RESEARCH ARTICLE

Bioethanol Production from Citrus Peel Waste in the absence of D-Limonene

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ABSTRACT: Citrus peel wastes were studied for bioethanol production using the H_2SO_4 pretreatment method and the fermentation of *Saccharomyces cerevisiae.* This study investigates the potential of acidic pre-treatment for 3 h at 100 °C to remove D-Limonene. The results obtained from FT-IR analysis confirmed that D-limonene was significantly decreased from 0.21% to below 0.01%. The density of the bio-ethanol produced was found to be 0.85 g/cc. The turbidity analysis results showed that there was no haze or turbidity developed. Further, the citrus peel wastes treated with 10% (v/v) of H_2SO_4 showed an increase in reducing sugar concentration at 42 hrs. An ethanol concentration of 70.94 % was obtained from citrus peel wastes during fermentation for 24 hrs. The results observed from the density, turbidity, and Benedict's test confirms that a good bioethanol yield was produced by removing D-limonene through the sequential acid hydrolysis and fermentation process.

KEYWORDS: Citrus peel wastes, D-limonene, Bioethanol, Fermentation, Hydrolysis

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1. INTRODUCTION

Hydrocarbon deposit is used as a primary energy source across worldwide. Combustion of hydrocarbons like non renewable energy source generates massive impact on environment and it is also not strewn throughout the globe [1]. Hence, alternative energy produced from bio-resources is required for melioration of ecosystem [2]. Bioethanol (ethyl alcohol, grain alcohol, or $CH_3–CH_2–OH$) is a renewable, economical, and eco-friendly alternative that can be reserved, and energy conservation techniques can be developed to fulfill the broad range of energy requirements [3]. More than 65,000 mega liters of ethyl alcohol were consumed across the world and it is already replaced by 5.4% of hydrocarbons. In the current scenario, the demand for biofuels will increase by 41 billion liters, or 28 %, between 2021 and 2026. The pre-Covid-19 has resulted in a high surge of up to one-fifth of normal consumption. The issues related to fossil fuels are obviously lessened by ethyl alcohol, an alternate energy source, either an octane enhancer or primary energy source [4, 5].

Citrus peel waste (CPW) materials are regarded as wastes since they must not enter the food chain. The presence of essential oils prevents the composting of citruses due to its low pH value. Further, its rapid biodegradation, which can lead to anaerobiosis issues in humus heaps, and also the disposal of non-hazardous materials often do not accept citrus peel left over for composting. These wastes have 75-85 % water content [2], which prevents other thermal treatments like incinerating, gasifying, or pyrolyzing it from being effective. Citrus peel material has traceable amount of D-limonene and bioactive molecules which hinders the effective fermentation process. Moreover, it creates bad odor which expedites to environmental pollution. Subsequently, energy and cost effective method is required for dehydration process, even if they would technically be possible. Besides, the disposal material is based on the harvest from the whole amount of fruit produced. In that, juice extraction from a ton of lemon peel will lead to generate around 500 tons of trash, which affects the ecosystem due to liberation of atmospheric photochemicals [6]. CPW materials are utilized for alternate energy production instead of using fossil fuels to rectify above-mentioned problems on top of increasing energy production for human needs. Many researchers investigated the use of orange, lemon, mandarin and grape fruit wastes to produce ethyl alcohol by

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undergoing pretreatments likes team explosion prior to enzymatic integration followed by fermentation process [7, 8]. However, there are few studies reported about the quality production of ethyl alcohol in short time and economical friendly. From the results, it was found that D-limonene present in the lemon peel could limit fermentation process. It is hard to remove D-limonene generated during the break down of biomass that obviously hinders the microbial growth. Among various pretreatment techniques such as ammonia fiber explosion, bio-cremation, oxidative delignification and biological methods, steam explosion followed by hydrolysis process was extensively explored to disintegrate lignocellulosic biomass of different citrus wastes [9]. However, the major limitations of many of these methods are longer extraction time, higher energy and elevated temperature. Therefore, the present work is aimed to develop an efficient and less time consuming pretreatment method to remove D-limonene and to convert CPW into value added product. The results confirmed that 70 % of ethanol concentration was achieved along with significant removal of D-limonene by low temperature hydrolysis method using H_2SO_4.

