Ethanol Production from Mahua (*Madhuca indica* J. F. Gmel) Flowers by Soil Bacteria

Tripti Agrawal[1], Afaque Quraishi[2], Kishan Lal Tiwari[3] and Shailesh Kumar Jadhav[4*]

[1, 2, 4*]. School of Studies in Biotechnology, Pandit Ravishankar Shukla University, Raipur 492010, Chhattisgarh, India
[3.] Department of Biotechnology, Guru Ghasidas Central University, Bilaspur 495001, Chhattisgarh, India
tripti.0221@gmail.com, dr_afaque@yahoo.co.in, kishan_tiwari37@hotmail.com, shailesh_07@sify.com

Abstract: In an effort to combat climate change, aid energy independence and counteract diminishing supplies of fossil fuels, there is a need to research on renewable fuel energy sources. Bioethanol is a sustainable and renewable biofuel that is a promising substitute to gasoline and represents an environment-friendly fuel because it reduces the amount of greenhouse gas emissions. The rise in prices and environmental problems caused by fossil fuels has contributed to this recent interest in biofuel research from economical and ecological perspectives. The biofuel that is expected to be most widely used around the world is bioethanol. Bioethanol is ethanol derived from biological feedstocks utilizing microbial fermentation processes. Bioethanol has a low toxicity and is readily biodegradable than petroleum fuel. Production of ethanol by fermentation from cheap carbohydrate materials for use as an alternative liquid fuel is the current focus of research worldwide. Mahua (*Madhuca indica*) belonging to the family *Sapotaceae*, is a multipurpose forest tree which is widely distributed in the South Asian countries. Mahua flowers are rich source of reducing sugar and nutrient content. The lack of industrially suitable microorganisms for converting biomass into ethanol has been cited as a major technical barrier to a developing bioethanol industry. In this context, production of ethanol from mahua flowers by bacterial fermentation was investigated. Bacteria were isolated from compost and kitchen waste dumped soil. A total of 16 bacteria were isolated on Nutrient agar medium, identified primarily on morphological characteristics, screened for their fermentation ability by carbohydrate fermentation test and positively screened bacterial isolates were subjected to ethanol production using mahua flower as fermentation medium under incubation period of 120 hours in shaker incubator at 80 rpm and 30 ºC. Ethanol concentration of the fermentation liquid after distillation was determined by specific gravity method. The highest ethanol yield of 8.94 % was obtained from fermentation by bacterial isolate S_4B_3.

[Tripti Agrawal, Afaque Quraishi, Kishan Lal Tiwari and Shailesh Kumar Jadhav. **Ethanol Production from Mahua (*Madhuca indica* J. F. Gmel) Flowers by Soil Bacteria.** *Researcher* 2013;5(12):102-106]. (ISSN: 1553-9865). http://www.sciencepub.net/researcher. 14

Keywords: bioethanol; fermentation; mahua; specific gravity

1. Introduction

Energy is the lifeline of global economy, diminishing fossil fuel reserves and increased concerns over environmental pollution accelerated the need to look for renewable and environmentally sustainable energy sources. In this context, ethanol derived from biomass is means to meet our energy needs. Bioethanol is a sustainable and renewable transportation fuel that is a promising substitute to gasoline and represents an environment-friendly fuel because it reduces the amount of greenhouse gas emissions, which is a major cause of global warming. The development of alternative fuel and energy from biomass has therefore, resurfaced as a research priority in recent years. It is expected that demand for fuel ethanol will rise from current estimates of 4 billion to 22.7 billion gasoline-equivalent gallons (or 20% market share) by 2020 (BRDI, 2006). The future risks of global warming and shortage of petroleum, as well as the superior environmental characteristics of ethanol as an oxygenated additive to gasoline that improves the knocking resistance of gasoline, promote the production and usage of bioethanol in the fuel market. (Millati et al., 2005)

Bioethanol is fermentation alcohol, which refers to ethyl alcohol produced by microbial fermentation processes as opposed to synthetically ethanol from petrochemical sources. It is produced through distillation of fermented biomass. In general bioethanol can be extracted from every sort of carbohydrate material. The main cost element in bioethanol production is the feedstock. These can be divided into three main groups: sugary, starchy and lignocellulosic biomass. First generation feedstocks for bioethanol production primarily refer to plant biomass sources that are also sources of human and animal nutrition, namely cereal starches and sugar crops. Sucrose based materials are predominantly derived from sugarcane and sugar beet; while starch based materials are predominantly derived from cereal crops such as maize, wheat and other cereals. Second generation raw materials for bioethanol production typically refer to non-food biomass sources, mainly lignocellulosic biomass. This represent the most abundant form of carbon on Earth, and encompasses two categories of feedstocks: waste materials like straws, corn residues, wood chippings, forestry residues, spent grains, MSW, agricultural residues and Energy crops such as basket willow, switchgrass, reed canary grass, ryegrass etc. (Walker,2010)

