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Optimization of chemical pre-treatment methods for production of bioethanol from dry fallen Neem leaves

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Abstract

During the recent years ethanol derived from biomass, popularly known as bioethanol is grabbing attention due to incessant spike in petroleum prices. Ethanol derived from Corn and sugar are the most popular substitute for ethanol. However, the feedstock is not sufficient and poses the menace food versus fuel. Hence cheaper and inedible sources need to be investigated for the production of bioethanol. In the current study lignocellulosic biomass derived from waste, dried neem leaves was used as a source for bioethanol production. The powdered leaves were pre-treated with conc. H_2SO_4 followed by fermentation with yeast *S. cereviciae***.**On completion of the fermentation process the broths obtained were distilled to obtain bioethanol. The effect of pre-treatment on the bioethanol yield was studied by varying the concentrations of H_2SO_4 as 0.5 N, 1 N, 2 N, 3 N and 5 N, temperature as100°C,120°Cand 140°C and pre-treatment time as 15, 30 and 60 minutes**.** H_2SO_4 conc. of 1N, temperature 120° C and pre-treatment time of 60min was found to be the most optimum condition for liberating reducing sugars from neem leaves under the present experimental conditions. The FTIR studies of the neem leaves before and after pre-treatment showed breakdown in lignocellulosic biomass structure due to pre-treatment. The fermentation of pre-treated solution produced at optimum conditions resulted 24.14 g/L ethanol after 5 days of fermentation.

Keywords: lignocellulosic biomass; neem leaves; acid pre-treatment; bioethanol; Saccharomyces cerevisiae

1. Introduction

The soaring demand for fossil fuel has led to its rapid depletion as well as added to the environmental woes [1]. The increase in global green house emissions due to extensive use of fossil fuels has become a major concern. Thus development and use of alternate renewable source of energy with negligible carbon footprint is the need of the day [2,3,4,5]. Recently energy derived from biomass is grabbing the attention worldwide as a potential renewable energy source. Lignocellulosics are such renewable carbon sources that can be used to produce biofuels with reduced green house emissions [6,7,8]. Lignocellulosic biomass is widely available as agricultural and forest wastes and its use for generation of bioethanol can avert the food versus fuel crisis as well as help in efficient waste management [9,10,11,12,13]. Lignocellulose is made up of cellulose, hemicellulose and lignin in different percentage depending upon the plant type and its parts [12]. Till date cellulose and hemicelluloses are utilized for producing bioethanol [5,13] and lignin still remains as a waste. The recalcitrance of lignocellulose towards degradation is an important and challenging aspect of bioethanol production. Hence different pretreatment methods are adopted to increase the liberation of fermentable sugars from such biomasses [10,11,12,13]. The different pre-treatment methods are: physical, chemical, and biological pretreatment methods. The physical pretreatment method decreases the particle size and thus enhances the enzymatic accessibility of biomass. Similarly the chemical pretreatment also increases the surface area by converting the crystalline structure of lignocellulose into amorphous form. The biological pretreatment causes the degradation of lignin and reduces the degree of polymerization in both cellulose and hemicellulose. The chemical pre-treatment step is easy to perform and highly efficient [3,12]. Following the pretreatment step, the cellulose and hemicellulose are hydrolyzed either enzymatically or chemically producing simple fermentable carbohydrates/sugars [12]. These sugars are then fermented to ethanol by yeast or bacteria. *Saccharomyces cerevisiae* (the baker's yeast) is mostly used on an industrial scale production of bioethanol due to high ethanol yield [6]. Many studies have been carried out to produce bioethanol from diversified cellulosic biomass such forestry

wastes, agricultural residues etc. [14]. However, very few studies have carried out on production of bioethanol from leafy biomass, which is abundantly available. Moreover, fallen dried leaves do not have further applications. The low lignin content in the lignocellulosic biomass favours the conversion efficiency of cellulose to fermentable simple sugars and in this regard, neem leaves have comparatively low lignin content in comparison to other leafy biomass sources [14], and hence can be a better choice for bioethanol production. The easy and ample accessibility of neem leaves in the state of Odisha, India envisaged interest in exploiting them as substrate for bioethanol production. Moreover very few studies have been reported on the potential of neem leaves to produce bioethanol. Hence, the current study explores the potential of fallen waste neem leaves to produce bioethanol by fermentation using the yeast *Saccharomyces cerevisiae*.