2. MATERIALS AND METHODS

Citrus (*Citrus. limon L.*) peel wastes was collected from local market (Salem, India). After collection, the sample was cut into small pieces between 2 and 6 mm in size. Distilled water, H_2SO_4, Benedict's reagent and aluminum foils were purchased from Mercury lab, Salem. *Saccharomyces cerevisiae* was purchased from Mercury lab, Salem. Lemon peel has distinctive composition of bioactive components and also a natural source of numerous enzymes as mentioned in Table 1 and 2 [10-12].

2.1 Pre-treatment

Lignocellulosic biomass was pretreated prior to disintegration process, which is a prerequisite for the bioethanol production that determines the yield of bioethanol. During pretreatment, 40 g of lemon peel was mixed with 10 % (v/v) of concentrated H_2SO_4 in 200 ml distilled water and kept at 100 °C for 3 h. The obtained solid residues were separated from the mixture and dried at 45 °C followed by washing for several times using distilled water. The pretreated lemon peel waste samples before and after the treatment were shown in the Fig. 1 and Fig. 2, respectively.

Table1.Lemon Composition

Composition of Lemon Peels	
Moisture content	70.0%
Cellulose	21.6%
Hemicellulose	6.0%
Lignin	8.9%
Reducing Sugars	1.8%

Table 2 Monosaccharide composition of citrus peels [10-12]

Monosaccharide	Orange peel (% of dry matter)	Mandarin peel (% of dry matter)
Rhamnose	0.9	3.8
Arabinose	8.4	11.2
Mannose	3.0	2.0
Galactose	6.4	4.2
Glucose	25.8	24.8
Xylose	3.7	3.1

Fig 1: Lemon peels for pretreatment

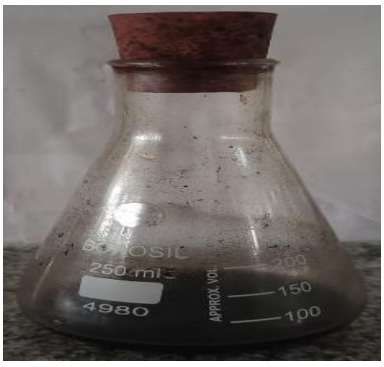

Fig 2: Sample after acid pretreatment

2.2 Hydrolysis

Biomass disintegration was performed using H_2SO_4 (10 % v/v) as acid catalyst at a pH of 2.5. Sulphuric acid was utilized to catalyze the hydrolysis reaction of citrus peel wastes. Mainly, it was used to accelerate the breaking down process of polysaccharides into reducing sugars. As a result of acid catalysis, the chemical bonds between the sugar molecules were cleaved into individual sugar units which will lead to achieve high ethanol yield. So far, cellulose hydrolysis of CPW was investigated by many researchers, but hydrolysis using H_2SO_4 at low temperature have been rarely used.

The biomass disintegration can be represented by:

$$C_6H_8O_7 + H_2SO_4 \rightarrow \text{Sugars } (C_6H_{12}O_6)$$

This process will enhance the ethyl alcohol production when *Saccharomyces cerevisiae* is used for fermentation process. H_2SO_4 solution (10 % v/v) was added with pretreated lignocellulosic biomass. Then the solution was heated at 100 °C for 3h as depicted in Fig. 3. When it reaches the room temperature, the hydrolysis treatment was performed at 0 h, 24 h and 42 h.

2.3 Sugar Analysis

The presence of simple sugars was performed using Benedict's test. For that, 5 ml of biomass sample were heated and cooled. Then, mixing and heating of sample was done for 5 min after addition of Benedict's reagent. The color change from blue to green which is shown in the Fig. 4 indicated the presence of reducing sugars in the sample. The color changes occur based on the concentration of simple sugar present in the sample during the test, as given in Table.3. From that, it was observed that green color indicates the presence of very low-level concentration of simple sugars, yet possessing a potential to produce bioethanol. The result obtained through Benedict's test and the concentration of simple sugar at 0 h, 24 h and 42 h was shown in Fig. 4 and Fig. 5.

