison to other starch and sucrose-based raw materials because of its profusion and low cost [6][7].

According to numerous studies, acid hydrolysis was carried out in two stages, the first stage is at the relatively milder condition at which hemicellulose fraction is hydrolyzed and the second stage is dilute acid hydrolysis at a higher temperature during which cellulose is hydrolyzed [8][9][10]. Previous studies show that simultaneous saccharification and fermentation (SSF) is greater than the traditional saccharification and succeeding fermentation in the production of ethanol from rice straw [11][12][13]. Technology for conversion of lignocellulosic raw biomass feed to fuel such as ethanol has been developed for decades and much more advanced technology is needed for its commercial application. Conventional processes for ethanol production cited in different literature generally use dilute acid hydrolysis followed by enzymatic hydrolysis and fermentation of the TRS extracted from the preceding stages. The chemical pretreatment of rice straw for bioethanol production is optimized by Anu et al, 2020[14]. The conversion of polysaccharides to monosaccharides should be boosted up, from lignocellulosic waste by post hydrolysis ultrasonic sound treatment [15][16][17], which was the most innovative part of this work. In many fields such as biological science, medical science, engineering, etc. ultrasonic sound has been applied, which is extremely useful [18], but with our best information, it is still a very exceptional application of the sonication after hydrolysis of rice straw for enhancement of the production of TRS. A previous study showed the fermentation of xylose of rice straw hydrolyzate to ethanol by *Candida shehatae* [19][20] and *Saccharomyces cerevisiae* was efficient [21].

The focal objective of this research was to improve the TRS production by utilizing the ultrasonic sound after dilute acid pretreatment with different acid concentrations. Therefore, more importance was given to the extent of sugar extraction under different prevailing conditions of sonication, and an empirical correlation was developed to optimize the different sonication parameter which was applied during this experiment. Another important goal of this research was to check the concentration of ethanol production from dilute acid hydrolyzed without sonicated and sonicated rice straw by two fermenting microorganisms.

2. Materials and Method

2.1. Raw Materials:

The rice straw employed in all the experiments was obtained from the East Medinipur field (West Bengal, India) and dried at 60-75°C for one day. Full-length rice straws were cut into a smaller length of 10 to 20 cm by the straw cutter. Then pre-milled rice straw was passed through a 2-5 mm screen, and it had been reduced to a size of 20-48 mess (0.833~0.295 mm) by using a ball mill before hydrolysis and was stored in the refrigerator (4°C) for further use (Figure 1). The moisture content of the milled rice straw was found to be 6.21% (based on total dry biomass content). Milling of straw increases the accessible area, size of the pores and reduces the crystallinity, degrees of polymerization of cellulose before going to hydrolysis. DNS reagent (Batch no. -MD8M572045) was purchased from Merck, Mumbai, India. De-ionized water, used for preparing different solutions and cleaning the various glassware, was obtained from the ultrapure water system (model Arium 611) of Sartorius AG, Germany. Supply for the de-ionized water system was water, produced by reverse osmosis (RO) system (model Arium 61315 S/N 17106017, make Sartorius AG Germany).

Fig.1. Size reduction of rice straw before hydrolysis

2.2. Dilute acid hydrolysis

Milled rice straw was primarily soaked in three different dilute sulfuric acid (0.25, 0.5, and 0.75% v/v) solutions. These were maintained at a solid to liquid ratio of 1:60 (v/v) for 18± 2h at the ambient conditions. Then, each of these soaked samples was hydrolyzed for 30 minutes. The hydrolysis apparatus is primarily an autoclave, which was maintained at the pressure of 2 bar (15 psig approx.or1.03×10^5 Pascal) and 121°C.

2.3. Neutralization

Acid hydrolysis is followed by the pH neutralization process of samples, which is required for downstream processes. The pH of different samples was adjusted to 5-6 by adding 40×10^3 mol/m^3 (1 N) sodium hydroxide [22].

