Validation of Pre - Treatments for Cost Effective Production of Bioethanol From Floral Wastes

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Abstract

The present study has focused on validating pre- treatment methods for cost effective production of bioethanol from discarded and otherwise waste flowers which are renewable, abundantly available and eco-friendly. Floral waste was collected from various dumpsites and banquet halls and subjected to physical, chemical and biological pre- treatments. Biological pre- treatment by enzymatic hydrolysis using crude cellulase enzyme (5%) yielded 39.4 ± 0.03 g/L of alcohol which is 24.20% and 31.60% more than the alcohol obtained by physical pre- treatment (thermal hydrolysis) and chemical pre- treatment (1% KOH), therefore simultaneous saccharification and fermentation was optimised. A maximum of 396 ± 6.48 g/L bioethanol was obtained after 96 hrs of fermentation with the isolated yeast, *Pichia kudriavzevii* CY 902 at pH 5.5 and 37°C. The minimum ethanol selling price (MESP) of bioethanol produced in our study was enumerated to be 30.43 Rs/ L which is 68.31% lesser than the market price of ethanol in India today, making our methodology for production of bioethanol from mixed floral wastes very competitive and cost effective to the existing methodologies.

Introduction

Owing to the increscent world population and to their standards of living, there is an inflated demand for fuels. However, their supply is in contrast, as the sources for these fossil fuels are limited and nonrenewable. It is due to this disproportion that the fuel prices are mounting day by day. Unless met by an alternative, we will contribute to both depletion of fossil fuels and also to climate change through global warming. Fortunately, ethanol has met all the parameters to be used as fuel (Prasad et al. 2007). Moreover, it is renewable, cost effective and ecofriendly. Bioethanol is an alternative term used for ethanol indicating its origin from a biological source. Thus far, bioethanol was reportedly produced from various sources. The bioethanol obtained from sugar and starch containing crops are grouped under first generation (1G) bioethanol (1G) (Kaur Singh et al, 2021). Due to this, more than 20 LMT (Lakh metric tonne) of sugar from sugar factories had been diverted for ethanol production during sugar season 2020-21 in India, and diversion of about 50–60 LMT is projected by 2025 (Ministry of Consumer Affairs, Food & Public Distribution,2021). As a consequence this reflected upon the prices of sugar (Jayashree, 2021). Hence to avoid the conflict of food vs fuel, using non food and abundantly available lignocellulosic biomass of agricultural origin as 2G substrates were proposed (Adeolu and David, 2021). Such a biomass must be subjected to expensive and time consuming pre- treatments to break down the complex sugars to simple and fermentable sugars. These pre- treatments vary depending upon the types of sugars present in the biomass. Physical pre- treatments alter the physical barriers of the biomass by reducing the particle size thereby making it more vulnerable to succeeding saccharification methods. Chemical pre- treatment by using acids and alkali's attack the structural linkage between the lignocellulose and carbohydrates facilitating more access by enzymatic hydrolysis (Harinder et al. 2012). Simultaneous saccharification and fermentation (SSF) involves the process of enzymatic hydrolysis followed by fermentation within the same vessel thereby reduces the cost of production as well as minimising chances of contamination.

In the present study using mixed floral wastes as simple and abundantly available substrate for the production bioethanol was explored. India is a land of culture and celebrations. Flowers are an inevitable part of every small and large occasion due to their immediate and abundant availability. However the problem arises during their disposal. Our country is the world’s second largest producer of flowers (Nikhila and Ranjit, 2021), therefore making these one time use material an excellent non food, ecofriendly and abundantly available substrates for ethanol production. Converting this waste to energy would not only aid in solid waste management but also contribute to the economic growth of the country (Heera Lal, 2022) Although in the recent past floral waste have been utilised to make products such as incense sticks, biosurfactants; sugar syrup etc (Meghmala et al. 2009), production of bioethanol has been less emphasized on. Flowers are a good source of simple sugars (Fernandi et al. 2020). Hence they can be used as a promising substrate for ethanol production. According to our literature survey, previous studies reported using single species of flowers as biomass and *Saccharomyces cerevisiae* to ferment ethanol (Gethara Gowri et al. 2018; Omprakash. 2021; Phitchaphorn et al. 2021; Shreya and Omprakash, 2022; Sujit et al. 2009; Tripti et al. 2009) but using *Pichia kudriavzevii* to ferment mixed floral wastes was not been reported thus far. Hence the present study was conducted to validate the effect of various pre-treatment methods for production of bioethanol from mixed floral wastes using *Pichia kudriavzevii.* Fermentation economics were also calculated and compared to the existing market prices of ethanol to understand the feasibility of the methodology for cost effective production of bioethanol.

Materials and methods

Collection of substrate

Floral wastes (FW) were collected from various banquet halls, dump yards and household in and around Secunderabad area. The moisture in the FW was removed by drying them under direct sunlight at 42–45°C for 5–7 days. Dried FW was ground to coarse powder using an electric kitchen blender and stored.

Extraction

Solvent extraction of substrate which are of agricultural origin impacts the analysis of various components present in that substrate (Khamphet et al. 1997). Therefore, solvents such as ethanol, methanol and distilled water were employed to extract the components present in FW. 1gm of FW was suspended in 10ml of each of the solvents and kept on an orbital shaker at 120 rpm at room temperature (RT) for 48 hrs. Following extraction, the slurry was filtered. Components present in the floral extract (FE) were estimated. The ability of each of the solvent in extracting maximum reducing sugars from the FW was determined.

Analysis of components in FE

Components in FE were qualitatively analysed for flavanoids, saponins, tannins (Nisreen 2016),carbohydrates, phenols, alkaloids were checked (Prabhavathi et al. 2016). Subsequently they were also quantitatively analysed (Karthikeyan and Vidya 2019)

Pre- treatment

Pre- treatment helps in increasing the concentration of fermentable sugars from the biomass which aid in increasing the yield of ethanol. Accordingly, FW was subjected to three different types of pre-treatment methods such as physical, chemical and biological.

Physical pretreatment

a. Thermal hydrolysis: 1gm FW was suspended in 10ml distilled water and kept in a boiling water bath

(100°C) for varying time period of 10–30min (Mai et al. 2009).

b. Microwave treatment: 1gm FW was suspended with 10ml distilled water (1:10), was exposed to dry heat in a

microwave of 100PW (Power watts) for 5-30min (Ooshima and Harano 1984)

c. Autoclaving: 1gm FW was suspended with 10ml distilled water (1:10) was autoclaved at 121°C, 15lbs pressure for 21 min (Marvin et al.2020).

Chemical pretreatment

The FW was subjected to chemical pretreatment using acid and alkali.

a. Acidic hydrolysis: The FW (1gm) was suspended in 10ml of various diluted acids (1%) such as Sulphuric acid

(H2SO4), Hydrochloric acid (HCl) and Nitric acid (HNO3) (Juan et al. 2019)and kept on an orbital shaker at 120rpm overnight.

b. Alkaline hydrolysis: The FW (1gm) was suspended in 10 ml of various dilute alkali solutions (1%) such as Sodium hydroxide(NaOH), Potassium hydroxide (KOH) and Calcium hydroxide Ca(OH)2 (Praveen et al. 2009) and kept on an orbital shaker at 120rpm overnight.

Biological pre-treatment: Pre-treatments using extracellular enzymes produced by fungi are highly efficient in degrading complex biopolymers to simple and fermentable form (Hem Kanta et al.2019) It is known that biomass of agricultural origin contain cellulose. This cellulose has to be converted to simple and fermentable monosaccharides to increase the yield of ethanol. Hence cellulase producing fungi were isolated from rotten wood over PDA (Potato dextrose agar) (Reddy et al.2014) Antibiotic streptomycin (1mg) was added as bactericidal agent. The isolates were then screened for their ability to produce cellulase enzyme on CMC agar plates suspended with Congo red indicator (Uttam Kumar et al,2014). Fungal colonies showing clear zones of hydrolysis around them were recognised as cellulase producers. Measuring these zones of hydrolysis indicates fungi with highest cellulase enzyme activity. The fungi thus isolated was identified as *Apergillus niger*, was suspended in 250ml Czapekdox peptone broth suspended with cellulose (Amaeze et al. 2015)and incubated for 7–10 days until the mycelia was fully grown, at RT and pH 5. The media was centrifuged at 10,000 rpm for 5mins. The supernatant contained crude cellulase enzyme. Enzyme activity was evaluated (Ghose, 1987) and its effectivity on the biomass was studied by suspending it into FW (1:10) at 5% and 10% (w/v) concentrations, incubated on an orbital shaker at 120rpm overnight.

