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# Improvement over Traditional Brewing Techniques for Production of Bioethanol from Mahua Flowers

## (*Madhuca indica*)

**Tandrima Banerjee**[*]
**Abhijit Samanta**[**]

**Abstract**

An initiative has been taken to develop bioenergy from some tribal forest biomass as the alternative energy resources. The present study is mainly focused on the development of traditional brewing techniques used by some tribes of West Bengal (India) for production of rice beer using wild herbals, rice grains and local *Mahua* flowers. Our current research is based on the production of first generation biofuels as bioethanol derived from *Mahua* flowers (*Madhuca indica*) enriched with high amount of fermentable sugars. The biochemical analysis of *Mahua* flowers revealed that it contained moisture (10-15%), sugar (64-68%), reducing sugars (50-55%), invert sugars (10-14%), ash (2-4%), crude protein (4-5%), crude fat (0.8-1%), $Fe^{2+}$ and $Ca^{2+}$. The present investigation was used for the development of bioethanol production from *Mahua* flower extract by two types of fermentation process (batch and fed-batch) using yeast strain *S. cerevisiae-3078* culture. About twenty five treatment combinations were used for fermentation under same nutrients conditions maintaining pH= 4, 5 and 5.7 and two temperatures 30$^{o}$ and 33$^{o}$C over a period of fermentation (5, 7, 14, 18 and 21 days). The concentration of produced alcohol increased with aging and prolonged fermentation until it reached a steady concentration. The maximum yields of ethanol at 33$^{o}$C and pH=5.7 after 14 days was found 18% and 15% (using batch) while it is 22% and 16% (using fed-batch fermentation) from fresh and 6-month-stored *Mahua* flowers, respectively. Thus bioethanol production would be increased by developing the traditional methodology, as it is expected to benefit the people of tribal areas as well as the bioenergy demand in India in the long run.



***Keywords:***

Bioenergy;
Bioethanol;
Mahua;
Brewing Techniques;
Fermentation.

***Author correspondence:***

Abhijit Samanta
Department of Chemistry, The Neotia University
Kolkata- 743368, India.

### 1. Introduction

The global primary energy demand is projected to be increased of over 35% oil equivalent from around in 2008 to 2035. On global basis, it is estimated that renewable energy accounted for 18% of the total oil equivalent of primary energy supply in 2014 [1]. The largest contributor to renewable energy with 14% points was bioenergy whereas hydropower represented 3% points and other renewable energy sources accounted for 1% point (Figure 1). The bioenergy consumption includes about 68% from fuelwood, 10% from charcoal, 7% from black liquid, 4% from bioethanol and rest parts come from other biomasses which

[*] Department of Chemistry, Pailan Technical Campus, Bengal Pailan Park, Kolkata-700104, India
[**] Department of Chemistry, The Neotia University, Kolkata-743368, India.

include MSW, biodiesel, biogas etc. (Figure 2) [2]. Therefore to fulfill the energy demand modern renewable biomasses are to be revealed. The total volume of modern biomass consumption contributed an estimated 3-4% of global primary energy [3]. Biomass used for energy purposes is derived from a number of sources like residues from forests, wood processing, and food crops dominate. The contribution of renewable energy to primary energy supply varies substantially by country and region.

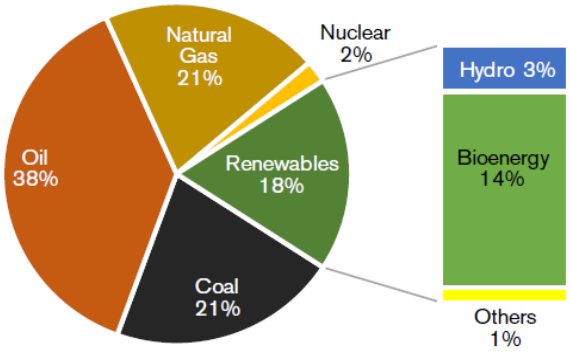


Figure 1: Gross final energy consumption globally in 2014 (Source: IEA Key World Energy Statistics, 2016)