2.4. Sonication

One part of the dilute acid hydrolyzed, and neutralized sample was kept for the direct fermentation process. The other part of that hydrolyzed samples was sonicated at varying conditions in a laboratory sonication apparatus (Sartorius LABSONIC® M, Cat. -No 8535027). The parameters like amplitude, cycle, and treatment time were measured automatically as well as manually. The ultrasonic processor is equally suitable for stand mounting additionally as for manual operation [17]. It creates longitudinal vibrations with a frequency of 30,000 oscillations per second (30 kHz). The tip diameter of the probe is 2mm, which is made of titanium alloy. This probe was submerged centrally in the 20ml of hydrolyzed rice straw suspension in the 50ml cylindrical glass container. The extreme amplitude applied by the probe was 140 µm. Probe transferals minimal output ultrasonic energy by its front surface with total intensity level ~100 W and acoustic intensities ~600 W/cm^2 (in this probe) at the horn's surface into the sample medium [17].

2.4.1. Experimental design of sonication

According to Bhattacharyya et al. 2011, there were three sonication parameters, viz., amplitude, cycle, and treatment time, which were identified as controlling factors. As the variation of these parameters produces optimal sugar extraction and their levels were fixed accordingly for different hydrolyzed rice straw samples at different acid concentrations (0.25%, 0.5%, 0.75%) as shown in Table 1 [15]. The experiment was planned to use 3^3 factorial designs. For three parameters and three levels full factorial set up, the total number of experimental runs conducted was 27. The cycle of the sonicator means the marked space ratio between the switched on and switched off time of the output power.

Table 1. Sonication parameters and their levels (Bhattacharyya et al. 2013)

Sonication parameters	**Levels**		
	Low (-1)	Medium (0)	High (+1)
Time(min)(X1)	5	10	15
Amplitude (%) (X2)	60	80	100
Cycle(X3)	0.6	0.8	1.0

2.5. Purification

After the sonication process, a little fraction of the sonicated and without sonicated samples were purified by cold centrifugation at the temperature of 10° C where centrifuge was maintained at a rotating speed of 10,000 rpm for 10 minutes. Supernatants were separated carefully for the analysis purpose of extracted sugar. The dilute acid hydrolyzed, neutralized and sonicated wheat straw sample was kept for further fermentation procedure.

2.6. Fermentation

2.6.1. Microorganisms and media

The yeast strain *Saccharomyces cerevisiae* ATCC 24702 (NCIM- 3494) and *Candida shehatae* ATCC 34887 (NCIM- 3500) were supplied as mother culture on agar slant by the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, Maharashtra, India. These strains were used in the fermentation process in all the experiments. A few subcultures were prepared from those mother cultures on agar slants, which contains (g/l) malt extract 3, glucose 10, yeast extract 3, peptone 5, and agar 20 at pH 5.5± 0.1 and 30°C for 3 days. The whole inoculation process was done in laminar flow apparatus, which provide a total aseptic environment. After incubation, the growth appeared on the agar slant, and these were stored at 4°C for future use.

2.6.2. Cell biomass preparation

Before fermentation, the cell biomass of both strains was prepared from the subculture. These strains were inoculated in a 250 ml conical flask, which contains 50 ml broth of malt extract (3 gl^{-1}), glucose (10 gl^{-1}), yeast extract (3 gl^{-1}), peptone (5 gl^{-1}), and distilled water at pH 5.5± 0.1 and 30°C for 2 days. These conical flasks were incubated at 150 rpm on a rotatory shaker incubator. After two days of incubation, cell suspensions were isolated from broth medium by refrigerated centrifugation at 4°C and the supernatant was discarded. The precipitated biomass was washed two to thrice with sterile distilled water. These cell biomasses were diluted with sterile deionized water to obtain an inoculum level of 1 g/l, which was utilized in the process of fermentation.

2.6.3. Fermentation of dilute acid hydrolyzed and sonicated raw materials

The aerobic cultivation of *Saccharomyces cerevisiae* and *Candida shehatae* was carried out in a 250 ml conical flask containing 4 ml of inoculums and 100 ml of medium consisting of sonicated hydrolyzate as well as without sonicated hydrolyzate. These were supplemented with (g/l): yeast extract, 5; NH_4Cl, 2; $MgSO_4$. $7H_2O$, 0.3; K_2HPO_4, 1; and the pH was adjusted to 5.5 with NaOH, 4M. The temperature of this fermentation process was maintained at 30°C at constant mode, at which the growth of the fermenting strain was maximum. It was incubated in cotton plugged conical flasks on a rotator shaker at 150 rpm. Samples were collected in 24 hours intervals three times.