The FW was directly subjected to pre- treatment without extraction. The control flask maintained for each of the pre- treatment contained distilled water added to the FW (1:10) and kept on an orbital shaker at 120 rpm as long as the pre- treatment was carried out, from few minutes to overnight. After pre-treatment the FW slurry both in pre- treated and control flasks were filtered using whatmann filter paper. The concentration of reducing sugars in the filtrate (FE) was estimated and compared for both controls and pre- treated samples to draw conclusions on effectiveness of each pre-treatment.

Isolation of microorganisms

A total of 3 yeast strains were isolated from sugarcane juice (SJ) and grape skin (GS) and labelled accordingly as SJ 4 SJ 5 and GS 5 were used for fermenting the FW. Strain SJ 4 showed a maximum fermentation efficiency (96%) as compared to the strain isolated from grape skin (GS- 5) (82%). Fermentation efficiency was calculated using the formula

Fermentation efficiency (%) = Practical yield / Theoretical yield x 100

Hence a pure culture of this strain was established and maintained over YPD (Fig. 2A). Microscopic examination using methylene blue staining showed long elongated oval shaped budding yeasts (Fig. 2B). The strain was identified as *Pichia kudriavzevii* CY 902, by 16s rRNA method (Shuo-Fu et al. 2017; Kolawole et al.2020). The cultural conditions for optimal growth of yeast was identified to be 37°C, pH 5.5 and 48 hrs of incubation.

Optimization of pre- treatments

For optimization, the FW was first subjected to extraction with distilled water for 48hrs follows by pre - treatment methods showing highest concentration of reducing sugars in each of the physical, chemical and biological pre- Subsequently fermentation for 96 hrs using 10% inoculum of *Pichia kudriavzevii* CY 902 (V/V) The concentration of ethanol fermented was estimated using potassium di chromate method. The pre- treatment yielding the highest amount of ethanol was optimised. The process was subsequently scaled up to obtain the product bioethanol.

Scale up and downstream processing

The production volume was scaled up from 10 ml to 250 ml (25gms FW + 250ml distilled water) with extraction and enzymatic hydrolysis with 5% (v/v) crude cellulase enzyme followed by SSF for 96 hrs at RT and pH 5.5. Bioethanol obtained was separated by simple distillation at 80 °C, followed by dehydration by salting out using salts like KH_2PO_4, K_2CO_3 and rock salt at the rate of 0.3g/ml (Irene and Asker 2020; Shaoqu et al. 2017).

Evaluation of the purity of the product

The purity of bioethanol produced was evaluated by Gas Chromatography and Mass spectrometry (GC-MS) studies were performed at Centre for Microbial fermentation and technology, Department of microbiology, Osmania University using GC-MS QP2020 NX, Shimatzu, Japan equipped with a SH- Rsi-5 Sil MS (30x 0.25 x 0.25 mm) coloumn. Similarly, the fuel properties were evaluated according to the ASTM (American standards of testing materials) and IS (Indian standards) at Zeal Biologicals. Pvt. Ltd, Maredpally, Secunderabad. These properties were compared to commercially available anhydrous ethanol revealing its purity and performance as fuel.

Techno- economical evaluation

Although the production of bioethanol is now well known and explored, making the production cost effective is challenging. The expenditure for production bioethanol for our study was calculated in three categories namely, the cost of chemicals (C_{Cm}), the cost of equipments (C_{Eq}) and the cost of operation (C_{Op}) (Johann et al. 2011; Sonica et al. 2020)

The cost of chemicals (C_{Cm})

This cost involves expenditure on Media, Reagents and Chemicals. All chemicals were procured form Hi-media or Sigma Aldrich (Merck). The expense are calculated to interms of obtaining 1L bioethanol. For our study, the media used were YPD broth for *Pichia kudriavzevii* CY 902, Potato dextrose agar (PDA) and Cazepkdox broth for isolation and production of crude cellulase enzyme from *Aspergillus niger* respectively. Besides reagents such as DNS and Potassium di chromate were used for estimations of reducing sugar and alcohol respectively to observe the progress of fermentation. The salt potassium carbonate was added to dehydrate the distilled bioethanol obtained. The prices of each of these chemicals used were taken according to the latest price list (2022–2023) as declared by the respective brand of chemicals. Thus the cost of chemicals were calculated.

The cost of equipments (C_{Eq})

This cost includes the price of the equipments used at various stages for production of bioethanol. Such as Incubator, Autoclave, Centrifuge, Distillation unit and Orbital shaker. The price of these instruments were obtained from their respective manufacturers. The cost calculated also includes the life time of the equipment and the number of days these equipments were involved in the study for the production of maximum amount of bioethanol (D_{max}). For, example, orbital shaker was used for 48 hrs for extraction of the floral waste. Accordingly, number of cycles taken for producing the maximum yield of bioethanol is calculated annually viz; 365/ 2 = 182.5. This usage is compared against the life time of the equipment which interprets the longitivtiy of using the instrument. Therefore, the cost of equipments were calculated using the formula:

Cost Equipment (C_{Eq}) =. Price of equipment

365/ D_{max} X Life time X Capacity

The cost of operation (C_o)

The cost under operations comprise the expenses due to electricity consumed by the equipments used. The cost for electricity for each of the equipment is taken from the tariff released by the Electricity board of Telangana state (TSSPDCL) for the Financial year 2023–2024. Accordingly, the cost were calculated using the formula:

E (hrs)

Capacity

Total cost

The total cost was enumerated by substituting the values of each of the expense in the formula

Total cost for bioethanol production (Rs/L) = C_{Cm}+ C_{Eq}+ C_O

Production cost per unit (Rs/g/L) = C_{Cm}+ C_{Eq}+ C_O

Maximum bioethanol obtained (g/L)

Analytical methods

The effect of pre-treatment on the FW was analysed by estimating the amount of reducing sugars by 2,5 Dinitro salicylic acid (DNS) method (Miller 1959) Glucose (1mg/ml) was used as standard. Concentration of reducing sugar was determined using a colorimeter by noting the absorbance at 540nm against blank. Similarly, the concentration of alcohol was determined by potassium dichromate method (Williams and Reese 1950) using ethanol as standard (1mg/ml). Concentration of alcohol was determined using a colorimeter by noting the absorbance at 600nm against blank.

Data analysis

The experiments were conducted in triplicates. All data are expressed as mean standard error (±). Statistical analysis was performed using Origin Pro 2023b software. Single factor ANOVA analysis tool was used for comparing the means of more than two groups together. Results with p value < 0.05 were considered significant.

Results

The substrate

The floral wastes collected contained mixed flowers of *Transvaal daisy*, *Rosa Rubiginosa*, Chrysanthemum, *Dianthus caryophyllus*, *Tagetes*, *Jasminum multiform, Crossandra infundibuliformis, Artemisia pallens*. The floral wastes weighed about 5.2 kgs. After sun drying for 5–7 days the moisture was completely removed. The dry floral waste was blended to a coarse powder using electrical blender and weighed to be 3.9 kgs. The FW powder was transferred to and maintained in zip lock bags until use.

Extraction

Components in the FW powder was extracted using various solvents (1:10). Since sugars are converted to alcohol by fermentation, the filtrate (FE) obtained after filtering the FW, was analysed for reducing sugar concentration. The concentration of sugars in the FE extracted using distilled water was (34.37 ± 0.03 g/L) which is 25.41% and 43.96% more amount of sugar than in the FE extracted by methanol (25.41 ± 7.5g/L), and ethanol (19.26 ± 1.5g/L) after 48 hrs of extraction (Fig. 1).

Analysis of components in the FE

As revealed in (Table 1) the components present in FE obtained from 1gm of FW were tannins (3.2mg/ml) and phenols (3.6mg/ml). While flavonoids (0.17mg/ml) and proteins (0.25mg/ml) were present in negligible concentrations, saponins were absent. The concentration of total carbohydrates were (34.37 ± 0.03 g/L). Subsequent qualitative analysis using Molish's test, Fehling's test revealed the presence of simple sugars such as glucose, fructose, disaccharide (sucrose). The concentration of polysaccharide cellulose was estimated using anthrone reagent (Updegraff 1969) and was found to be 2.14 ± 0.002 mg/ml, which is 6.23% of the total sugar content found in the FE. Converting this concentration of polysaccharide to simple and fermentable sugar monomers would add to the amount of bioethanol yield.