Madhuca indica, commonly known as mahua, belongs to the family *Sapotaceae*, is a tropical tree found abundantly in the central and north Indian forests. It is adapted to arid environments and is predominantly found in tropical mixed deciduous forests of India. The cream coloured corollas of the flower are commercially known as mahua flower. Mahua flowers are rich source of protein 4.4%, Fat 0.5%, Total sugar 72.9%, Fibre 1.7%, Ash 2.7% (Wealth of India, CSIR, 1962). Mahua flower is abundant in India and it is having good keeping qualities. If mahua flower can be utilized as a substrate for the production of bioethanol, it will become a great advantage for Indian economy (Benerji et al., 2010). Non - Timber Forest Products (NTFPs) are backbone to the Indian forest economy contributing over 50% to the forest revenues.Some of the major NTFPs of India are sal seeds, mahua flowers and fruits, myrobalans, bamboo shoots etc (Patel and Naik, 2010). Among these, mahua flowers are one of the important NTFPs which can be a potential substrate for bioethanol production. Mahua flowers represent a readily fermentable sugar source comprising mainly sucrose, fructose and glucose, which can be fermented without accomplishment of prior hydrolysis or other pretreatments because the sugar is available in disaccharides which can be metabolized directly by enzymes present in microorganisms.

Biotechnology has played a vital role in the fermentation of raw materials by microorganisms. Microorganisms are used by fermentation biotechnologists for the conversion of sugar into ethyl alcohol. The scarcity of industrially suitable microorganisms for converting biomass into fuel ethanol has traditionally been a major technical barrier to a developing bioethanol industry. In the last two decades, numerous microorganisms have been engineered to produce ethanol (Dien et al., 2003). Due to severe energy crisis in world, ethanol is considered to be an important alternate energyfuel amongst different fossil fuels (Benerji et al., 2010). For this reason, the conversion of sucrose containing feedstocks like mahua flowers is convenient, efficient and the cost of the process is relatively low. Based on the above advantages and necessities of the usage of raw material for the production of ethanol, mahua flower was used for ethanol production in the present work.

2. Materials & Methods

2.1 Collection of Mahua Flower and Soil Samples

Mahua flowers used for ethanol production in this study were collected from local market of Ghortalao village of Rajnandgaon district of Chhatisgarh state, India. Soil samples were collected from 4 different sampling regions viz. soil beneath mahua tree(S_1), kitchen waste dumped soil site 1(S_2), compost soil(S_3), kitchen waste dumped soil site 2(S_4).

2.2 Isolation of Bacteria

Bacteria were isolated from the different soil sample by serial dilution technique on Nutrient Agar Medium (Prescott et al., 2000) and all the inoculated plates were incubated at 37±1˚C for 24-48 hours. The isolates were purified by repeated subculturing and preserved in slants of the same medium and stored at 4°C.

2.3 Morphological Characterization of Soil Isolates

Isolated microbes were preliminary characterized on the basis of colonial morphological characteristics and methylene blue staining.

2.4 Fermentation Test

Each of the bacterial isolate was screened for fermentation ability by carbohydrate fermentation test (Prescott et al., 2000). Bacteria producing acid and gas production indicating positive fermentation test were selected for bioethanol production.

2.5 Fermentation Process

Twenty grams of thoroughly washed dried mahua flower and distilled water (flower: water ratio 1:10, w/v) was taken in a 250 ml Erlenmeyer flask and autoclaved at 121˚C for 30 minutes and inoculated with bacterial cultures and incubated at 30˚C in a shaker incubator at 80 rpm for incubation period of 120 hours. All the experiments were carried out in triplicate. After incubation, fermentation flasks (in triplicate) were removed from shaker incubator and analyzed for ethanol production.