2.7. Analysis

Total reducing sugars (TRS) were determined by the dinitro salicylic acid (DNS) method using glucose as the standard [23]. Free carbonyl group (C=O) of the reducing sugars were reacted with 3, 5- dinitrosalicylic acid (DNS) and reduced to 3- amino, 5-nitrosalicylic acid under alkaline conditions. The supernatant liquid was analyzed to estimate the amount of total reducing sugar (TRS) extracted from rice straw by UV-visible spectrophotometer (Cary50 Bio, S/N EL07113760) using 3,5-dinitrosalicylic acid as reagent (DNS method) at 575 nm wavelength.

Ethanol was analyzed by Thermo-Fisher gas chromatograph (S/N- 745093) apparatus equipped with Chemito GC 8610 series injector, a flame ionization detector

(FID), and a Chemito capillary column (S/N- 11065 B07) with length 50 m and diameter 0.32mm. For the detection of the ethanol concentration, the initial oven temperature was maintained at 40°C and after then the temperature rise 10°C per minute up to 200°C. The temperature of the injector and detector was maintained at 180°C and 220°C, respectively.

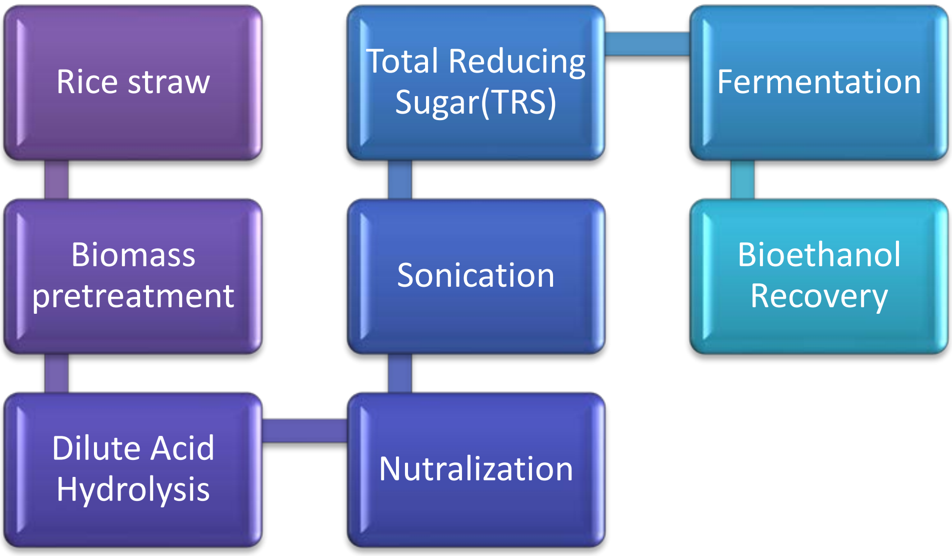

Fig. 2. Flow chart of bioethanol producing pathway.\

3. Results & discussion

Results obtained from the spectrophotometric analysis have been shown through graphical presentations. Figure 3 shows the effect of acid concentration on sugar extraction without implementing sonication. It is evident from the plot that optimum extraction occurs at 0.5% acid concentration, which is a quite significant observation.