Table 1
Components present in FE after extraction

Component	Concentration (mg/ml/g)
Tannins	3.2 ± 0.005
Phenols	3.6 ± 0.24
Saponins	-
Flavinoids	0.17 *
Proteins	0.25*
Carbohydrates	34.37 ± 0.03
Cellulose	2.14 ± 0.002
Values depicted are mean ± SD for n−3	
(*variance is less than 0.001)	

Table 2
Comparison in the concentration of reducing sugars in control and pre- treated FW.

S.No.	Pre- treatment	Condition	Concentration of reducing sugar in control	Concentration of reducing sugar after pre-treatment	p - value
1.	Thermal hydrolysis	1gm FW + 10ml water in boiling water bath for 30 mins	9.5 ± 0.6	7.05 ± 0.02	< 3.12 E−09
2	Acidic	1gm FW + 10ml (1%Sulphuric acid) + overnight incubation	13.8 ± 3.64	5.2 ± 0.10	< 2.32E−06
3.	Alkaline	1gm FW + 10ml (1%KOH + overnight incubation	13.8 ± 3.64	12.8 ± 1.62	< 2.1E−04
4.	Enzymatic hydrolysis	1gm FW + 5%crude enzyme + + overnight incubation	11.7 ± 0.12	16.04 ± 0.21	0.04
Values depicted are mean ± SD for n−3					

Table 3
Cost validation for production of bioethanol (Rs/L)

S. No.	Cost of	Cost calculated (Rs/L)
1	Cost of operation (C_o)	0.0045
2	Cost of equipments (C_{Eq})	2.33
3	Cost of chemicals (C_{Cm}	15.76
4	Total Cost (A)	18.09
5	Maximum amount of bioethanol produced (g/L) (B)	396
6	Cost/Unit (Rs/g/L) (A) / (B)	0.045
Depicting the enumeration of total cost involved in the study and the cost per unit		

Table 4
Determination of minimum ethanol selling price (MESP) of bioethanol produced in the study

S. No.	Cost of	Cost calculated (Rs/L)
1	Labour @ 5%	0.90
2	Maintainance @ 3%	0.54
3	Rentals @ 5%	0.90
4	Transportation @ 2000 Rs/ Ton of biomass	10 (for 5 Kg)
5	Production cost (Table 2)	396
6	Minimum ethanol selling price (MESP)	30.43
Depicting the production cost including the charges applicable at industrial scale.		

Pre- treatment

The study has also focussed on reducing the cost of production by validating various pre- treatment methods so as to yield high amounts of bioethanol with minimal pre- treatment.

Physical pre- treatment

Physical pre- treatment by thermal hydrolysis for 30min at 100°C showed a maximum of 7.05 ± 0.04 g/L reducing sugars from the FW when compared to same treatment for lesser time. Autoclaving could yield 5.5 ± 1.5 g/L of sugars. Exposure of FW to 100 watt hour power (189.56 Celsius heat units) [Kyle's converter] in a microwave for 5 mins could yield maximum amount of 5.05 ± 0.04g/L reducing sugars. However it was observed that control showed a maximum of 9.5 ± 0.6 g/L/ of reducing sugars which was 27.22%, 42.10% and 46.84% more than the optimum amount of reducing obtained by thermal hydrolysis, autoclaving and microwave treatment respectively **(**Fig. 3**).**

Chemical Pre- treatment

The FW when treated overnight with dilute acids (1%) of H2SO4, HNO3 and HCl, the concentration of reducing sugars were estimated to be 5.2 ± 0.10 g/L, 4.1 ± 0.08 g/L and 3.6 ± 0.02 g/L respectively. Similarly, when the FW was treated with dilute alkaline solutions (1%) of KOH, NaOH and Ca(OH)2, the concentration of reducing sugars were estimated to be 12.8 ± 1.62 g/L, 8.8 ± 1.28g/L and 4.3 ± 0.24g/L respectively. The control flask showed a maximum sugar concentration of 13.8 ± 3.64 g/L **(Fig. 4).** Pre- treatment with 1% sulphuric acid and 1% KOH could hydrolyse highest amount of sugar for acidic treatment and alkaline treatment respectively. It is likely that alkali being more milder than dilute acid retained better concentration of reducing sugars which could have been degraded by acidic conditions. Yet they were 65.15% and 8.69% lesser than the sugar concentration in the control.

Enzymatic hydrolysis

15 Cellulase producing fungi were isolated from rotten wood. Efficiency of the cellulase enzyme produced by each of these fungi was identified by measuring the zones of hydrolysis on CMC agar plates. The fungal strain (CP- 6) showed the highest cellulase activity with a zone diameter of 52mm. It was identified microscopically as *Aspergillus niger* by staining with lactophenol cotton blue. Pure culture was maintained on PDA agar plates and slants **(**Fig. 5**)**. Enzyme activity of crude cellulase enzyme was found to be 1.82 IU/ml/min and 0.92 IU/ml CMCase and FPase respectively. The enzyme was added in 5% and 10% (v/v) concentrations to the FW slurry. An overnight enzymatic hydrolysis at RT and 120rpm showed that 5% enzyme could hydrolyse 22.25% more sugars (16.04 ± 0.21 g/L) than 10% enzyme (12.4 ± g/L) and 27.05% more than control (11.7 ± 0.12 g/L). Therefore enzymatic hydrolysis was optimized to using 5% (V/V) crude cellulase enzyme.

A summary of the effect of all the pre- treatments, physical (thermal hydrolysis) chemical (acidic, alkaline), biological pre – treatment (enzymatic hydrolysis) and the maximum amount of reducing sugars obtained from each of them in comparison to the control along with the p- value is described in **Table.2**.

Optimization of pre- treatments

It is notable from **(**Fig. 6**)**, that enzymatic hydrolysis could produce 39.56 ± 0.08 g/L alcohol which was 20.22%, 24.01% and 19.79% more amount of alcohol as compared to, pre- treatment by thermal hydrolysis (31.56 ± 1.2 g/L) 1% KOH (30.06 ± 0.02 g/L) and control (31.73 ± 0.01g/L) respectively. It can thereby be concluded that, the FW produced maximum alcohol after extraction with water (1:10) followed by simultaneous saccharification and fermentation with 5% (v/ v) crude cellulase and 10% (V/V) *Pichia kudriavzevii* CY 902 for 96 hrs at RT and pH5.5.

Scale up for fermentation

For optimisation of pre- treatments, only 1g of floral waste powder was used in 1:10 dilution. Once the pre- treatment had been optimised, the study was scaled up to 250ml (25gms floral waste 250 ml distilled water) in batches to acquire maximum amount of the end product, ethanol. Sample was withdrawn every 24 hrs and analysed for the concentrations of both sugars and alcohol. Initial sugar concentration 1068g/L/Kg yielded a maximum of 396 ± 6.48 g/L bioethanol after 96 hrs of fermentation, the maximum fermentation efficiency recorded was 96%.

Evaluating the purity of bioethanol obtained

The initial concentration of bioethanol after fermentation was 396 ± 6.48 g/L increased to 852.8 g/L after simple distillation, equivalent to concentration of 70% ethanol. For our study, potassium carbonate (0.3g/ml) showed instant dehydration yielding anhydrous ethanol separated into and organic phase in less than 5mins. The bioethanol thus obtained was collected by carefully transferring it into separating funnel. Its concentration was equivalent to absolute ethanol indicating its purity. The bioethanol obtained was checked for its purity by comparing fuel properties with that of commercially available absolute ethanol is discussed in our previous paper (Shailaja and Shalini 2024) Additionally GC- MS analysis was done which revealed the purity of the bioethanol indicated by peak formed with retention time 1.47. Besides two more peaks of hydrazine and propanol were identified with retention time 1.40 and 1.51 respectively **(**Fig. 7**).**

Cost analysis

Enumerating the total cost for production of bioethanol in our study was found to be 18.09 Rs/ L and the cost/unit (ml) was 0.045 Rs/g/L **(Table.3)**. Breaking up the expense showed that about 87.11% of this cost was towards procuring chemicals, 12.88% for equipments and 0.002% was the operational cost. Escalating this methodology of bioethanol production to industrial scale involves additional costs such as labour, rentals, maintainance and transportation. Including these costs to the production cost, we arrive at minimum ethanol selling price (MESP) which was calculated to be 30.43 Rs/L **(Table.4).**

Discussion

As production of ethanol is gaining importance, unlike employing conventional food crops like sugarcane and corn, using floral waste would help in reducing land and water pollution and in turn add to the yield of bioethanol, thereby significantly reducing the mounting prices of both fuels and food crops. The aim of this study was to effectively use the abundantly available floral waste to produce bioethanol.