2.4 Fermentation Process

Saccharomyces cerevisiae is a species of yeast that has been extensively studied and is widely used in various fields, particularly in baking, brewing, and fermentation process. It converts ethanol and carbon dioxide while breaking down the simple sugars using yeast. The simple sugar present in the CPW was converted to ethyl alcohol by the addition of *Saccharomyces cerevisiae*.

Break down of simple sugar is given by,

$C_6H_{12}O_6$ (Glucose) $\longrightarrow$ CH_3 CH_2 OH (Ethyl alcohol) + CO_2 (Carbon dioxide)

After hydrolysis, fermentation was performed at aerobic conditions with 2 g of *Saccharomyces cerevisiae* in a 250 ml baffled flask at 30 °C for 3-6 days and pH of 5.2 in a shaking incubator at 100 rpm. The yeast growth was noticed after 3 days of fermentation process. Then, the total volume of ethanol produced in each day was recorded in day wise after distillation was shown in the Fig. 6.

Fig 3: Hydrolysis process set-up

Fig 4: Color change during Benedict's test

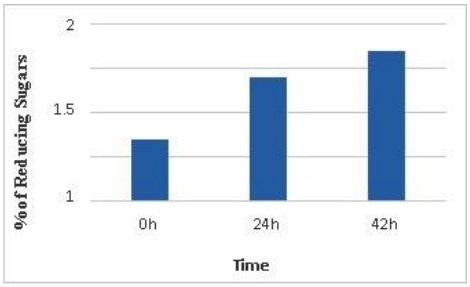

Fig 5: Percentage of reducing sugars at 0 h, 24 h and 42 h

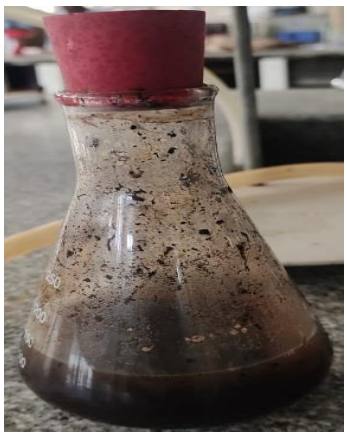

Fig 6: Fermentation Process

Fig.7. Distillation process set-up

2.5 Distillation Process

The separation of ethyl alcohol from the mixture was done by distillation operations. For that, the sample mixture was taken in a round bottom flask for the distillation operation as shown in Fig. 7. The process was maintained at 80 °C to prevent the evaporation of ethyl alcohol along with the water present in the sample. The volume of the ethanol obtained after distillation of the samples recorded in 3rd, 4th , 5th and 6th days were shown in Fig. 8. The volume of ethyl alcohol obtained from distillation operation in the 4th day was high compared to other days.

2.6 Ethanol Concentration Calculation

Ethanol concentration was calculated using a procedure reported by Feinstein [13,14]. The value of ethanol concentration can be estimated from the obtained density values. Let's assume that the concentration of ethanol in the mixture is denoted by "x" (in grams of ethanol per gram of solution).

Density of Mixture =

(x * Density of Ethanol) + ((1 - x) *Density of Water)

The density of the mixture was found to be 0.8504 g/cc using pycnometer, and the known densities of ethanol and water are used. Using these values:

0.8504 g/cc = (x * 0.789 g/cc) + ((1 - x) * 0.9998 g/cc), Now, you can solve for "x":

0.8504 = 0.789x + 0.9998 - 0.9998x

0.8504 - 0.9998 = 0.789x- 0.9998 x

- 0 .1494 = - 0.2108 x

Now, divide both sides by - 0.2108 to solve for "x"

Then, x = (- 0.1494) / (- 0.2108); $x \approx 0.7094$

So, the concentration of ethanol in the mixture is approximately 0.7094 grams of ethanol per gram of solution. Therefore, % Concentration of Ethanol = 0.7094 * 100 = 70.94 %