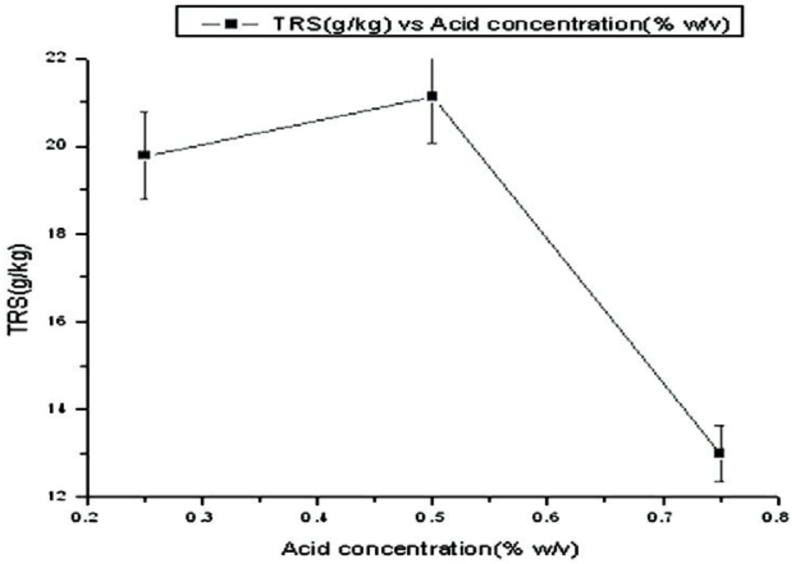

Fig.3. The effect of acid hydrolysis on yields of TRS.

Over the years, dilute acid hydrolysis has become a remarkably successful and popular method for extracting sugars from lignocellulosic materials. Despite being an immensely powerful reagent for cellulosic hydrolysis, it was not used for hydrolysis since concentrated acids are hazardous, toxic, and corrosive. These characteristics are harmful to reactors. Therefore, the importance of dilute acid hydrolysis has grown gradually over the years, and an optimum acid concentration was required to be identified for better yield of fermentable sugars, and to make the process economically feasible. Now, it was important to see whether sonication results follow the same trend and produce optimum yield at the same concentration of acid. Figure 4 shows the two conical containers, which contain the dilute acid hydrolyzed without sonicated and sonicated rice straw samples. These samples were further treated by two different fermenting microorganisms for ethanol production.

Mathematical model development

The purpose of mathematical modeling is to relate response variable sugar extraction with the process parameters, mathematically to get an estimation of responses and to facilitate the optimization of sugar extraction from rice straw at appropriate conditions. The model can be represented by

$$Y=f(X1, X2, X3\ldots..) \quad (1)$$

Where, Y is the response variable, and X1, X2, X3.... are process parameters which influence Y. Equation (1) represents Y (dependant variable) as a function of process parameters (i.e. independent variables). Method consists of defining a suitable response function which can represent the experimental observations quite accurately. Success of a model depends on how closely it matches with the experimental results. Various mathematical models have been studied to represent the experimental data appropriately. In this study, following form of mathematical equation (Eq.2) has been proposed to express the experimental data suitably.

$$Y=A_0+A_1X_1+A_2X_2+A_3X_3+A_4X_1^2+A_5X_2^2+A_6X_3^2+A_7X_1X_2+A_8X_1X_3+A_9X_2X_3 \quad (2)$$

Where A_n is coefficient associated with nth parameter, and X_1, X_2, X_3 represent the sonication parameters in the model. Combination of parameters (i.e. X_1X_2) in the model represents interaction between individual parameters.

Evaluation of the fitted model can be done by

(a) Graphical indicators of testing model (i.e. residuals analysis)

(b) Overall numerical indicators for testing model and model terms (i.e. ANOVA analysis, R-squared and R-squared adjusted) (Statistical Handbook,).

Fig. 4. Acid hydrolyzed of rice straw sample before sonication and after sonication