Using distilled water for extraction could extract 25.41% and 43.96% more amount of reducing sugars than using methanol and ethanol respectively. Similar results on water extraction from *Clitoria ternatea* flowers was reported (Ethel et al. 2021). This is the first step for cost effective production of bioethanol since cost of distilled water is minimal as compared to the cost of ethanol and methanol.

The effect of heating on the biomass varies depending upon the kind of carbohydrates present in them (Abraham et al.2019). Therefore, exposing the FW to increasingly high temperatures from 100°C, 121°C and 189.56°C during thermal hydrolysis, autoclaving and microwave treatment respectively denatured the simple monomers, while the complex polysaccharide were broken down to simple sugars. Hence the control which retained both and showed higher concentration of sugars than the pre- treated FW.

Pre- treatment of lignocellulosic biomass with dilute acids results in weakening the hemicellulose and thereby making it more available for degradation during enzymatic hydrolysis (Chen-Guang et al. 2019; Niju et al 2020). However the FE obtained after extraction with water when analysed qualitatively for the presence of hemicellulose (Esther 1930) did not show any detectable change in colour indicating the absence or negligible presence of hemicellulose. The concentration of hemicellulose is less as compared to cellulose concentration in flowers (Buxton and Hornstein. 1986). Hence when the FW was subjected to chemical pre-treatment targeted towards hemicellulose, there was no significant increase in the concentration of reducing sugars. Furthermore, treating the biomass with either acid and alkali require subsequent neutralisation to remove any inhibitors formed due to pre- treatment (Haghighi et al. 2013) SSF using crude cellulase enzyme obtained from *Aspergillus niger* isolated from rotten wood and fermentation by *Pichia kudriavzevii* CY 902 produced 396 ± 6.48 g/L after 96hrs of fermentation.. This amount of ethanol with minimal pre- treatment in the present study were better than the results reported on ethanol production from flowers by previous authors (Gethara Gowri et al. 2018; Omprakash. 2021; Phitchaphorn et al. 2021; Shreya and Omprakash, 2022; Sujit et al. 2009; Tripti et al. 2009; Ravi et al.2016]. Moreover there was no further additional components added to the FE to enhance ethanol production during fermentation. This also contributes towards production of cost effective ethanol. Considering the starter culture, while most of the previous studies used *Saccharmyces cerevisiae*, we used *Pichia kudriavzevii* CY 902 . The bioethanol yield surpassed the amount of ethanol produced from *Madhuca latifolia* using the same culture (Tripti et al. 2009*).* Although simple distillation aided in increasing the concentration of bioethanol, anhydrous bioethanol was obtained by dehydration using salting out wherein hydrogen bonds between ethanol and water were disrupted in the presence of salt potassium carbonate

(Shakhashiri, 1983) This method of purification also adds to cost effective production compared to other sophisticated extractive distillation (Elhan and Kimberley 2017; Yuelei et al. 2012; Vicente 2007). The purity of ethanol thus obtained was checked by gas chromatography which revealed 3 peaks corresponding to Hydrazine, propanol and ethanol. Hydrazine is a common ingredient present in chemicals and fertilizers used for agricultural crops (Ivan and Vincent. 2023). They can also be carried from flower to flower by insects or bees (Shrabani Saha et al. 2021) Propanol is a common by product formed during ethanol fermentation (Timothy et al. 2021). Hence these two components are expected to be identified during bioethanol fermentation using floral wastes.

The cost for production of bioethanol is mainly dependent on the biomass utilised. Bioethanol produced from the first generation biomass was priced between 49.41–65.61 Rs/L, as declared by the Ministry of Petroleum and Natural gas, Government of India during financial year 2021–2022. A majority of this price was invested to procure the biomass itself (Simon et al. 2013). In India the cost for procuring biomass is priced at 3532 and 2225 Rs/ Tonne for wheat / rice straw and sugarcane baggage respectively (Obiora 2022). Using the waste flowers as substrate is no cost biomass. Water is used as solvent for extraction of maximum components from the biomass which is again a negligible expense as compared to using other solvents for extraction. The pre-treatment method was restricted to enzymatic hydrolysis using crude cellulase enzyme which was produced from *Aspergillus niger* isolated from rotten wood. This reduced the cost as compared to using ready made cellulase enzyme priced at 2965 Rs (Himedia price list 2023–2024).

The cost for production of bioethanol from kitchen waste 0.62 Rs/ G (Sonica et al. 2020). However they did not claim to purify the bioethanol obtained. The cost for production of 396 ± 6.48 g/L bioethanol in our study including dehydration was 0.045 Rs/G. Which means more amount of bioethanol could be produced from floral wastes for lesser cost in comparison. Ethanol produced in Brazil, USA and China using sugarcane is priced between 0.30–0.53 USD/L (Gunter et al. 2013; Lili et al. 2015; Mustafa and Havva 2009). In India, ethanol predominantly produced using sugarcane/sugar syrup is priced at 65.61 Rs/L [Ministry of Petroleum and Natural gas, Government of India, Financial year 2021–2022]. The minimum selling price of bioethanol produced in our study was 30.43 Rs/L which is cheaper than the cost of all the previously reported studies and commercial cost of ethanol both at national and international markets. Therefore proving the feasibility of using mixed floral wastes for cost effective production of bioethanol as well as contributing to solid waste management.

Conclusion

This is probably the first study reported on using mixed floral wastes for the production of bioethanol using *Pichia kudriavzevii* CY 902. The study demonstrated the efficacy of using distilled water for extraction. Since physical and chemical pre- treatment methods were not effective in increasing the concentration of reducing sugars in the biomass as compared to the untreated control, they were excluded in the methodology. Simultaneous saccharification and fermentation using 5% crude cellulase enzyme obtained from *Aspergillus niger* and *Pichia kudriavzevii* CY 902 yielded a maximum of 396 ± 6.48 g/L bioethanol after 96hrs of fermentation. It is notable that both saccharification and fermentation were carried out at 37 °C and no additional components were added to enhance the yield of ethanol. Thereby making the process involve minimal cost, enumerated to be 30.43 Rs/ L as the MESP which is 68.31% less than the market price of bioethanol (65.61 Rs/ L) in India. Thus using floral wastes for bioethanol production contributes to achieve the vision of the country in using 20% ethanol blended fuel (E20) by 2025, faster with cheaper investment. The study also caters to producing bioethanol, as a value added product from floral wastes, as well as contributing to solid waste management.

Declarations

Conflict of Interest

The authors hereby declare that they have no conflicts of interest.

Ethical approval

Not applicable.

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Author Contribution

The present research work was done under the guidance of Dr. M. Shailaja Raj (Research guide). The entire process of pre- treatments, validation and cost analysis studies was done by research scholar, Shalini Rachel. Maria Shajan contributed in culturing and maintaining the cultures involved in the study.

Data Availability

All the required links or identifiers of the data are available in the manuscript as described.