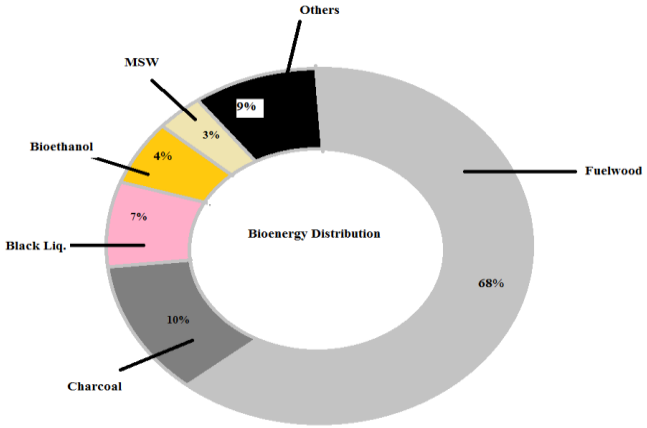


Figure 2: *Primary energy supply of biomass resources globally in 2014 (WBA Global Bioenergy Statistics 2016)*

Bioenergy also offers the opportunity to reduce not only the fossil greenhouse gas (GHG) emissions but also the dependence of energy imports [4-5]. Accoring to United Nations Organization for Food and Agriculture, 2008, it reduces the diversifications of the energy matrix as well as reduces the oil dependence. This becomes an interesting alternative to reduce competition with the food industry and to generate an added value to the agroindustrial residues [6]. Bioethanol productions from agricultural materials offer a solution to some of the recent environmental, economic, and energy problems facing worldwide. The bioethanol production also offers promising economic potential through diversified value chains and low feedstock costs. Partly immature technologies, challenging logistics for sourcing waste, and hesitating investors pose barriers to using this potential. However, in order to unlock its potential towards cost-competitive waste-based bioethanol production and the use of biofuel in transport, support for research and development, as well as an enabling political framework, are needed [7]. Nationally, energy costs are on the rise and forecasts of petroleum supply disruptions are once again making news. Fermentation has contributed significantly to different industrial activities in the present world today. Bioethanol production by fermentation has received special attention to solve world energy crisis [8-12].

*Madhuca indica*, commonly known as *Mahua*, is a tropical tree, found largely in various parts of Andhra Pradesh, Maharashtra, Chhattisgarh, Jharkhand and West Bengal and also some tribal communities cultivate and harvest mahua flowers for alcoholic beverages using traditional methods [13-14]. *Mahua* flowers are rich source of sugars [15] and the component analysis of *Mahua* flowers revealed that it contained high amount of sugars and some inorganic nutrients like $Fe^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ [16-17]. Sarkar et al. (1984) [18] studied structural feature of polysaccharides of *Mahua* flowers. Some researchers have been studied that the *Mahua*

flowers have been utilized as a substrate for the production of ethanol through submerged fermentation [13, 19]. These facts have given new dimension and orientation on research towards preparation of bioethanol as an alternative biofuel in India.

Literature lacks sufficient works on increase in production of bioethanol from *Mahua* flowers of West Bengal. Thus significant works are still required for economic implementation of this technology in practical field. The design of production of maximum bioethanol from *Mahua* flowers extract must achieve two main objectives: propagation and the biochemical analysis of the *Mahua* flowers collected from tribal areas in West Bengal in an active mode and the production of enough bioethanol depending on different factors. Achieving these objectives is significantly affected by the selection of the biomass, the composition of different nutrients and the other physical and chemical factors. Therefore, in this paper we have studied at the first step the biochemical analysis of *Mahua* flowers and then a complete study of maximum yield of bioethanol by fermentation technique in various batch reactors depending on different factors like biomass, innocolum periods, pH and temperature have been perform.

## 2. Research Method

### *2.1 Materials*

The substrate *Mahua* (*Madhuca indica*) flowers were collected mainly from some areas of Paschim Midnapore and Purulia District of West Bengal. The flowers were washed in tap water and sun-dried for 2 days to reduce moisture content to 10-15 %. After washing and sun drying the mahua flowers are sterilized by autoclaving at pressure 10 lb/inch$^2$ for a period of 20 minutes.

Yeast strain *S.cerevisiae-3078* was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Others required chemicals were collected from the Department of Chemistry and Biotechnology, The Neotia University as well as from the Department of Chemistry at Pailan Technical Campus, Kolkata.

### *2.2 Methods*

#### *2.2.1 Extraction and Characterization of Mahua Juice*

***Extraction***

The *Mahua flower* juice was extracted in Soxhlet apparatus using water as solvent [20]. About 20gm of *Mahua flowers* was placed in the extraction thimble for each set of extraction and about 1L of *Mahua* juice was prepared for analysis. The extract is then dried, concentrated as necessary.