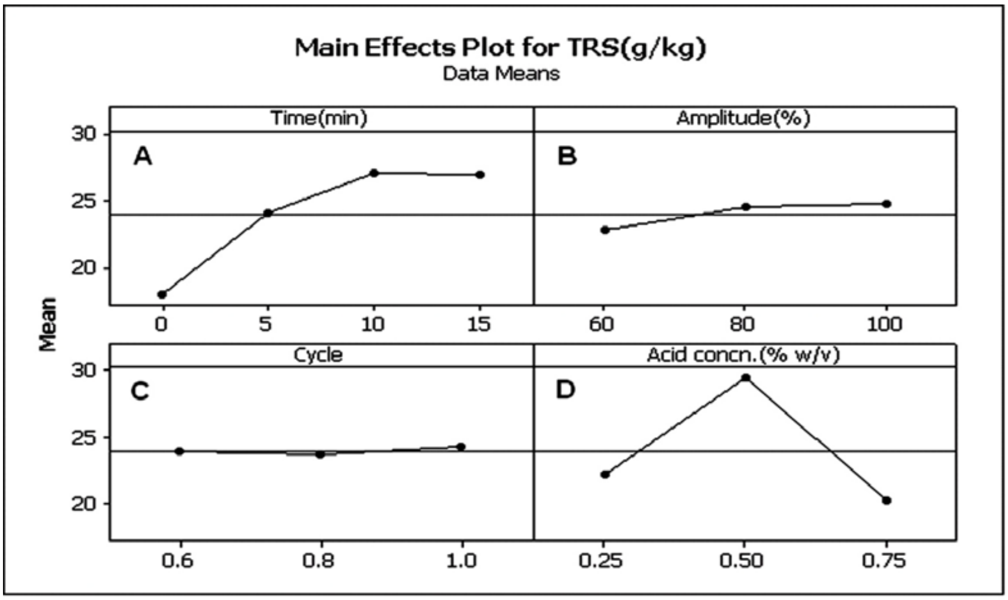

Fig. 5. Effect of sonication on TRS extraction from rice straw (main effects).

Observations from spectrophotometric data of sonicated samples are shown in Fig.5 with main effects and in Fig.6 with interactive effects of different sonication parameters on the sugar yield.Figure 5 showed the main effects of the sonicated samples, which were analyzed through a spectrophotometer. Almost similar kind of observation obtained in previous work where 0.5 % diluted sulfuric acid with sonication gives optimum sugar extraction [17].

It also confirms the previous work results that optimum sugar extraction is obtained when the biomass is hydrolyzed by 0.5% sulfuric acid concentration (Karimi et al, 2006, Bhattacharyya et al, 2012b,). More interestingly, the higher concentration of acid has worked reversely by reducing yield even lower than the value observed before sonication in a few observations. Beginning of formation of sugar degrading product i.e., furfural (from pentoses), hydroxymethylfurfural (HMF) (from hexoses), acetic acid (from hemicelluloses, mainly), etc., at severe conditions like higher acid concentration could be responsible for this observation [24].

After 10 minutes the yield of TRS gets improved with sonication time (Fig.4A) and finally reaches a steady value where no further improvement has been observed, which is probably due to the completion of all possible cell rupture because of high shear development on the cell wall in the hydrolyzed biomass samples by the ultrasonic sound wave.