References

1. Abraham Kusi Obeng,Duangporn Premjet and Siripong Premjet (2019) Combining Autoclaving with Mild Alkaline Solution as a Pretreatment Technique to Enhance Glucose Recovery from the Invasive Weed Chloris barbata, Biomolecules. 9(4):120. 10.3390/biom9040120
2. Adeolu A, Awoyale, David Lokhat (2021) Experimental determination of the effects of pretreatment on selected Nigerian lignocellulosic biomass in bioethanol production. Sci Rep 11:557. https://doi.org/10.1038/s41598-020-78105-8
3. Amaeze NJ, Okoliegbe IN, Francis ME (2015) Cellulase production by Aspergillus niger and Saccharomyces cerevisiae using fruit wastes as substrates. Int J Appl Microbiol Biotechnol Res IJAMBR 3:36–44 ISSN 2053 – 1818
4. Liu C-G, Li K, Wen Y, Geng B-Y, Liu Q (2019) Yen-HanLinDiluteacidpretreatmentisthemostcommonandefficientmethodforlignocellulosicfeedstockstodissolvethehemicelluloseofbiomass,ChapterOne-Bioethanol:Newopportunitiesforanancientproduct,https://doi.org/10.1016/bs.aibe.2018.12.002
5. Buxton DR, Hornstein JS (1986) Cell-Wall Concentration and Components in Stratified Canopies of Alfalfa, Birdsfoot Trefoil, and Red Clover. Crop Sci 26(1):180. 10.2135/cropsci1986.0011183x002600010043x
6. Lim EJJYY, Wee Sim C (2021) EffectofOrganicSolventsandWaterExtractiononthePhytochemicalProfileandAntioxidantActivityofClitoriaternateaFlowers,ACSFoodScienceTechnology,1,9,1577,September,2021,https://doi.org/10.1021/acsfoodscitech.1c00168
7. Esther Martha Mischelle,((1930) AMicrochemicalstudiesofhemicellulosesofendospermandcotyledons,Americanjournalofbotany,February,Volume17,No.2pages117–138,10.2307/2435649
8. Elhan E, Kimberly LO (2017) Simulation and Cost Analysis of Distillation and Purification Step in Production of Anhydrous Ethanol from Sweet Sorghum. ACS Sustain Chem Eng 5(8):6854–6862. 10.1021/acssuschemeng.7b01082
9. Fernando Carlos Gómez-Merino Maribel Ramírez-Martínez, Ana María Castillo-González, Libia Iris Trejo-Téllez, (2020). Lanthanum Prolongs Vase Life of Cut Tulip Flowers by Increasing Water Consumption and Concentrations of Sugars, Proteins and Chlorophylls. Sci Rep March 10(1),10.1038/s41598-020-61200-1
10. Gethara GRY, Vijayalakshmi S (2018) Production and Optimization Techniques of Bioethanol from Withered Flowers of Allamanda schottii L. by Activated Dry Yeast. J Pure Appl Microbiol June 12(2):943–952
11. Ghose TK (1987) Measurement of cellulase activities, Pure and applied chemistry. 59:257–268. https://doi.org/10.1351/pac198759020257
12. Gunter Festel M, Bellof M (2013) ModellingProductionCostScenariosforBiofuelsandFossilFuelsinEurope,DiscussionpaperNo.13–075.Mannheim,Germany:ZEW–CentreforEuropeanEconomicResearch;.Availablefrom:http://ftp.zew.de/pub/zew-docs/dp/dp13075.pdf
13. Haghighi Mood S, Hossein Golfeshan A, Tabatabaei M, Salehi Jouzani G, H NG,Gholami.M,andArdjmand.M (2013) Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renew Sustainable Energy Reviews Volume 27:77–93. 10.1016/j.rser.2013.06.033
14. Oberoi HS, Babbar N, Sandhu SK, Dhaiwal SS, Kaur U, Chadha BS (2012) Ethanol production from alkali- treated rice straw via simultaneous saccharification and fermentation using newly isolated thermotolerant Pichia kudriavzevii HOP-1, Bioenergy Biofuels Biochemicals. J Industraial Microbiol Biotechnol 39:557–566. 10.1007/s10295-011-1060-2
15. Heera Lal Atal (2022) Sustainable Management of Floral Waste to Produce Bioenergy and Valuable Products. J Indian Association Environ Manage Volume 42(2):26–34
16. Sharma HK, Xu C, Wensheng Qin (2019) &BiologicalPretreatmentofLignocellulosicBiomassforBiofuelsandBioproducts:AnOverview,WasteandBiomassValorization,28August2017,Volume10,pages25.https://doi.org/10.1007/s12649-017-0059-y
17. Irene Finkeldej, Asher Newsome G (2020) Saltingout,Asimpleandreliablemethodtodistinguishbetweencommonfluidpreservativesandestimatealcoholconcentration,CollectionForum2020;34(1):11–31,SocietyforthePreservationofNaturalHistoryCollections,https://doi.org/10.14351/0831-4985-34.1.11
18. Ivan I, Vincent R (2023) HydrazineToxicology,NationallibraryofMedicine,NationalcentreforBiotechnologyinformation,StatpearklspublishingsBookshelfID:NBK592403
19. Jayashree Bhosale (2021) Domestic sugar price increase slows down pace of India's sugar export deals, Economic times bureau. Last Updated: Sep 09:09:56AM
20. Johann F (2011) Cost analysis in laccase production. J Environ Manage 92(2907e2912). 10.1016/j.jenvman.2011.06.052
21. ,Juan Carilo Solarte-Toro, Romero-García JM, Martínez-Patiño JC (2019) J.C,EncarnacionRuiz-Ramos,EulogioCastro-Galiano,&CarlosArielCardona-Alzate, Acidpretreatmentoflignocellulosicbiomassforenergyvectorsproduction:Areviewfocusedonoperationalconditionsandtechno-economicassessmentforbioethanolproduction.RenewableandSustainableEnergyReviews.10.1016/j.rser. 2019.02.024
22. Nehra KS, Jangra MR, Sharma P, Aggarwal M, Mishra P, Bharti R, Sachdeva H, Poonia P, Sumit Jangra (2021) Production of Bioethanol from Sugarcane Juice, Molasses and Paddy Straw using *Saccharomyces cerevisiae*. Bioscience Biotechnol Res Commun 14(2). http://dx.doi.org/10.21786/bbrc/14.2.22
23. Karthikeyan.G AK (2019) Phytochemical analysis, antioxidant and antibacterial activity of pomegranate peel, Research journal of life sciences, bioinformatics, pharmaceutical and chemical sciences, Jan- Feb. 5. 10.26479/2019.0501.22
24. KhamphetThammasouk D, Penner MH (1997) Influence of Extractives on the Analysis of Herbaceous Biomass. J Agric Food Chem Febr 45:2,437–443. https://doi.org/10.1021/jf960401r
25. Kolawole Banwo K, Temitayo OF, Ayoinka O (2020) *Potentials of Lactobacillus plantarum and Pichia kudriavzevii in Co-Fermentation of Sourdough from*. Millet Int J Food Sci Technol. 10.1111/ijfs.14729