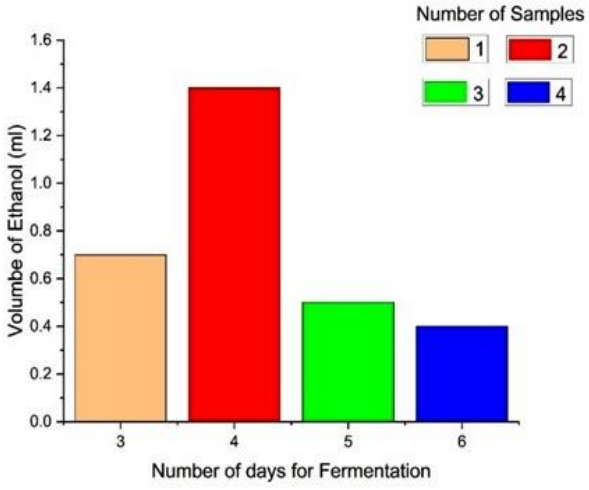

Fig 8: Volume of ethanol obtained from different times of fermentation process

3. RESULTS AND DISCUSSION

It is crucial to choose an efficient pretreatment method to disintegrate the biomass since the selection of a suitable method majorly impacts the production of bioethanol. It helps to effectively breakdown the biomass and also to obtain efficient fermentation and hydrolysis. After the acid pretreatment, reducing sugars were generated in large amount while processing the lemon peels using H_2SO_4 indicates that the disintegration was achieved effectively [15].

Hydrolysis can be achieved in two ways, either with the use of acids or enzymes. The main purpose of hydrolysis is to convert the components of lignocellulosic biomass into readily available reducing sugars and can be utilized as substrate by *Saccharomyces cerevisiae*

during fermentation. Then, the mixtures were heated for 3 hrs at 100 °C and hydrolyzed with 10 % (v/v) sulfuric acid. After that, the samples were tested using Benedict's to determine the presence of sugars before fermentation process [16]. It was observed from the Benedict's test results that the hydrolysis time also played a significant role in the production of reducing sugars.

As shown in Fig. 5, the lemon peels on enzymatic treatment with *Saccharomyces cerevisiae* increased the total reducing sugar content. The enzymatic treatment of lemon peels on different reaction time from 0 hrs, 24 hrs, and 42 hrs at 2 % enzyme concentration, pH 5.2, temperature 30 °C with *Saccharomyces cerevisiae* showed an increasing trend in the total reducing sugar content with 7.24, 10.25, and 19.63 g/L respectively.

After distillation operations, about 20-25 ml of ethanol was produced from 40 g of biomass at 100^{o}C. It may vary depending on the processing parameters. To confirm the presence of ethanol, density and turbidity test was performed by the standard procedures. According to the ASTM4806 standard, the density of bioethanol was determined using pycnometer and the density value can't be greater than 0.99 g/cc at a temperature of 15 °C [17,18]. The value of density indicates that whether the sample contains ethanol or any other compounds. The density value was found to be 0.85 g/cc for the sample prepared in this work, which lie between the values of density of water and ethanol. The calculations were given in the section 2.6. This result indicates that the sample consists of ethanol with the presence of water molecules.

The ethanol concentration of the four bioethanol samples was estimated using pycnometer at periods between 3 to 6 days. As shown in Fig. 8, the ethanol concentration values obtained in each day for the four bioethanol samples showed a higher bioethanol production on the 4th day of fermentation and lowered significantly as the fermentation days increased. A lower ethanol concentration may be attributed to the higher osmotic pressures created in the yeast cell membranes which can retard the microbial growth and decreases the bioethanol production. The varied bioethanol concentration may be due to a strong inhibition effect of the yeast growth, whereas longer fermentation days above 4 days become detrimental for the yeast growth.

The turbidity of the sample was analyzed by IS 3025 (Part 10) 1984 (RA 2006) testing method. Turbidity is the measure of relative clarity of a liquid and the amount of light scattered by materials in the water sample. The higher intensity of scattered light explains that the turbidity was higher. The turbidity test result of the sample was found to be less than 1 NTU, which indicates that the sample was free from impurities. The results were shown in Table 4 [19].