2.6 Estimation of Ethanol

Crude fermented sample was qualitatively tested for the presence of ethanol by Jones Reagent test (Jones, 1953). Crude fermented sample, 2% $K_2Cr_2O_7$, conc. H_2SO_4 in 1:2:1 ratio was added, appearance of blue green colour indicates presence of ethanol in fermented sample. The fermented filtrate exhibiting positive result in Jones test was finally distilled to collect the distillate to quantify the ethanol concentration the fermented sample. Ethanol concentration of the recovered distillate was determined by measuring the specific gravity of the distillate according to procedure described by Pharmacopoeia of India (1985). Ethanol percentage was calculated by specific gravity method (Yadav, 2003) and percentage in v/v was obtained from the standard table correlating percentage volume of ethanol with specific gravity at 25˚C.

3. Result & Discussion

For screening the soil bacterial flora capability for bioethanol production, sixteen bacteria were isolated from soil collected from four different decomposing regions and preliminary identified on the basis of their morphological characteristics (Table 1). Bacteria were unidentified and designated as S_1B_1 (isolates from S_1), S_2B_1, S_2B_2, S_2B_3, S_2B_4, S_2B_5, S_2B_6 (isolates from S_2) S_3B_1 S_3B_2 S_3B_3 S_3B_4 S_3B_5 S_3B_6 (isolates from S_3) S_4B_1, S_4B_2, S_4B_3 (isolates from S_4).Individual cells of S_1B_1 , S_2B_1,S_2B_3, S_2B_4, S_2B_6, S_3B_1, S_3B_2, , S_3B_5, S_3B_6, S_4B_1, was coccus, S_2B_2, S_2B_5 S_3B_3, S_3B_4 was coccobacillus, S_4B_2 and S_4B_3 was bacillus.

Amongst sixteen bacterial isolates, thirteen were screened positive in carbohydrate fermentation test with both acid as well as gas production in fermentation tube. Positively screened bacterial isolate was subjected to fermentation in fermentation medium comprising of sterilized mahua flowers. Fermented samples were qualitatively tested for bioethanol production by Jones reagent test, all the thirteen bacteria fermented samples showed appearance of blue green colour in the test indicating positive test for the presence of ethanol. Oxidation of ethanol to acetic acid with an excess of potassium dichromate in the presence of sulfuric acid, gives off a blue - green colour (Brooks *et al.,* 2008).

Quantitative estimation of ethanol was done by specific gravity method and ethanol yield was expressed as percentage volume of ethanol/volume of fermented liquid. Ethanol production in mahua sample inoculated with bacteria was in the range of 7.67 - 8.94 % (Figure 1). Bioethanol production by S_2B_2, S_3B_3 & S_2B_5 was 7.67%. S_1B_1 7.76%, S_2B_1, S_2B_4 & S_4B_2 8.03%, S_2B_3 8.13%, S_4B_1 8.31%, S_3B_1 & S_3B_6 8.49%, S_2B_6 8.76% and S_4B_3 exhibiting highest ethanol percentage of 8.94% amongst all the isolates. Tiwari et al. (2012) investigated production of bioethanol from Jatropha oil cake and obtained bioethanol yield of 8.04%.Brooks (2008) obtained ethanol yield of 3.6% and 5.85% from local yeast strain isolated from ripe banana peels. Behera et al. (2011) studied ethanol fermentation of mahula flowers using free and immobilized bacteria *Zymomonas mobilis* MTCC92. Jadhav et al. (2011) worked on bioethanol production by four gram-positive bacteria on substrate Mahua flowers. Tiwari et al. (2011) studied bioethanol production from some carbohydrate sources by Gram positive bacteria. Bioethanol production from mahula (*Madhuca latifolia*L.) flowers by solid-state fermentation was reported by Mohanty et al. (2009). Swain et al. (2007) reported Mahula flowers (*M. latifolia* L.) for bioethanol production using free and immobilized yeast.