26. LiliZhao X, Wu M (2015) Techno-Economic Analysis of Bioethanol Production from Lignocellulosic Biomass in China: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover. Energies 8:4096–4117. 10.3390/en8054096
27. Mai ØstergaardPetersen J *Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals*,Biomassandbioenergy33834–840.https://doi.org/10.1016/j.biombioe.2009.01.004
28. MarvinScherzinger T (2020) Autoclave pre-treatment of green wastes – Effects of temperature, residence time and rotation speed on fuel properties, Fuel. 273 1 August, 117796. https://doi.org/10.1016/j.fuel.2020.117796
29. MeghmalaWaghmode A, Neha Patil (2009) ManagementofFloralWastebyConversiontoValue-AddedProductsandTheirOtherApplications,Springerresearchgate,January2018,WasteandBiomassValorization9(1):33–43,10.1007/s12649-016-9763-2,https://doi.org/10.1007/s12649-016-9763-2
30. Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem 31(3):426–428. https://doi.org/10.1021/ac60147a030
31. MustafaBalat H (2009) Recenttrendsinglobalproductionandutilizationofbio-ethanol,Applied Energy,Volume86,Issue11,November,Pages2273–2282.https://doi.org/10.1016/j.apenergy.2009.03.015
32. Niju SM (2020) Chap. 10-Pretreatmentoflignocellulosicsugarcaneleavesandtopsforbioethanolproduction,Lignocellulosicbiomasstoliquidbiofuel,pg301–304,https://doi.org/10.1016/B978-0-12-815936-1.00010-1
33. Nikhila Vaagdevi Anumala & Ranjit Kumar (2021) FloriculturesectorinIndia:currentstatusandexportpotential,TheJournalofHorticulturalScienceandBiotechnology,Volume96,-Issue5Pages673–680|March2021.https://doi.org/10.1080/14620316.2021.1902863q
34. NisreenHusain AK (2016) Phytochemical Analysis of Flower, Leaf and Root of Achyranthes Aspera from Durg District of Chhattisgarh-A Comparative Study, International Journal of Science and Research (IJSR), ISSN online:2319–7064. Int J Sci Res (IJSR), 5,Issue 3, March,2016.
35. Obiora Chiemerie Martins (2022)), Optimal Cost Of Production Of Bioethanol: A Review, Research Gate. SSRN Electronic Journal, August
36. Omprakash Sahu (2021) Appropriateness of rose (Rosa hybrida) for bioethanol conversion with enzymatic hydrolysis: Sustainable development on green fuel production, Energy, 232, 1 October. 120922. 120922.https://doi.org/10.1016/j.energy.2021.120922
37. Ooshima H, Aso K, Harano Y (1984) Microwave treatment of cellulosic materials for their enzymatic hydrolysis, Biotechnology Letters. 6:289–294. https://doi.org/10.1007/BF00129056
38. PhitchaphornKhammee Y, RameshprabuRamaraj (2021) Appropriatenessofwastejasmineflowerforbioethanolconversionwithenzymatichydrolysis:sustainabledevelopmentongreenfuelproduction,Springer,3Biotec021-02776-x
39. Praveen Kumar DMB,MichaelJ.Delwiche,&, PieterStroeve (2009) Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Industrial Eng Chem Research(48(8):3713–3729. 10.1021/ie801542g
40. AnoopSingh 1 PS (2007) Ethanolasanalternativefuelfromagricultural,industrialandurbanresidues,Reviewarticle,Elsevier,Resources,ConservationandRecycling,Volume50,Issue1,Mar39,https://doi.org/10.1016/j.resconrec.2006.05.007
41. Prabhavathi RM, Prasad MP, Jayaramu M (2016) Studies on Qualitative and Quantitative Phytochemical Analysis of Cissus quadrangularis. Adv Appl Sci Res 7(4):11–17
42. Ravi Gedela RT, Naidu A (2016) *Madhuca longifolia* flowers for high yields of bio-ethanol feedstock production. Int J Appl Sci Biotechnol 4(4):525–528. 10.3126/ijasbt.v4i4.16272
43. Reddy PLN, Radhaiah A, Sreeramulu A (2014) Screening,identificationandisolationofcellulolyticfungifromsoilsofChittoordistrict,India, InternationalJournalofCurrentMicrobiologyandAppliedSciencesVol.3No.7pp.761–771ref.43
44. Xie S, Song W,ConghuaYi,&, XuequingQiu (2017) Salting-out extraction systems of ethanol and water induced by high-solubility inorganic electrolytes. J Ind Eng Chem 56:145–150. 10.1016/j.jiec2017.07.006
45. Shailaja Raj M, Shalini Rachel N (2024) Evaluation of bioethanol production from Floral wastes, Bioinfolet, Indian Journals, (MS 23/ 6739) 21, N o.1,Pages70–73,January- March.
46. Shakhashiri BZ (1983) ChemicaIdemonstrations,UniversityofWisconsinpress,MaldisonW.I,Volume3,pp266
47. Saha S, Das S, Sarkar O Ansuman Chattopadhyay,Kari RissanenandPrithidipa Sahoo,*Introduction of a luminescent sensor for tracking trace levels of hydrazine in insect pollinated cropland flowers*, NewJournalofChemistry
48. ShreyaDoda O (2022) Production of bioethanol from biomass (Marigold flower), Science direct journals and books, 48, Part 5. 932–937. https://doi.org/10.1016/j.matpr.2021.05.309
49. Yuan S-F, Guo G-L, Hwang W-S (2017) Ethanol production from dilute-acid steam exploded lignocellulosic feedstocks using an isolated multistress-tolerant Pichia kudriavzevii strain, Applied microbiology international. https://doi.org/10.1111/1751-7915.12712
50. Chovau S, Van der Degrauwe D (2013) BruggenCriticalanalysisoftechno-economicestimatesfortheproductioncostoflignocellulosicbio-ethanol,RenewableandSustainableEnergyReviews,Elsevier,vol.26(C),pages307–321,10.1016/j.rser.2013.05.064
51. SonicaSondhi PS (2020) Techno-economic analysis of bioethanol production from microwave pretreated kitchen waste. SN Appl Sci 2:1558. https://doi.org/10.1007/s42452-020-03362-1
52. Mohanty SK, Swain SBMR, Chandra Ray R (2009) Bioethanolproductionfrommahula(MadhucalatifoliaL.)flowersbysolid-statefermentation,AppliedEnergy,Volume86,Issue5,May,Pages640–644,https://doi.org/10.1016/j.apenergy.2008.08.022

53. Timothy J, Martin JT (2021) Value-Added Products from Ethanol Fermentation—A Review. Fermentation 7(4):267. https://doi.org/10.3390/fermentation7040267
54. Agrawal T (2019) Bioethanol production from Madhuca latifolia L. flowers by a newly isolated strain of *Pichia kudriavzevii*, Sage journals, energy and environment. 30 Issue 8,May 24. https://doi.org/10.1177/0958305X19852475. Afaque Quraishi and Shailesh Kumar Jadhav
55. Updegraff DM (1969) AnalyticalBiochemistry,December,32(3):420-4.doi:10.1016/s0003-2697(69)80009-6,Semimicrodeterminationofcelluloseinbiologicalmaterials,PMID:536139610.1016/ s0003-2697(69)80009-6
56. Kumar U, Tapwal A 1, Kalkal P, Varghese S (2014) Isolation and Screening of Cellulase Producing Fungi from Forest Waste. Int J Pharm Biol Archives 5(1):56–59
57. VicenteGomis R (2007)), Dehydration of Ethanol Using Azeotropic Distillation with Isooctane, Industrial and engineering chemistry research. May 46 13:4572–4576. https://doi.org/10.1021/ie0616343
58. Williams MB, Reese HD (1950) Colorimetric Determination of Ethyl Alcohol. Anal Chem 22(12):1556–1561. https://doi.org/10.1021/ac60048a025
59. YueleiYang KBoots,&, DanZhang (2012) ASustainableEthanolDistillationSystem.Sustainbility,4(1),92–105.10.3390/su4010092

Figures

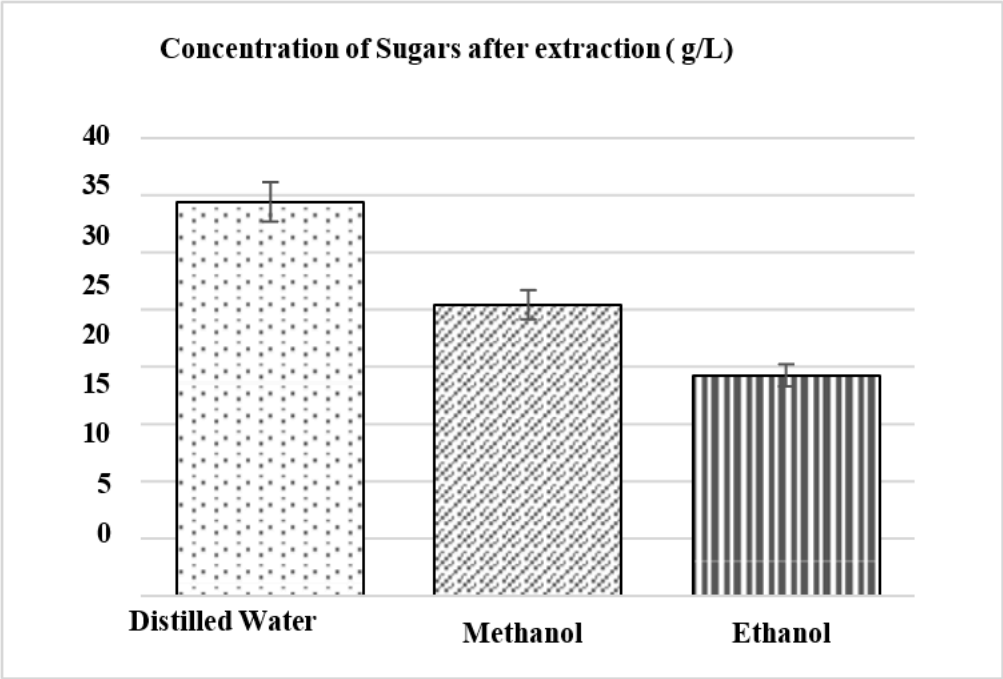

Figure 1

Showing the quantitative analysis of reducing sugars liberated from the FW using various solvents. Water (34.37± 0.03g/L/g), methanol (25.41± 7.51 g/L/g) and ethanol (19.26± 1.5g/L/g.