***Determination of proximate composition of Mahua flowers***

Proximate compositions of the *Mahua flower* samples were determined using the methods of Association of Official Analytical Chemists (AOAC) International [21-23]. The percentage of moisture content was determined by heating 1.0 g of the sample at 135$^o$C for 2 hours [24]. Ash content was determined by drying 1.0g of honey samples in porcelain crucibles at 105°C for 3 hrs in hot air oven. The dried samples were ignited in a furnace at 550-600°C to constant weight, cooled and weighed.

The Micro-Kjeldhal procedure was used to estimate the total nitrogen content and the protein content was calculated using the 6.25 conversion factor for protein nitrogen. The total protein content was estimated by the standard Biuret assay method using bovine serum albumin of 10 mg/mL stock as standard [25].

Crude fat content was determined following extraction using rob ring tube or Majonnier fat extraction apparatus [21]. Five grams (5.0 g) of the mahua extract was weighed in the extraction apparatus and mixed thoroughly with 2.0 mL of 99% ethyl alcohol. Then 10.0 mL of dilute HCl (prepared by adding 11 volumes of water to 25 volumes of concentrated HCl) was added and mixed well. The tube was then set in a water bath held at 70-80°C and shaken frequently at intervals for 30 minutes. The fat extraction apparatus was then filled to half its volume capacity with alcohol and cooled. 25.0 mL of ethyl ether was then added, shaken vigorously and allowed to stand until the upper liquid was practically clear. The ether extract was then drawn off by passing through a filter (using a plug of cotton in the stem of the funnel just enough to allow free passage of ether extract) into a pre-weighed 125 mL beaker, and was then dried on a water bath. The liquid remaining in the tube was re-extracted twice each with only 1.0 mL of ether. A similar pre-weighed beaker was then used as counter poise at 100°C. The beakers were then cooled in desiccators to constant weight and the fat content calculated.

The amount of total carbohydrate in the sample was determined by phenol-sulfuric acid assay method using glucose (stock concentration of 2 mg/mL) as standard [26].

Carbohydrate contents of the honey samples could be determined by calculation (by difference) as follows:

$$\%\ Carbohydrate = 100\% - (\%\ Moisture + \%\ Crude\ Fat + \%\ Crude\ Protein + \%\ Ash)$$

***Determination of $Ca^{2+}$ and $Fe^{2+}$***

The presence of inorganic metal ions like $Ca^{2+}$ and $Fe^{2+}$ were determined by standard methods [27].

***Determination of reducing sugars and sucrose contents:***

Total sugar of *Mahua flower* is estimated by Anthrone method. The amount of reducing sugar was estimated by the standard biochemical method using 3,5-dinitrosalicylic acid reagent and glucose (stock concentration of 1 mg/mL) as standard [27]. The nonreducing sugar was calculated by subtracting the amount of reducing sugar from the amount of total carbohydrate in the sample [28]. Invert sugar content was determined by inversion, adding 10 mL of dilute HCl, 50 mL of diluted extract solution and water in a 100 mL volumetric flask. The solution was then heated in a water bath, cooled and diluted to the mark. Finally, the Layne-Enyon method was applied and the sucrose content was obtained by difference.

Total acidity and volatile acidity were determined by titration of the samples against sodium hydroxide solution using phenolphthalein as an indicator and expressed as grams of tartaric acid present per 100 mL of the samples and grams of acetic acid present per 100mL of the samples [29].

***Determination of glucose content:***

Glucose content of the samples was determined by enzymatic oxidation with glucose oxidase reagent (Merck Life Science Private Limited, India). 20µL of the extract sample and standard was allowed to react with 2.0 mL of the reagent in two different set of reaction, mixed well and incubated for 10 min at 37°C. The absorbance of the sample ($A_{sample}$) and standard ($A_{standard}$) was read against a reagent blank within 60 min. Glucose concentration was calculated as follows:

$$Glucose\ content\ (mg/dL) = (A_{sample}\ /A_{standard}\ ) \times Conc.of\ standard$$
$$= (A_{sample}\ /A_{standard}\ ) \times 100\ (\%)$$

***Determination of fructose content***

Fructose content was determined using the resorcinol reagent method [22]. To a solution of the extract sample, 1.0 mL resorcinol reagent was added and mixed thoroughly, and then 1.0 mL of dilute HCl was added. Standard solutions containing 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ mL and made up to 2 mL with distilled water was also treated with 1.0 mL of the resorcinol reagent and 1.0 mL of diluted HCl as above. A blank solution was also prepared along with the standard and treated in the same manner. The test solution, the standard and blank were then heated in a water bath at 80°C for about 10min, the solution was then removed from the water bath, cooled by immersing in tap water for 5min and then the absorbance of both the test and standard solution were read against the blank solution at 520 nm within 30 min. The fructose contents of the samples were then extrapolated from a standard curve prepared using the absorbance of the standard.