Although, lignocellulosic biomass is a potential feedstock for production of bioethanol, the pretreatment of the biomass poses as one of the major challenges and hurdle in developing a cost effective method for production of bioethanol. Additionally, during pretreatment various products are derived from the lignocellulosic biomass that affect the sugar yield adversely [5, 15]. Various factors such as the temperature, residence time and sulfuric acid concentration have been reported to influence the yield of from lignocellulosic biomass pretreatment [8]. Thus, the present study investigates the potential of dried waste neem leaves in production of bioethanol and optimizes the conditions of pre-treatment to obtain a maximum yield of fermentable sugar.

2. Experimental

2.1. Materials

Dried Neem leaves were collected locally during the months of November and December (during this time the leaves dry and fall in Odisha, India). The leaves were properly cleaned and dried at room temperature. After drying it was pulverized using a blender and then sieved with a sieve No 20(850μm). All the chemicals used are of analytical grade.

2.2 Methods

The major components of neem leaves are cellulose (approx. 21%), hemicelluloses (approx. 51%) and lignin (19%) [14]. Such lignocellulosic biomass are hydrolysed to fermentable simple sugars prior to fermentation to produce bioethanol. In order to produce fermentable sugar, the impermeable structure of lignocellulosic biomass needs to be degraded. The impermeability of lignocellulosic biomass is attributed to the presence of complex structured cellulose that provides strength to the cell walls, the hemicellulose that acts as a wire mesh encircling the cellulose and the lignin that fills the remaining space. This prevents the hydrolyzing agent from interacting with the polysaccharide and forming fermentable simple sugars [5]. In this context, pre-treatment of such complex structured biomass with dilute sulphuric acid is reported to be more effective and simple in comparison to others [7]. During pre-treatment the lignin part is removed and the crystallinity of cellulose decreases along with degradation of hemicelluloses [5]. In the present study the conditions of chemical pre-treatment such as conc. of H_2SO_4, reaction time and temperature have been optimized to obtain maximum yield of fermentable sugar. 30g of accurately weighed powdered Neem leaves was taken in a conical flask and 300ml of H_2SO_4 solution of varying concentrations (0.5N, 1N, 2N, 3N and 5N) was added to it and stirred continuously with a magnetic stirrer for different time durations (15, 30, 60, and 90 minutes) and at different pre-treatment temperatures (100, 120 and 140^0C). Post pre-treatment, the sample in the flask is cooled down to room temperature, followed by filtration and centrifugation. The resulting liquid after centrifugation is neutralized to pH 4.5 by adding ammonia buffer and then subjected to fermentation to produce bioethanol. The high acid concentration of the resulting filtrate inhibits the fermentation process, for which the neutralization step was carried out before fermentation [10].

The amount of reducing sugar in the pre-treated samples was estimated by using the Fehling's method. In the first step the Fehling's solution was standardized using a standard glucose sample. 10 ml of Fehling A reagent was mixed with an equal amount of Fehling B reagent and diluted with distilled water upto 40ml. The Fehling mixture solution was then heated on a hot plate to a temp. of 70^0C. Glucose standard from the burette was added until the blue colour of the mixture of Fehling solutions disappeared. At this point methylene blue indicator was added and the titration was continued with the standard glucose sample till the solution becomes colourless. After standardization of Fehling's solution, the reducing sugar in pre-treated sample of Neem leaves was determined following the same procedure.