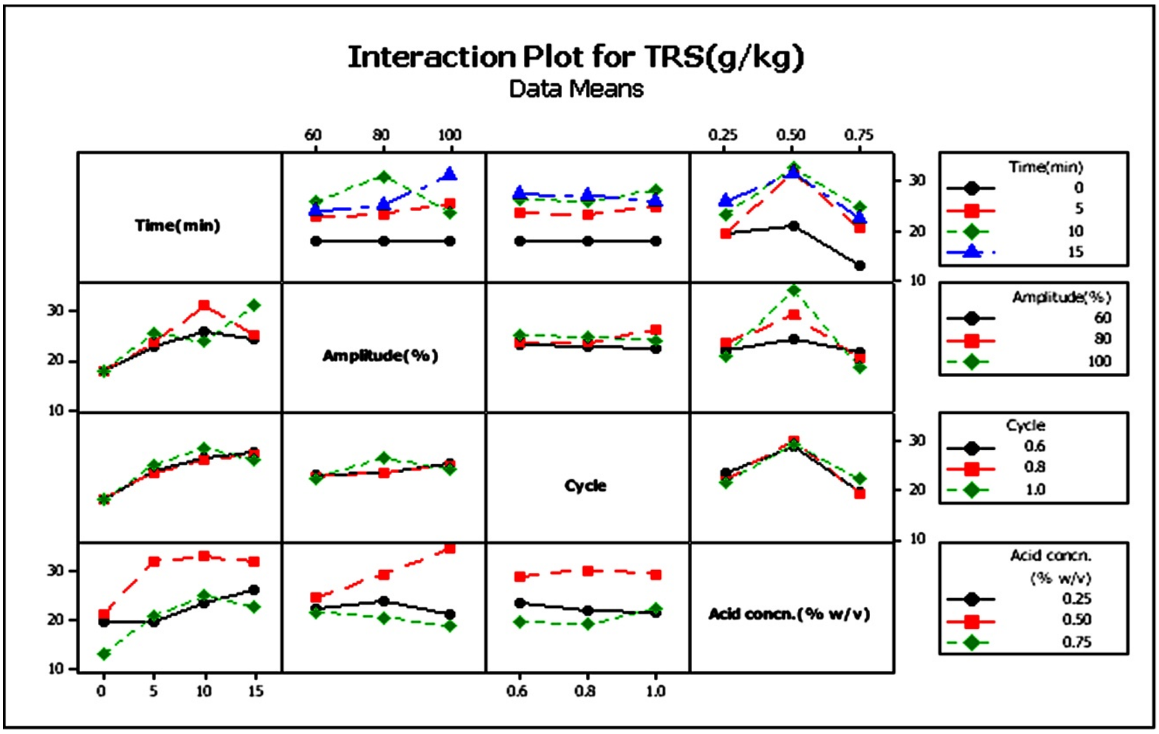

Fig.6. Effect of sonication on TRS extraction from rice straw (interaction effect).

A periodic compression and depression of biomass substances were generated by ultrasonic effect created by sonicator probe, which leads to the formation of unstable microscopic bubbles inside the sample. During compression, these bubbles implode under extreme conditions due to cavitation accompanied by adiabatic heating of the vapor phase inside the bubbles. It may generate a high pressure of around 313 atm. and a localized, transient high temperature of around 3360 K for an aqueous solution of biomass as reported by Sehgal et al. (1977)[25]. These processes tear up the cell walls. Microscopic view (Fig.7) was shown that the rigid rice straw wall was broken down and increases the surface area after the ultrasonic sound was applied.

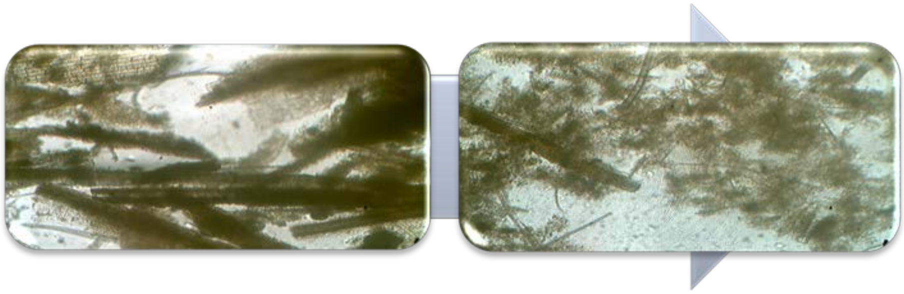

Fig.7. Microscopic (10 X) view of dilute acid hydrolyzed before and after sonicated rice straw sample

The yield of TRS increases nonlinearly with the increase of sonication amplitude (Fig. 5B) and it reaches the peak at 100% amplitude. The sonication cycle (Fig. 5C) did not show much variation in this study. The major reason for controlling the cycle is to regulate the excess heat generation during sonication, which may affect the yield by damaging organic cells of biomass due to temperature rise in the sample observed during experimentation. Therefore, it becomes very necessary to maintain an operation cycle of less than 1 to reduce the heating effect even after exposing samples at 100% amplitude [26].

Table. 2. Analysis of variance table for sonication parameters and acid concentration, where X_1- time, X_2-cycle, X_3-Amplitude, X_4-Acid concentration.

Source	**Sum of sq.**	**d.f.**	**Mean sq.**	**F**	**Probability (p)>F**
X1	1317.15	3	439.05	18.19	0
X2	18.36	2	9.05	0.38	0.6852
X3	83.67	2	41.834	1.73	0.1845
X4	1578.42	2	789.209	32.69	0
X1*X2	183.38	6	30.564	1.27	0.2846
X1*X3	527.81	6	87.969	3.64	0.0034
X1*X4	451.49	6	75.249	3.12	0.0093
X2*X3	88.1	4	22.026	0.91	0.4618
X2*X4	60.49	4	15.123	0.63	0.6452
X3*X4	731.92	4	182.279	7.58	0
Error	1641.55	68	24.14		
Total	6682.35	107			

3.1. ANOVA analysis

The effects of the sonicator parameters and their interactions on TRS extraction have been evaluated using ANOVA analysis. Table.2 shows the results of the analysis which has been accomplished using MATLAB 7.0 Software. Results of this analysis show how effects of different factors and interaction terms in the table are statistically significant; where the level of confidence has been chosen as 95%, i.e., p-value is less than 0.05. If the p-value is less than 0.05, it shows doubt on the null hypothesis, which says all sample means equal and the effect of the factors on the result is statistically significant. The last column of the table shows the contribution of each factor, like total variation, indicating its influence on the TRS yield. It also gives a statistical explanation of the cycle having the least influence on the yield which has been observed from experimental results depicted in Fig. 5.