***Medium for Seed Culture***

Yeast strain *S. cerevisiae-3078* culture is maintained on the yeast extract, glucose, malt extract, peptone and some inorganic salts for nutrition at *pH* 4, 5 and 5.7. This nutrient agar medium containing malt extract 15 g/L, glucose 50 g/L, yeast extract 15 g/L, peptone 25 g/L and seed culture for fermentation is prepared as about 5% of fermentation slurry and the medium is autoclaved at 15 lbs pressure, 2-3 loops of original culture were transferred and incubated at 30°C for 48 hours [19].

***2.2.3 Fermentation process***

The traditional brewing techniques of preparation of liquor from rice grain and Mahua at some of the tribal area of Paschim Medinipur has been shown in Figure 3. Generally tribal peoples are using Mahua flowers as flavor in rice alcohol [30].

Figure 3: *Traditional brewing techniques for preparation of ethanol at Paschim Medinipur, West Bengal.*

The investigation has used for the development of bioethanol production from *Mahua* flower extract (both fresh and 6-months old) by different types of fermentation process (batch and fed-batch) using yeast strain *S. cerevisiae-3078* culture by inoculating. These experiments have been tracked four parameters throughout the fermentation: biomass concentration, pH, temperature and time of fermentation. Bioethanol concentrations have been analyzed with changing of these parameters. The mixture of bioethanol and hot water were separated by simple distillation methods using temperature of 78-96$^{o}$C. In this method 85% of pure bioethanol is obtained, which rectified by using rectifier units to obtain 99.2% pure bioethanol. The alcohol percentage of the nondistilled product was determined by titration of the samples against sodium thiosulfate solution using dichromate oxidation method and expressed in percentage volume by volume [31]. The distilled bioethanol concentration is estimated by potassium dichromate oxidation method and followed by spectrophotometric method using UV-spectrophotometer (JASCO, V630) [30]. About twenty one sets of batch in different batch reactors and four sets of fed-batch fermentations have been done in biofermentor (BRIO BT Series, Pisces Instruments, Chennai, India) depending on all optimum variables.

## 3. Results and Analysis

### *3.1 Biochemical Analysis of Mahua flowers*

### *3.1.2 Proximate composition*

The results of the proximate analysis of flowers samples obtained from different location in West Bengal states within the Paschim Mesdinipore and Purulia districts are presented in Table 1. The results showed no significant differences between the samples for moisture, ash, fat and carbohydrate contents of the flower samples from these two areas. In general, the results for the four samples from two districts of West Bengal showed that moisture contents ranged between 10% -15%, ash contents varied from 0.4% -0.47%, fat content lied between 0.27%-0.32% while the total carbohydrate contents from 78.09%-82.20% respectively.

Moisture content is an important quality parameter of mahua flowers shelf-life. The significance of moisture in mahua flowers derives from the fact that there is a relationship between water content and yeast count. Thus, mahua having high water content is more likely to ferment. The ash contents of the flowers obtained in this study were all within the limits specified by international norms [32-33]. There were no significant differences between the ash contents of the sample from all the districts in the sate. The ash contents of flowers represent their mineral and trace element contents and it was found that Mahua flowers contain inorganic ions like $Ca^{2+}$ and $Fe^{2+}$.

The total carbohydrate contents of the Mahua extract samples from all the different parts of States were not significantly different from each other. Carbohydrates are the main constituents of Mahua extract comprising about 80-82% of Mahua dry weight. The monosaccharides, fructose and glucose, are the main sugars found in the extract, these hexoses are products of the hydrolysis of sucrose.