The pre-treated solution containing the maximum sugar was taken for fermentation to produce bioethanol. Fermentation was carried out by using commercially available yeast, Saccharomyces cerevisiae. Before fermentation the pH of the pre-treated sample was adjusted to 4.5 by ammonium

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buffer. 3g of *S. cereviciae* was added into the flasks containing the pre-treated sample and then it incubated at 37°C with 100 rpm shaking speed for 3 days. After that the fermented broth was distillated with the round bottom distillation column enclosed with a running tap water and the temperature was set up to 78.3°C. Then, the distillate was collected in the other end of the distillation column

Characterization of powdered neem leaves was carried out to analyse the total carbohydrate (cellulose and hemicelluloses content), moisture and ash content using a similar procedure reported elsewhere [3]. Besides the biomass sample was also characterized by FTIR spectroscopy for analyzing the presence of various functional groups along with lignin content in the samples [12]. The FTIR analysis was carried out through Thermo Scientific Nicolet iS5 FTIR Spectrometer in a wave number range from 4000 cm^{-1} to 500cm^{-1}.

After distillation of the fermented sample, the resulting bioethanol was collected and characterized by FTIR and UV-Vis spectrophotometry (GENESYS 10S Bio) [16]. In order to get an idea of the extent of ethanol conversion, the FTIR spectra of the produced bioethanol was compared with commercial (99.9% purity) and various compositions of ethanol mixture with water such as 1%, 2%, 5%, 10% and 20%. For determination of exact concentration of the resulting bioethanol, 3ml. of the distilled fermented sample was added to 10 ml. acidified 0.1 M $K_2Cr_2O_7$ solution and mixed properly. The tube containing the mixture was capped and then the tube was placed in a water bath at 60^0 C for 20 min. and then cooled to room temperature. The resulting solution was then analyzed through UV-VIS spectrophotometry. Beer-Lambert plot was plotted from the absorbance data of various compositions of ethanol water mixture like 1%, 2%, 5%, 10% and 20% and by using this law the concentration of the resulting bioethanol was determined.

3. Results and Discussions

Physical pre-treatment involving size reduction usually disrupts the lignocellulosic structure enhancing the subsequent hydrolysis process. In the present investigation the fallen dried neem leaves are powdered to increase the surface area that ultimately increases the accessibility of the aqueous reagent during chemical pretreatment for maximum conversion into fermentable simple sugars. Figure1a and 1b show the picture of dried Neem leaves and powdered neem leaves respectively.

The total carbohydrate (cellulose and hemicelluloses), moisture and ash content in the biomass samples are important parameters that affect the conversion into simple fermentable sugar during chemical pretreatment process. The high carbohydrate content and low ash content is favorable for efficient production of bioethanol. The high ash content in the cellulosic biomass absorbs the water, acid during chemical pretreatment than the cellulosic fibres, that ultimately affects the conversion process of cellulose to simple fermentable sugars [12]. The combined biochemical and proximate analysis provides information regarding total carbohydrate (cellulose and hemicellulose), protein, lipid, moisture, ash etc in the sample as shown in Table-1.

Table 1. Chemical composition and proximate analysis of Neem leaves

Biochemical	Content (%)	Proximate	Analysis (%)
Carbohydrate	22.87	Moisture	6.3
Protein	6.9	Volatile matter	65.4
Lipid	48.7	Ash	3.2
		Fixed Carbon	25.1

Figure-2 and 3 show the FTIR spectra of powdered neem leaves before and after pre-treatment respectively. The FTIR analysis of biomass provides information regarding the presence of various organic groups present in it [12]. Apart from this, such analysis provides information related to breaking of bonds along with modification in stretching vibrations with respect to structural integrity of lignocellulosic biomass [6]. The absorption band between 3200 and 3600 cm^{-1} is usually attributed to the O-H stretching vibrations of alcohols, carboxylic acids and hydroperoxides. The FTIR spectrum shows a fingerprint region of 1420-670 cm^{-1}. The band at 1457 cm^{-1} shows the C-H bending or scissoring of alkanes found in the biomass samples. A very sharp peak between 1006 to 1028 cm^{-1} corresponds to ether group. The hemicelluloses content of the biomass can be ascribed at 1671 and 1028 cm^{-1} [6,12] and reduced intensity of such corresponding peaks in pre-treated sample signifies the removal of hemicelluloses due to pre-treatment. The peak at 2917 cm^{-1} can be ascribed due to methyl group of alkanes. The band at 3283 cm^{-1}can be assigned to O-H stretching of hydrogen bonds that represents the significant properties of cellulose [6]. The band