3.2. Fermentation

From the above discussion, it was clear that the TRS production from rice straw was improved by post dilute acid hydrolysis treatment. The ultrasonic sound helped to improve the amount of reducing sugar from an acid hydrolyzed rice straw. The batch mode fermentation process was done in two groups. One group contain an acid hydrolyzed sample without sonication and another group contain an acid hydrolyzed sonicated sample. Now both groups were inoculated separately by two different microbial strains (*Candida shehatae* and *Saccharomyces cerevisiae*). Figures 8 and 9 show the time vs. bioethanol production from TRS by *Candida shehatae* and *Saccharomyces cerevisiae.* These figures demonstrate that 48 hours was the best time for maximum ethanol production. The maximum yields of ethanol were 0.43 g/g and 0.47 g/g by *Candida shehatae* and *Saccharomyces cerevisiae,* respectively. The single-step simultaneous delignification, saccharification, and fermentation (SDSF) of RS using a partially purified in-house enzyme cocktail with Saccharomyces cerevisiae MTCC-173 resulted in a sugar yield of 26.7 ± 0.2 g/L with an experimental ethanol yield of 6.47 ± 0.16 g/L [10]. Krishnan et al. (2000)[27] reported that the rice straw hydrolyzates were fermented by recombinant *Zymomonas mobilis,* which produced 0.46 g/g of ethanol per sugar utilized. Abbi et al. (1996)[28] also reported that ethanol yields of 0.37 – 0.50 g/g sugar were utilized by free and immobilized *Candida shehatae.*In this experiment ethanol production from dilute acid hydrolyzed sonicated rice straw by *Saccharomyces cerevisiae* and *Candida shehatae* was 78% and 65% respectively better than the dilute acid hydrolyzed rice straw without sonication.

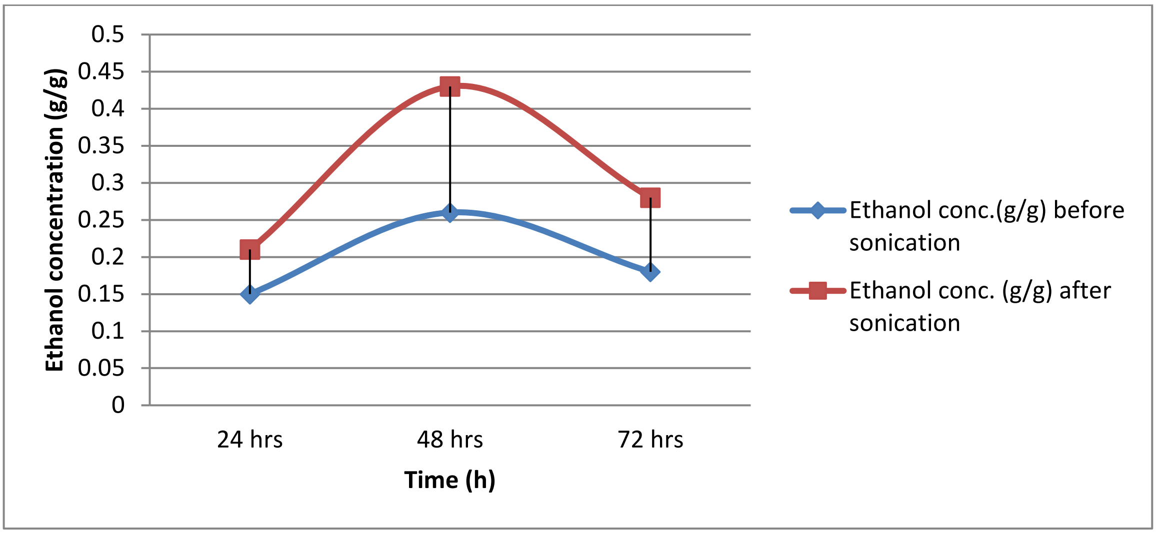

Fig.8. Ethanol production (g/g) of TRS from dilute acid hydrolyzed without sonicated and sonicated rice straw sample by *Candida shehatae*.