**Table-1:** *Proximate Composition of Mahua flowers samples from the different parts of West Bengal.*

| ***Sample.*** | ***Parameters*** | ***Paschim Medinipore*** | ***Purulia*** |
|---|---|---|---|
| ***Fresh Mahua flowers*** | Moisture (%) | 10.33 ± 0.82 | 10.1 ± 0.28 |
| | Ash (%) | 4.05 ± 0.004 | 3.92 ± 0.11 |
| | Fats (%) | 0.80 ± 0.09 | 0.77 ± 0.03 |
| | Carbohydrate (%) | 80.01 ± 0.07 | 79.26 ± 0.6 |
| | Crude Protein (%) | 4.81± 0.01 | 4.95± 0.001 |
| | $Ca^{2+}$ & $Fe^{2+}$ | + | + |
| ***6-month old Mahua flowers*** | Moisture (%) | 15.13 ± 0.58 | 14.83 ± 0.45 |
| | Ash (%) | 2.47 ± 0.07 | 2.04 ± 0.25 |
| | Fats (%) | 1.02 ± 0.10 | 1.06 ± 0.13 |
| | Carbohydrate (%) | 77.35± 0.14 | 77.76 ± 0.12 |
| | Crude Protein (%) | 4.03± 0.11 | 4.31± 0.05 |
| | $Ca^{2+}$ & $Fe^{+}$ | + | + |

### *3.1.2 Sugar contents*

The results of sugar analysis of the mahua flowers samples are presented in Table 2. There are no significant differences in the fructose, glucose, fructose+glucose and reducing sugar contents were observed in samples from the two districts regions. Similarly, no significant difference, in both fructose/glucose ratio and glucose/water ratio were observed between samples from all the four samples. The results of the sugar analysis of all the four samples (Table-2) showed that the fructose contents varied between 33.08% and 36.02% the glucose contents of the samples were within a range of 30.02% to 33.13%, the fructose contents of the samples were significantly higher than the glucose contents. The fructose/glucose ratio and

glucose/water ratio were within the range of 1.05 to 1.17 and 1.95 to 3.20 respectively. No significant difference was observed between the fructose/glucose and the glucose/ water ratios. The sum of fructose and glucose (fructose+glucose) contents ranged between 64.08% and 68.15% while the reducing sugar contents varied between 50.02% and 54.83%. There was no significant difference between the mean values of the fructose plus the glucose contents and the reducing sugar contents of the samples.

**Table-2:** *Sugar contents of Mahua flowers samples from the different parts of West Bengal*

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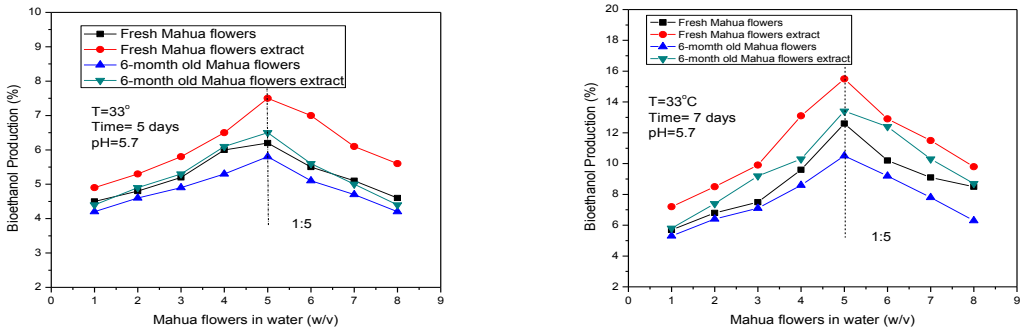
| ***Sample.*** | ***Parameters*** | ***Paschim Medinipore*** | ***Purulia*** |
|---|---|---|---|
| ***Fresh Mahua flowers*** | Fructose (%) | 35.02 ± 0.7 | 36.02 ± 0.31 |
| | Glucose (%) | 33.13 ± 0.32 | 30.81 ± 0.11 |
| | Fructose + Glucose (%) | 68.15 ± 0.02 | 66.83 ± 0.42 |
| | Reducing sugar (%) | 54.83 ± 0.15 | 52.46 ± 3.35 |
| | Invert Sugar (%) | 14.02 ± 0.40 | 12.37± 0.26 |
| ***6-month old Mahua flowers*** | Fructose (%) | 33.08 ± 1.05 | 34.06 ± 0.08 |
| | Glucose (%) | 31.36 ± 0.71 | 30.02 ± 0.35 |
| | Fructose + Glucose (%) | 65.44 ± 1.76 | 64.08 ± 0.43 |
| | Reducing sugar (%) | 50.02 ± 0.15 | 50.15 ± 0.25 |
| | Invert Sugar (%) | 11.01 ± 0.42 | 10.04 ± 0.97 |