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corresponding to CH_2 stretching in non-pretreated sample (2917 cm^{-1}) is reduced in pre-treated biomass sample (2919 cm^{-1}), suggesting a significant removal of aliphatic waxes due to pre-treatment. Moreover, the peak (1028 cm^{-1}) in non-pretreated sample is reduced in pre-treated sample (1030 cm^{-1}) suggesting the exposure of cellulose in pre-treated sample [6]. Hence, from the peak analysis of FTIR spectra of the samples before and after pre-treatment suggests that there is breakdown in the lignocellulosic biomass structure due to pre-treatment.

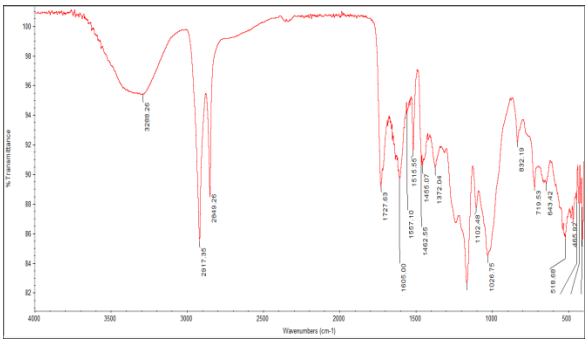

Figure-2 FTIR spectra of neem leaves powder before pre-treatment

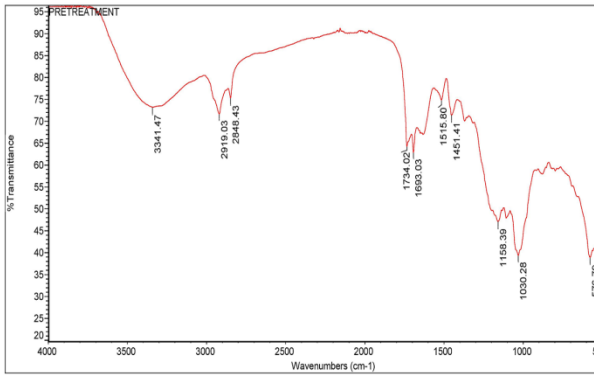

Figure-3 FTIR spectra of neem leaves powder after pre-treatment

In order to investigate the effect of acid concentration on the yield of reducible or fermentable sugar, the acid pre-treatments were performed at varying concentrations of H_2SO_4 solutions such as 0.5 N, 1 N, 2 N, 3 N and 5N at a fixed temperature of 100°C and pre-treatment time 60 min. The results of the study are shown in Fig 4 from which it is found that at 1N acid concentration highest sugar is produced. At higher acid concentration, the sugar content declines. Similar observations have also been reported by Sert et al. in the production of bioethanol from algae [3] According to Jutakridsada et al., when the conc. of acid increases, the hydronium ion not only decomposes the cellulose and hemi-cellulose but also the fermentable sugars that result into a low sugar yield [17]. High concentration of acids is detrimental to the formation of sugar whereas at lower concentrations, the pretreatment reaction rate is very high that subsequently improves cellulose hydrolysis [18]. Moreover, comparatively dilute acids effectively remove hemicellulose as dissolved sugars, and due to the removal of hemicellulose the glucose yield from cellulose also increases resulting in an enhanced yield of sugars [19].