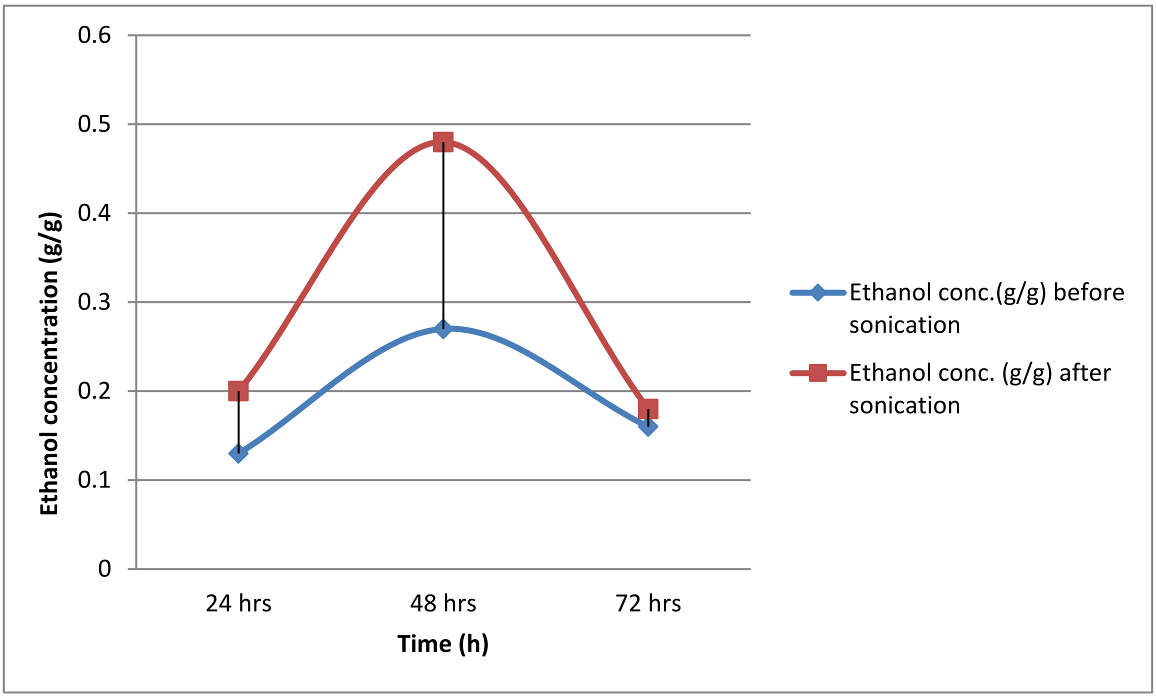

Fig.9.Ethanol production (g/g) of TRS from dilute acid hydrolyzed without sonicated and sonicated rice straw sample by *Saccharomyces cerevisiae*.

4. Conclusions

Effects of sonication in producing TRS for the downstream fermentation process have been studied in this work. It can be inferred from the above discussions, that sonication of hydrolyzed rice straw samples under 0.5% acid concentration and 100% amplitude gives optimum results where the improvement of TRS extraction from rice straw was about 90% better than that without sonicated sample. In this study treatment time of 10 minutes was sufficient to get the optimum yield of fermentable sugar. An additional processing time introduces additional cost for any industrial application, which makes it a very crucial process parameter to be optimized for an economically feasible process. Further, although the cycle has minimal influence on the yield, 0.8 cycles can be maintained for safe operation to avoid the excess heating effect. Sonicated rice straw was more capable for bioethanol than without sonicated sample. The capabilities of the ethanol production from dilute acid hydrolyzed sonicated rice straw by *Saccharomyces cerevisiae* and *Candida shehatae* was 78% and 65% respectively better than the dilute acid hydrolyzed rice straw without sonication.

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