### *3.2 Fermentation Process*

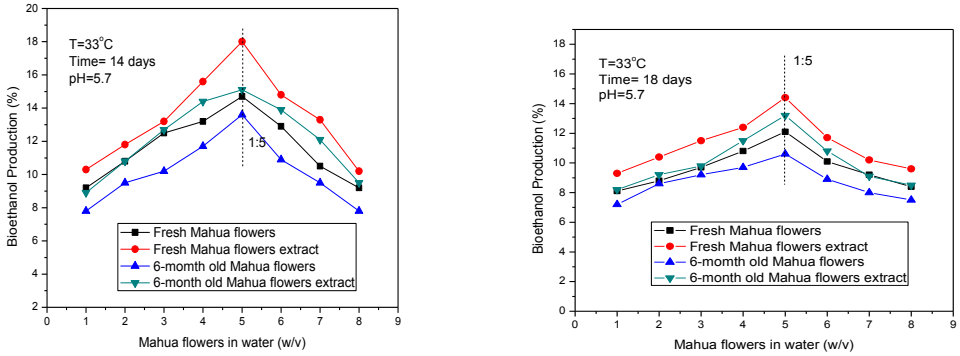
#### *3.2.1 Effect of Substrate Concentration*

Effect of substrate concentration for production of ethanol from 6-month old mahua and fresh mahua flower extract using *S.cerevisiae-3078* was carried out by varying substrate concentration from 1:1 to 1: 8 ratios (w/v) and shown in Figure 4 to Figure 6. Other parameters such as inoculums age (5, 7, 14, 18 and 21 days), agitation 50-70 rpm, and nitrogen sources i.e. urea 0.32 g (w/v) and pH at 5.7 at temperature 33°C were maintained. It has been found that the percentage of ethanol production increases with increasing the concentration ratio of mahua extract initially and it was reached at a maximum concentration for each fermentation periods. It can be seen from the figures that the maximum yield of ethanol is obtained at 1:5 (w/v) ratio of 6-months old and fresh Mahua flowers for each fermentation periods. It was observed that the percentage of ethanol production decreases further with increase in concentration ratio. In this view, substrate concentration at 1:5 (w/v) was taken as an optimum and the effects of other physico-chemical parameters such as *pH*, temperature, inoculums age on bioethanol production were studied furtheremore.




(a) (b)

Figure 4: *Bioethanol production from Mahua flowers extract varying biomass concentration after (a) 5 days and (b) 10 days at pH=5.7 and temperature= 33°C.*




(a) (b)

Figure 5: *Bioethanol production from Mahua flowers extract varying biomass concentration after (a) 14 days and 18 days at pH=5.7 and temperature= 33°C*

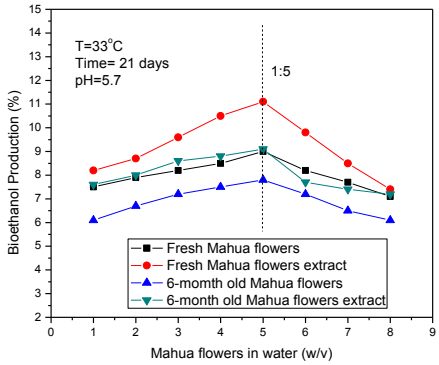



Figure 6: *Bioethanol production from Mahua flowers extracts varying biomass concentration after 21 days at pH=5.7 and temperature= 33°C*

### *3.2.2. Effect of pH and Temperature during the period of Fermentation (batch and fed-batch)*

The value of pH during the fermentation period gradually changes from 5.7-4.3 (Figure 7). This change in pH was adjusted by addition of aqueous solution of $NH_3$ and dil. $H_2SO_4$ after every 24 hours interval till the fermentation time reaches the first set of 5 days, next 7 days ans so on. It is clear from Figure 8 that if the fermentation time is kept for first slot of 5 days and then the second set after 7 days and so on, and the *pH* values was maintained between 4-5.7 and the production of ethanol was more as compared if the pH value is kept 5.7. Fermentation is a temperature dependent process and the variation of temperatures clearly shown that the maximum yield of bioethanol is obtained at 33°C.