Table 1. Colonial Morphology of Soil Isolated Bacteria

S.No.	Bacterial Isolates	Form	Elevation	Margin	Texture	Optical characters	Pigmentation /Colour
1.	S_1B_1	Regular	Flat	Entire	Slimy	Transparent	Creamish
2.	S_2B_1	Punctiform	Flat	Entire	Slimy	Opaque	Yellowish
3.	S_2B_2	Irregular	Flat	Irregular	Slimy	Transparent	Creamish
4.	S_2B_3	Circular	Convex	Entire	Slimy	Opaque	Off white
5.	S_2B_4	Circular	Flat	Entire	Slimy	Opaque	Creamish
6.	S_2B_5	Irregular	Flat	Irregular	Slimy	Opaque	Creamish
7.	S_2B_6	Rhizoidal	Flat	Irregular	Rough	Opaque	Creamish
8.	S_3B_1	Punctiform	Convex	Entire	Smooth	Opaque	Creamish
9.	S_3B_2	Filamentous	Raised	Filamentous	Smooth	Opaque	Creamish
10.	S_3B_3	Irregular	Convex	Lobate	Smooth	Opaque	Creamish
11.	S_3B_4	Irregular	Convex	Undulate	Smooth	Translucent	Creamish
12.	S_3B_5	Irregular	Flat	Lobate	Smooth	Opaque	Creamish
13.	S_3B_6	Irregular	Convex	Erose	Smooth	Opaque	Creamish
14.	S_4B_1	Regular	Convex	Entire	Smooth	Opaque	Creamish
15.	S_4B_2	Regular	Convex	Entire	Smooth	Opaque	Off white
16.	S_4B_3	Punctiform	Flat	Entire	Smooth	Opaque	Yellowish

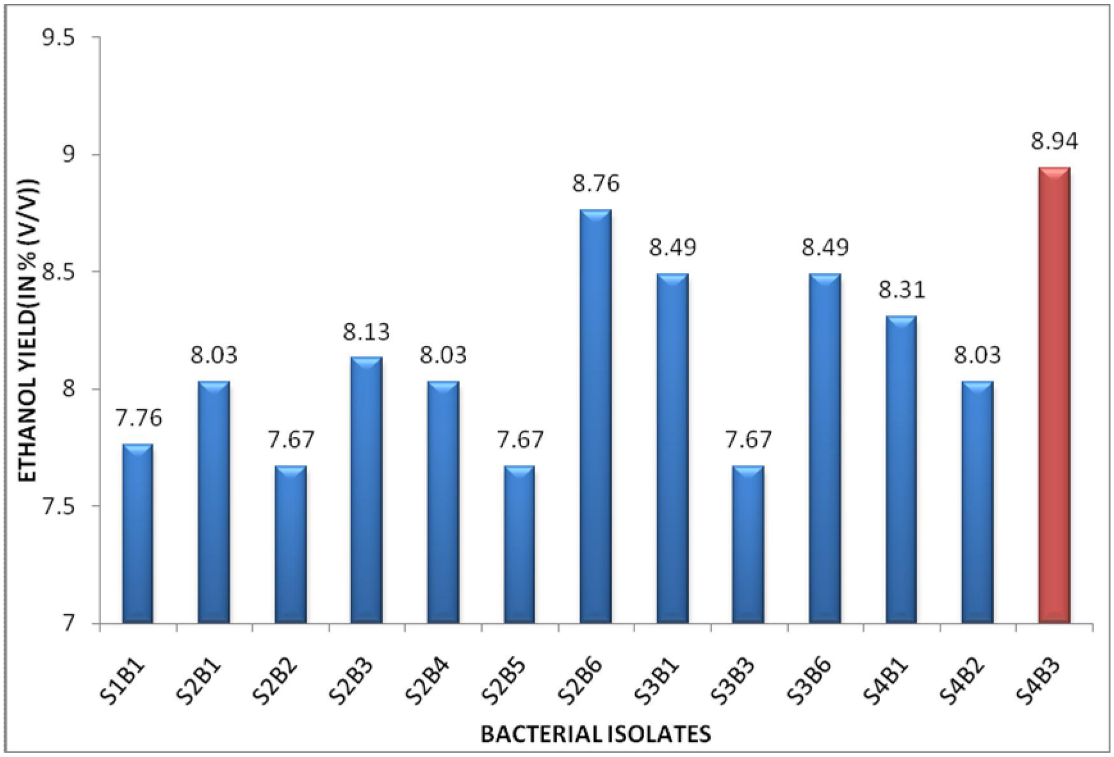

Figure 1. Bioethanol Production from Mahua Flowers by Soil Isolated Bacteria

4. Conclusion

Bioethanol production from mahua flowers, a renewable forest product with no extra cost for cultivation (except collection, transportation and storage) has an advantage over other sugar crops which are cultivated in fertile agricultural land with substantial input of fertilizers, pesticides and provision for irrigation, which together account for higher cost of ethanol production. Bioconversion of lignocellulosic biomass to sugars is a much more complicated process that require breakdown of lignin, cellulose and hemicelluloses fractions by application of a variety of physical and chemical or by biological means but for the production of ethanol from mahua flowers, no harsh pretreatment is necessary as it contains very little polymeric sugars, thereby also reducing the cost of ethanol production. The importance of this study is that it has been able to screen soil bacteria for production of bioethanol from a cheap carbohydrate substrate with appreciable fermentation ability naturally. The strain could be genetically manipulated under suitable environment for higher yield of ethanol.