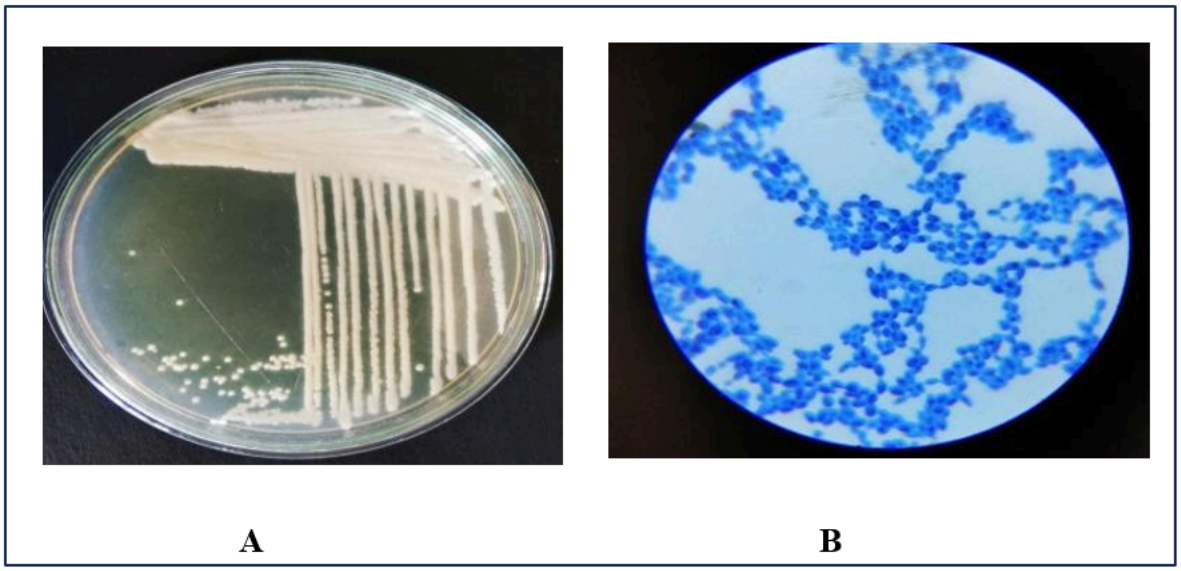

Figure 2

Pure Culture of *Pichia kudriavzevii* CY 902 on YEPD plate (A) and microscopic field view (B) after staining using Methylene Blue

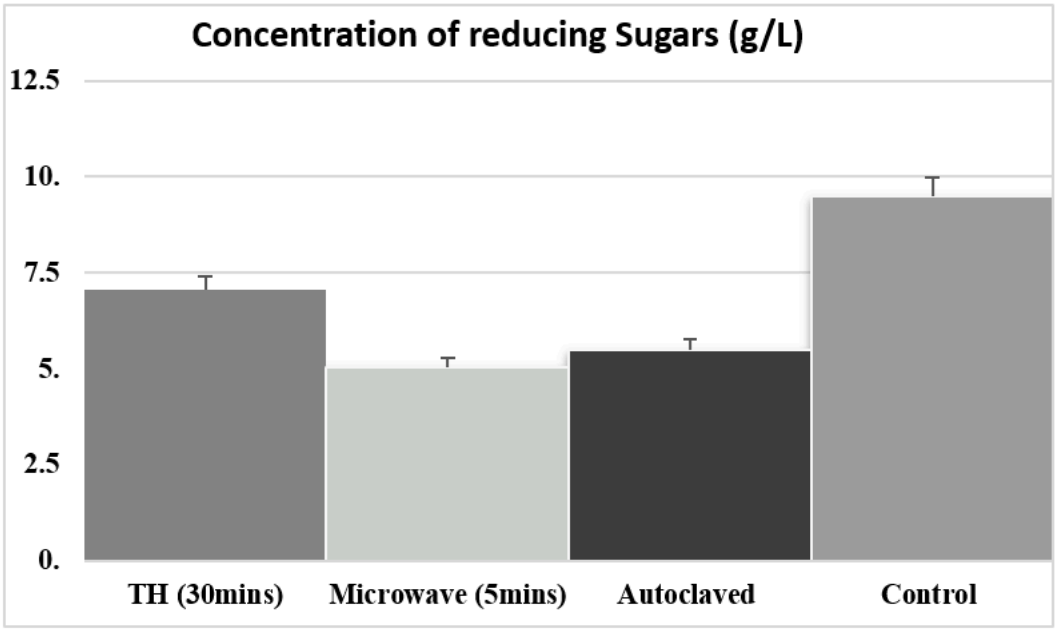

Figure 3

Maximum amount of reducing sugars hydrolysed through various physical pre- treatment by Thermal hydrolysis (TH) for 30 mins showed 7.05 ± 0.04 g/L/g, microwave treatment for 5 mins showed 5.05± 0.04 g/L/gand autoclaving showed 5.5 ± 1.5 g/L/g of reducing sugars respectively, in comparision to the untreated control which showed 9.5 ± 0.6 g/L/.g of reducing sugars.

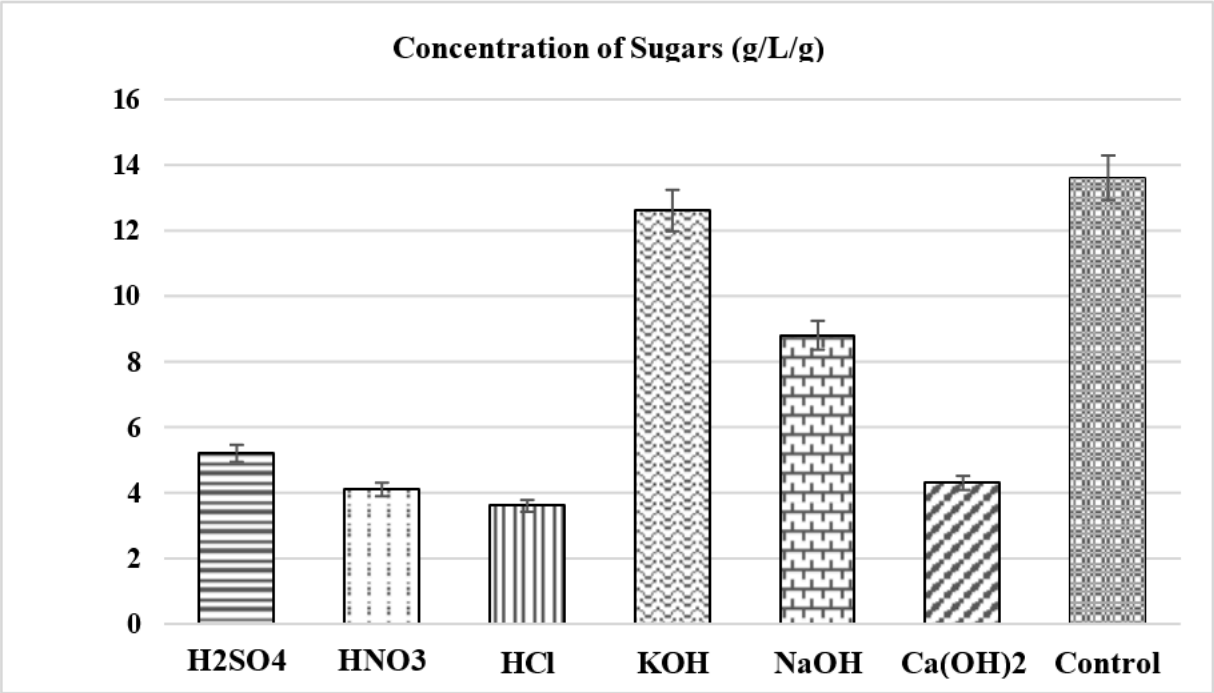

Figure 4

Depicting the effect of chemical pre-treatment method on the concentration of reducing sugars in the FW, Using dilute acids (1%) H2SO4 (5.2 ± 0.12 g/L/g), HNO3 (4.1 ± 0.08g/L/g) HCl (3.6 ± 0.02 g/L/g). Dilute alkali (1%) KOH (12.6 ± 1.62 g/L/g) NaOH (8.8 ± 1.28 g/L/g) Ca(OH)2 (4.3 ± 0.24 g/L/g) in comparision to the untreated control which showed of 13.8 ± 3.64 g/L. reducing sugars.