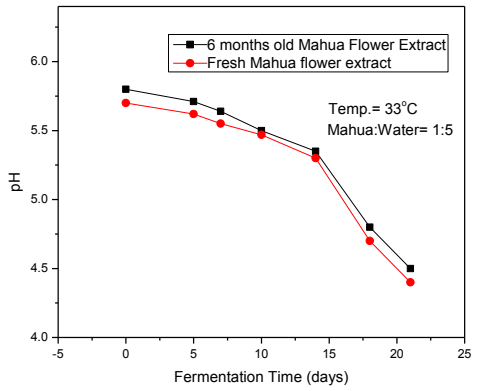
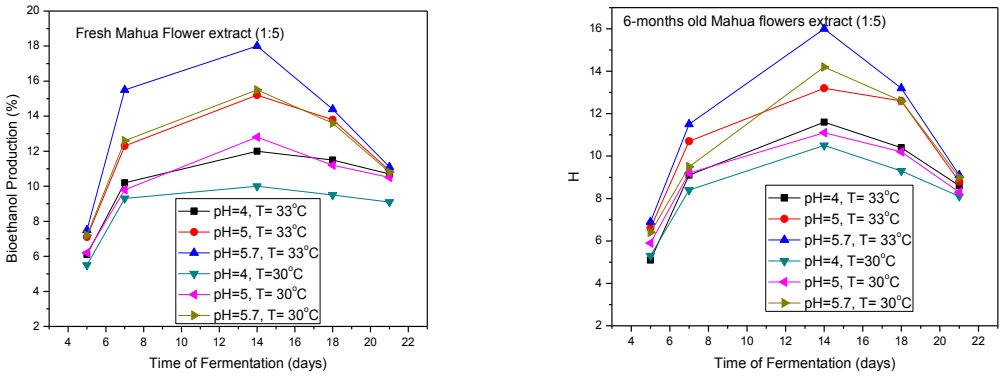



Figure 7: *Change of pH during the fermentation at bimass ratio 1:5 (w/v) and 33°C.*




(a) (b)

Figure 8: *Bioethanol production (%) at different pH (4, 5 and 5.7) of (a) fresh Mahua Flower and (b) 6-months old Mahua flowers extract by batch fermentation with time variation*

Four sets of different fermentation samples have been fermented in a biofermentor using fed-batch fermentation method maintaining the above optimum conditions the optimum concentration of Mahua (1:5) and pH=5.7 varying temperature 30° and 33°C. It has been found that the maximum ethanol yielded about 18% from 6-month old *Mahua* flower extract and 22% from fresh *Mahua* flower extract (Figure 9).

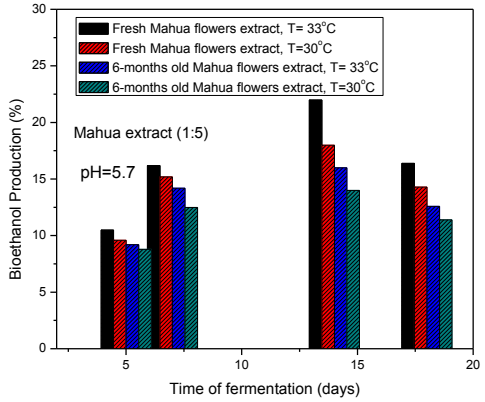



Figure 9: *Ethanol production (%) at 30° and 33°C and pH=5.7 of fresh Mahua Flower extract and 6-month old Mahua flower extract by fed-batch fermentation with time variation*

## 4. Conclusion

The present investigation was used for the development of ethanol production from *Mahua* flower extract by batch and fed-batch fermentation process using yeast strain *S. cerevisiae-3078* culture. Studies including physico-chemical parameters for the production of bioethanol were done in simplified way. The results show that mahua flowers proved to be cheap and suitable substrate for the production of biofuel in India. The concentration of produced bioethanol increased with aging and prolonged fermentation until it reached a steady concentration. The maximum yields of ethanol at 33°C and pH=5.7 after 14 days was found 18% from 6-month old *Mahua* flowers extract and 22% from fresh *Mahua* flowers extract respectively. Thus ethanol production would be increased by developing the traditional methodology, as it is expected to benefit the people of tribal areas as well as increase the industrial potentiability of *Mahua* flowers towards production the bioenergy demand in India in the long run.

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