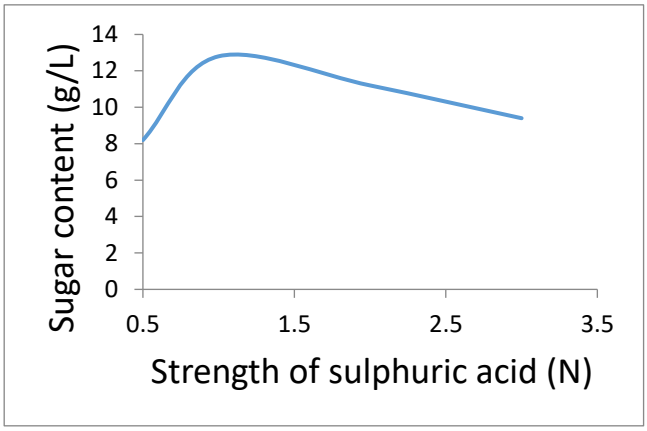

Figure-4: Effect of concentration of H_2SO_4 on sugar recovery during pretreatment

In order to study the effect of temperature on the fermentable sugar conversion during pre-treatment, the experiment was carried out at varying temperatures- 100 °C, 120 °C and 140 °C at fixed acid conc. of 1N and reaction time of 60 min. The results of the study are shown in Fig 5. The results suggest that with increase in temperature there is a simultaneous increase in the sugar content which is in agreement with the literature reports [2, 3]. For temperature higher than 120 °C, the yield of reducible sugar is found to decrease. Thus, high temperature favors the acid pre-treatment process, but very high temperatures decrease the reducible sugar content. This can be attributed to the formation of different inhibitory compounds such as acetic acid, furfural, HMF, phenols etc. that affect the sugar yield adversely and consequently the yield of bioethanol [5].

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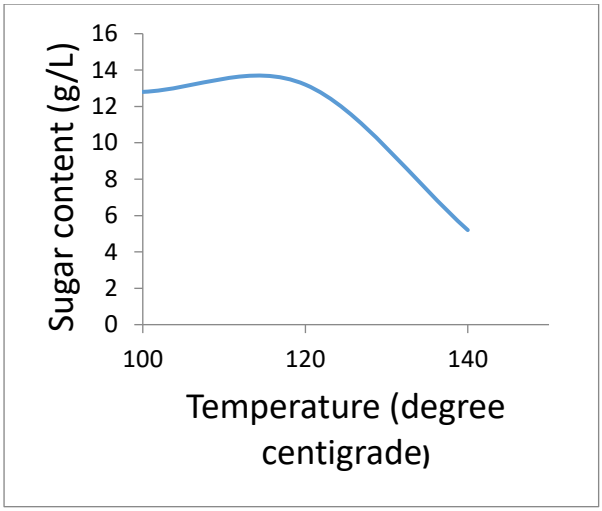

Figure-5 Effect of temperature on sugar recovery during pretreatment

To study the effect of residence time on the yield of reducible sugar, the study was performed for 15, 30 and 60 minutes respectively with 1N conc. of H_2SO_4 and temp.120^{0}C.The results of the study are shown in Fig 6. which clearly suggest that increasing the reaction time increases the yield of reducible sugars. The sugar content was found to rise steadily upto 60 minutes. With further increase in residence time, a decline in sugar concentration was noted.

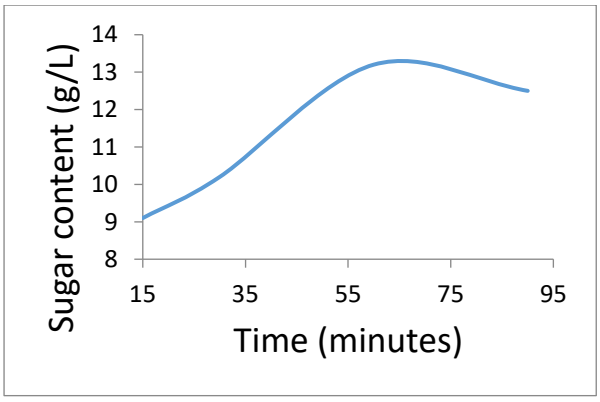

Figure-6 Effect of residence time on sugar recovery during pretreatment

Riansa-ngawong and Prasertsan reported that with the increase in reaction time and temperature, furfural production increases strongly that results in a low sugar content [20].