The above results indicated that a good yield was obtained due to high conversion of reducing sugars in to ethanol with an effective removal of D-limonene due the acid hydrolysis and fermentation method.

Table 3: Color changes and the concentration of reducing sugar present

BENEDICT'S TEST		
Color Changes	**Concentration of reducing Sugars**	**Percentage based on the color**
Blue	None	0g%
Green	Very Low	0.5-1g%
Yellow	Low	1-1.5g%
Orange-red	Moderate	1.5-2g%
Brick-red	Very High	>2g%

Table 4: Result obtained from Density and Turbidity Test [19]

Parameters	Test method	Result
Density @15.6 ^{o}C (g/cc)	IS3025P.3919 91RA 2019	0.8504
Turbidity (NTU)	IS3025P.1019 84RA 2006	< 1.0

In addition, FT-IR analysis was carried out to determine the functional groups presents in the obtained sample and the results were presented in Fig. 9. The band appears at 3347 cm^{-1} corresponds to O–H stretching and confirms the presence of alcohols and phenols [20, 21]. The absorption band at 1650 cm^{-1} corresponds to

C=C stretching vibrations was indicative of 1650 cm^{-1} indicates the presence of carboxylic acids and aldehydes [22]. The absorption band peak at 1380 cm^{-1} indicates the presence of CH_3 methyl groups and alkanes. The presence of aromatic esters and ethers were confirmed by the observed peaks between 1086 cm^{-1} and 1044 cm^{-1}. The peak at 879 cm^{-1} indicates that the compound was aromatic. These results confirm that the presence of ethanol yield while using the acid pretreatment. Moreover, the results confirm that the sequential acid hydrolysis and fermentation process has alkenes and aromatics. The observed peak at significantly reduced the D-limonene concentration from 0.021 to less than 0.01%. The process reported in this work requires less cost when compared to the steam explosion treatment process of citrus peel wastes to produce ethanol [23]. Also, this study paves a way for the efficient removal of D-limonene, since D-limonene can severely inhibit the yeast growth in the fermentation process which will lead to low conversion of bioethanol from reducing sugars [24-27].

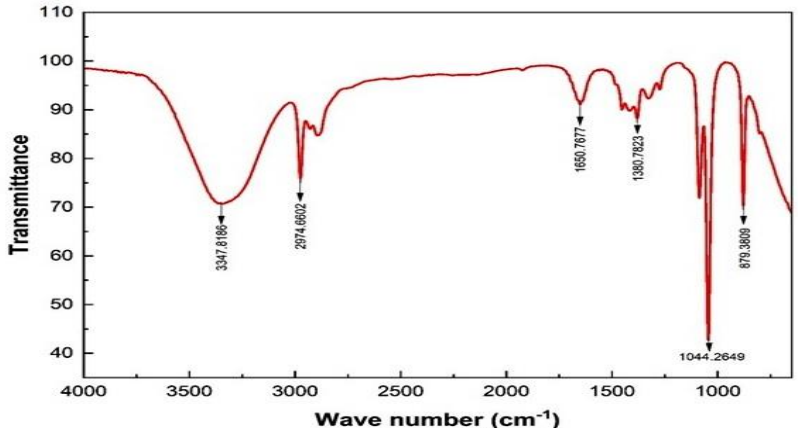

Fig.9. FT-IR analysis of biomass produced from citrus peel waste

CONCLUSION

In the present work, the citrus peel waste as a source of lignocellulosic biomass was utilized to investigate the feasibility of bioethanol production. By achieving an ethanol concentration of 70.94 % confirms that the acid catalyzed pretreatment needs very less time and temperature than the other methods to produce a good ethanol yield. The results suggest that when suphuric acid was utilized for hydrolysis, a significant removal of D-limonene was achieved with a high ethanol concentration. The density and turbidity measurements indicated that a good yield of ethanol was obtained. The results further confirms that citrus peel waste is a promising source to produce a good yield of bioethanol and pave ways to realize a large-scale bioethanol production.

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