Acknowledgement:

The authors are thankful to University Grant Commission, (Letter No. F. No. 39-217/2010 dated 06/01/2011) New New Delhi, India for providing financial assistance in carrying out the research work in School of Studies in Biotechnology, Raipur, India.

Correspondence to:
Dr. S.K. Jadhav
Professor and Head,
School of Studies in Biotechnology,
Pandit Ravishankar Shukla University,
Raipur-492010, Chhattisgarh, India
Email: shailesh_07@sify.com
Contact No: +919827114218

References

1. BRDI (Biomass Research Development Initiative). Vision for bioenergy and biobased products in the United States, 2006.
2. Brooks AA. Ethanol production potential of local yeast strains isolated from ripe banana peels. African Journal of Biotechnology 2008; 7(20):3749-3752.
3. Behera S, Mohanty RC, Ray RC. Ethanol production from mahula (Madhuca latifolia L.) flowers using free and immobilized (in Luffa cylindrical L. sponge discs) cells of Zymomonas mobilis MTCC 92. Ann Microbiol 2011; 61:469–474.
4. Benerji DSN, Rajini K, Srinivasa Rao B, Banerjee DRN, Swaroopa Rani K, Rajkumar G, Ayyanna C. Studies on Physico-Chemical and Nutritional Parameters forthe Production of Ethanol from Mahua Flower (Madhuca indica) Using

Saccharomyces cerevisiae – 3090 Through Submerged Fermentation (smf). Journal of Microbial & Biochemical Technology 2010;2(2):46-50.
5. Dien BS, Cotta MA, Jeffries TW. Bacteria engineered for fuel ethanol production current Status. Appl. Microbiol. Biotechnol. 2003; 63:258-266.
6. Jones ER. Jones reagent. J. Chem. Soc. 1953; 457: 2548-3019.
7. Jadhav SK, Tiwari KL, Renjini. Production of bioethanol from Mahua flower, Bioethanol a bioenergy resource. LAP Lambert academic publishing 2011;1-76.
8. Mohanty SK, Behera S, Swain MR, Ray RC. Bioethanol production from mahula (Madhuca latifolia L.) flowers by solid-state fermentation. Applied Energy 2009;86: 640-644.
9. Pharmacopoeia of India. The Indian Pharmacopoeia, controller of publications, 1985;3(2):113-115.
10. Prescott LM, Harley JP, Klein D. Microbiology. Wm. C. Brown publication. 2000; 1-992.
11. Patel M, Naik SN. Flowers of Madhuca indica J.F. Gmel. Present status and future Perspectives. Indian Journal of Natural products and resources 2010;1(4): 438-443.
12. Swain MR, Kar S, Sahoo AK, Ray RC. Ethanol fermentation of mahula (Madhuca latifolia L.) flowers using free and immobilized yeast Saccharomyces cerevisiae. Microbiology Research 2007;162: 93-98.
13. The Wealth of India. A Dictionary of Indian Raw Materials and Industrial Products - Raw Materials Series, Publications and Information Directorate. Council of Scientific & Industrial Research 1962; 6: 207-16.
14. Tiwari KL, Jadhav SK, Tiwari S. Studies of Bioethanol Production from Some Carbohydrate Sources by Gram Positive Bacteria. Journal of Sustainable Energy & Environment 2011; 2:141-144.
15. Tiwari S, Jadhav SK, Tiwari KL. Production of Bioethanol From "Jatropha Oil Cake." Researcher 2012; 4(7): 7-10.
16. Walker GM.Bioethanol:Science and technology of fuel alcohol. Graeme M. Walker and Ventus Publishing. 2010; 1-114.
17. Yadav JB. Advanced practical physical chemistry, Goel publishing house unit of Krishna prakashan media 2003; 52-56.
18. Millati R, Edebo L, Taherzadeh MJ. Performance of *Rhizopus, Rhizomucor*, and *Mucor* in ethanol production from glucose, xylose, and wood hydrolyzates. Enzyme Microbial Technology 2005; 36:294 - 300.

10/22/2013