Though various factors such as time period of fermentation, pH, agitation speed, temperature of fermentation and amount of sugar content in the pre-treated sample affect the bioethanol production, the effect of sugar content in the pre-treated sample has predominant effect on the bio-ethanol production [6]. In the present study, a maximum sugar content of 13.2 g/l was obtained at the optimized pre-treatment conditions- 1N H_2SO_4, temperature 120^{0}C and residence time 60 min. Hence, such pre-treated solution is taken for fermentation to produce bioethanol.

Figure-7 shows the FTIR peak of the distilled fermented sample. Upon comparison with the pure ethanol sample, it is found that many peaks (at) are absent in the produced ethanol sample (Figure-7) than that of the pure ethanol sample (Figure-8). This can be ascribed due to the presence of water in the produced ethanol sample. In order to get the approximate composition of the produced ethanol, the FTIR pattern is compared to various composition of ethanol and water mixture such as 1%, 2%, 5%, 10% and 20%. From such comparison, it is found that the produced bioethanol FTIR peak (Figure-7) is quite comparable to that of the 10% ethanol (Figure-8).

From the UV-VIS spectrophotometry study, the λ_{max} for all the ethanol samples (standard samples of 1%, 2%, 5%, 10% and 20% and produced bioethanol sample) are found at 590 nm. Using Beer-Lambert's law the concentration of bioethanol sample is found to be 11% which is in good agreement with that of the FTIR analysis.

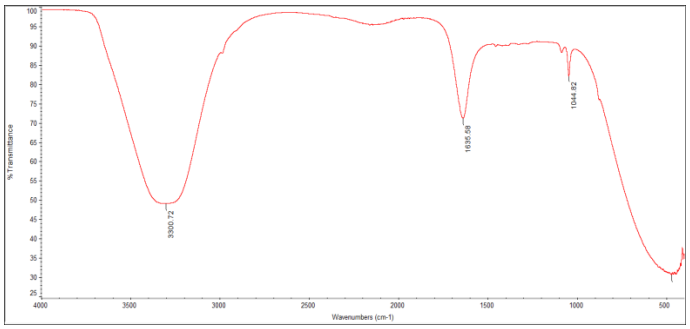

Figure-7 FTIR spectra of bioethanol (produced from 1M H_2SO_4 pre-treated solution)

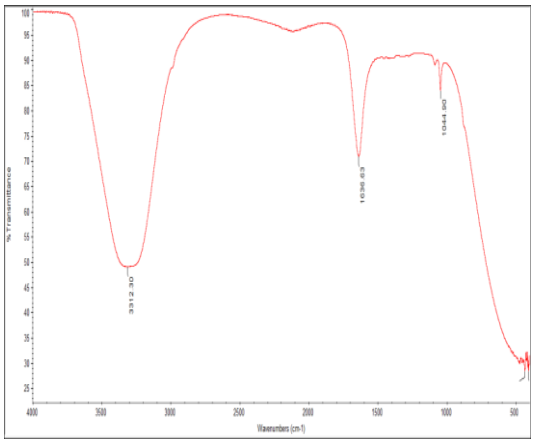

Figure-8 FTIR spectra of 10% ethanol

4. Conclusions

The exorbitant use of fossil fuels is leading to its depletion and adding to the environment problems. Thus, the use of environment-friendly and renewable source of energy is the need of the hour. Biofuels developed from biomass are a befitting solution to this problem. The current study investigated the potential of dried waste neem leaves for producing bioethanol by using the acid pre-treatment method. 1N H_2SO_4, temperature 120^0C and residence time 60 min was determined as the optimum condition of pre-treatment where a maximum yield of 13.2 g/l of fermentable sugar was obtained. The resulting sugar upon fermentation with yeast *Saccharomyces cerevisiae* resulted 11% bioethanol after 3 days of fermentation. Though neem leaves have medicinal applications, but fallen dry neem leaves does not bear any potential utilization. Thus such fallen neem leaves that is abundantly available in Indian subcontinent can be used as a potential feedstock for producing bioethanol.

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