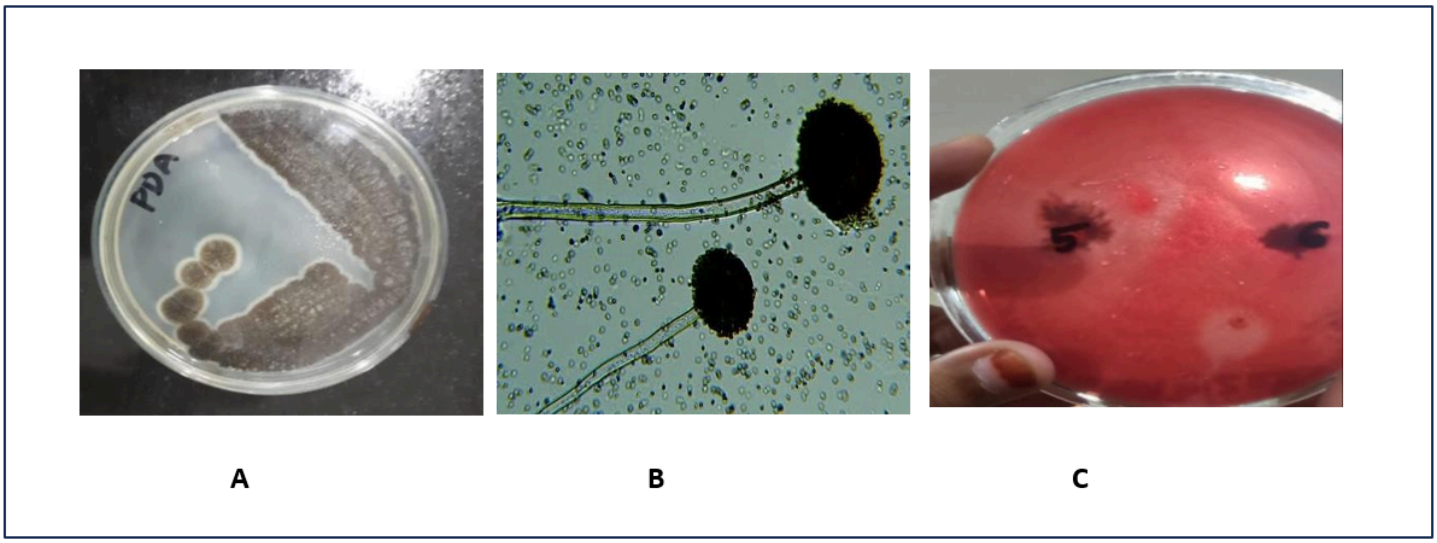

Figure 5

Cellulase producing fungi isolated from rotten wood to obtain crude cellulase enzymefor hydrolysing cellulose present in the FW. *Aspergillus* niger on PDA plate (A), microscopic field view (B) and zone of hydrolysis on CMC agar plate (C)

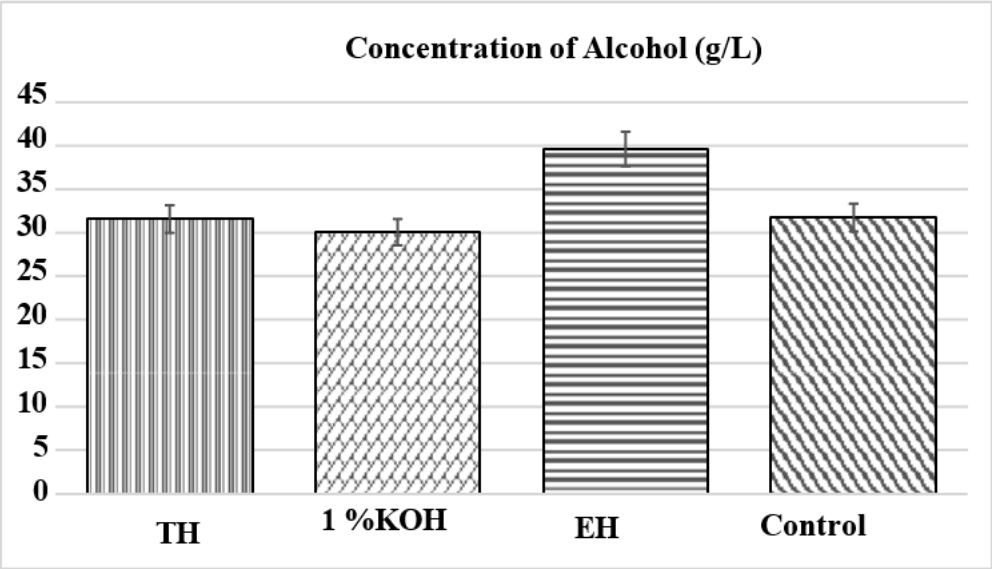

Figure 6

Illustrating the concentration of alcohol produced by optimised physical, chemical and biological pre-treatment methods. Thermal hydrolysis (30mins,100°C) (31.56g/L/g), 1%KOH (30.6 ± 0.02g/L/g) 5% crude cellulase enzyme hydrolysis (39.56 ± 0.08 g/L/g) Control (31.73 ± 0.01g/L/g)

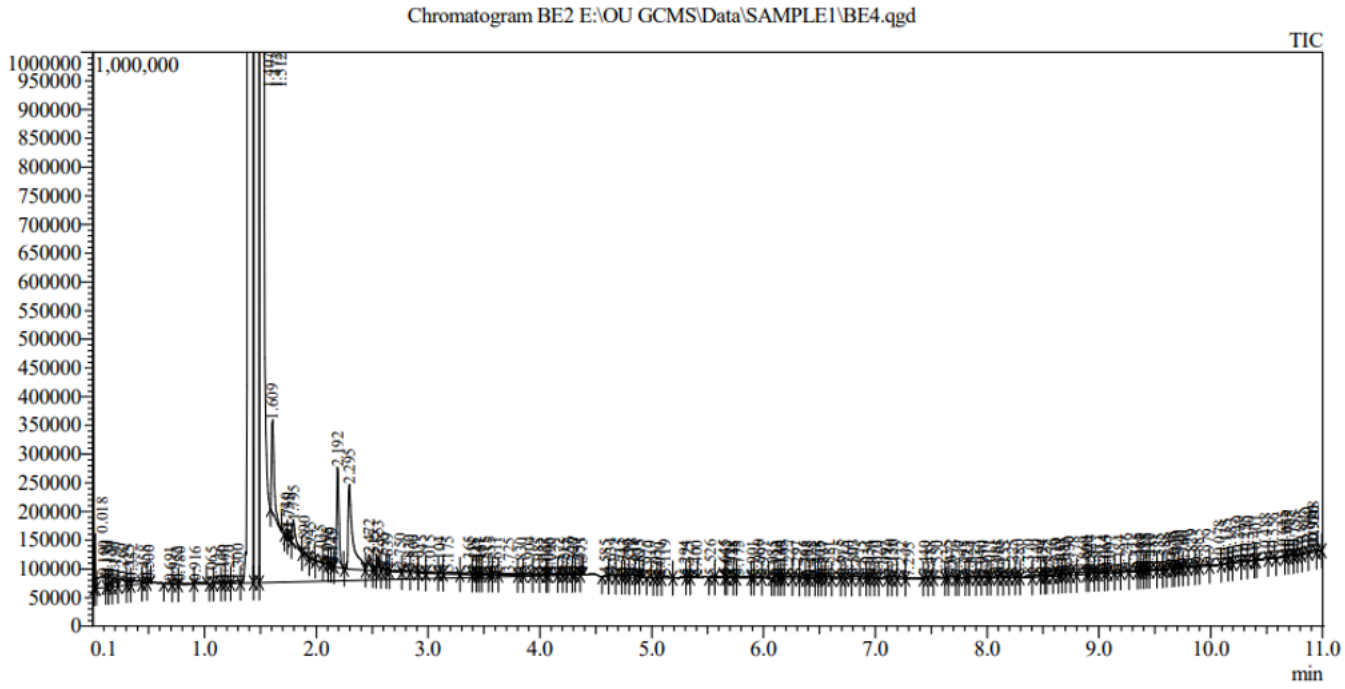

Figure 7

Chromatogram of GC- MS analysis showing peaks of Hydrazine, ethanol and propanol with retention time of 1.40, 1.47 and 1.51 respectively.