

Bioethanol Production from *Shorea robusta* (Sal) Seeds using *Zymomonas mobilis* MTCC92

Ankita Choudhary, Shubhra Tiwari*, Shailesh Kumar Jadhav and Kishan Lal Tiwari

School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh State, India

** Corresponding author: shubhratiwari238@yahoo.co.in*

Received July 17, 2015; Accepted April 8, 2016

Abstract

Bioethanol is a viable alternative to fossil fuels. It is an alcohol fermented from sugars, starches or from cellulosic biomass. Bioethanol is a renewable liquid fuel for motor vehicles because it displaces the use of fossil fuels by recycling the carbon dioxide that is released when it is combusted as fuel. The objective of the work is to produce bioethanol from *Shorea robusta* (Sal) seeds using *Zymomonas mobilis* MTCC92. The effects of incubation period, temperature, pH and nutrients were evaluated. It was found that after 72 hours of fermentation at temperature 37 °C, pH 4 the production of bioethanol was enhanced and addition of sulphur and phosphorus supported the bioethanol production.

Key Words: Bioethanol; Lignocellulose; *Shorea robusta*; *Zymomonas mobilis*

Introduction

Increasing uncertainty of petroleum supplies due to rising demand, decline in known reserves, and concerns over global warming and green house emissions associated with fossil fuel usage has driven interest in biofuels. The major obstacle of today's world is the global climate change. Carbon dioxide (CO₂) is the major greenhouse gas that traps the earth's heat and contributes to climate change (Fogarty and McCally, 2010). Bioethanol has emerged as the most suitable renewable alternatives to fossil fuel as their quality constituents match diesel and petrol (Tiwari et al., 2013). CO₂ emissions from road traffic worldwide will increase by 92% between 1990 and 2020 (Nejadkoorki et al., 2008). Bioethanol contains 35% oxygen that helps to complete combustion of the fuel which reduces particulate and NO_x emissions (Saini et al., 2014). Bioethanol has a higher octane number, higher flame speeds, broader flammability limits and higher heat of vaporization which give it a higher compression ratio and shorter burn time, which lead advantages over gasoline in internal combustion engine (Balat and Balat, 2009). Bioethanol can be categorized in different

generations, first generation bioethanol is made from carbohydrate content feedstock like corn, sugar beet, sugarcane, barley. Second generation feedstock includes the non-edible and non-food biomass such as stalks of corn, grass, wood chips, old paper, bagasse, municipal solid waste, agricultural residues. Third generation feedstock includes the microalgae and macroalgae seaweeds. Bioethanol production from the food crops develops concerns about its production, increased food prices, the large amount of arable land required for crops, as well as the energy and pollution balance of the whole cycle of ethanol production thus, insufficient to replace the considerable portion of fossil fuel demands thus making a negative impact on the biodiversity (Hagerdal et al., 2006). These concerns have encouraged searching for feedstocks that contribute to environmental sustainability (Tewfik, 2004). Lignocellulosic polymers are the most abundant raw materials that accounts for 90% of dry weight of plants and being outside the human food chain makes cellulosic materials relatively inexpensive inputs for ethanol production. Cellulosic materials are comprised of lignin, hemicelluloses, and cellulose and are thus sometimes called lignocellulosic

materials. Cellulose molecules consist of long chains of (β 1 \rightarrow 4) glucose molecules (6-carbon sugars). The structural characteristics plus the encapsulation by lignin makes cellulosic materials more difficult to hydrolyze than starchy materials. Cellulosic ethanol offers promise because cellulose fibers, a major and universal component in plant cell walls, can be used to produce ethanol and the recent developments and commercialization may allay some of these concerns (Demirbas, 2005). According to the International Energy Agency, cellulosic ethanol could allow ethanol fuels to play a much bigger role in the future than previously thought (Inderwildi and King, 2009). No other sustainable option for the production of transportation fuels can match ethanol from lignocellulosic biomass (Pimentel and Patzek, 2005). Therefore, efforts have been made to improve the existing technologies through the raw materials and alternate strains for bioethanol production.

Different lignocellulosic materials such as *Jatropha* oil cake (Tiwari et al., 2012), *Azolla* (Pandey et al., 2013), De-oiled rice bran (Beliya et al., 2013), fruit wastes (Tiwari et al., 2014), rice bran (Tiwari et al., 2015) has been utilized for bioethanol production. *Shorea robusta* (Sal) seeds have high content of carbohydrates which makes them a potential source for bioethanol production. *Shorea robusta* belongs to the family Dipterocarpaceae, has an important role in economy in Jharkhand, Bihar, Orissa, Chhattisgarh and Madhya Pradesh. Sal seeds contain fat and triglycerides and serve as an ingredient for products like vanaspati, oil, soap and cocoa butter. The chemical composition of *Shorea robusta* (Sal) seeds is 62% carbohydrates, 8% protein, 14.8% oil, 1.4% fiber, 2.3% ash and 10.8% water. The production of bioethanol greatly depends on the efficient strains used for the production. *Saccharomyces cerevisiae* is the major ethanol producing microorganism used worldwide. Despite of several advantages, its high aeration cost, high biomass production, low temperature and ethanol tolerances are some of the drawbacks (Saigal, 1993). Various bacteria like *Z. mobilis*, *Klebsiella oxytoca* and fungi like *Trichoderma* and *Aspergillus* sp.

can also produce bioethanol (Tiwari et al., 2013). *Z. mobilis*, gram negative anaerobic bacteria has emerged as a potential and unique bacterium reported for maximum bioethanol production (Thauer et al., 1977; Dumsday et al., 1997). *Z. mobilis* has a high specific rate of sugar uptake, high ethanol yield, low biomass production and non-requirement of controlled addition of oxygen to maintain the viability of the cells (Rogers et al., 1980; Gunasekaran et al., 1990). Ethanol production from lignocellulosic materials involves the degradation of the lignocellulosic structure to a fermentable substrate followed by fermentation and distillation of the fermentation broth to obtain 95% ethanol (Olsson and Higerdal, 1996).

The aim of present work is, bioethanol production from Sal seeds which is abundantly available in State Chhattisgarh, India. The optimization of leading parameters such as incubation time, pH, temperature and nutrients in production process.

Materials and Methods

Collection of sample

The sample *Shorea robusta* (Sal) seeds were collected from Village Sargipal, Bakawand range, Jagdalpur forest region, Chhattisgarh state, India. The seeds were grinded to form smooth powder which is used as a substrate for bioethanol production.

Media for inoculum

Z. mobilis MTCC92 culture was obtained from School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur Chhattisgarh state, India. The inoculum was maintained in *Zymomonas* Specific Medium (ZSM) (g/L): glucose 100 g, yeast extract 2 g, urea 1 g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g and agar 15 g and the pH was adjusted to 6.5. The culture was stored at 4 ± 0.5 °C for further use (Behera et al., 2010).

Preparation of starter culture

100 ml growth medium (as mentioned above but without agar) was taken in sterilized (at 121°C for 20 min) 250 ml Erlenmeyer flask. The flask containing the medium was inoculated with a loopful of *Z. mobilis* culture and incubated at 30 °C for 24 hours under stationary conditions. This served as the starter culture for ethanol production.

Fermentation

20 gm powdered seeds was taken in 200 ml of distilled water and autoclaved at 121 °C at 15 psi. 5% (v/v) of *Z. mobilis* MTCC92 was inoculated and incubated for the fermentation process.

Estimation of Bioethanol

Qualitative estimation

(a) Qualitative estimation was done by Jones reagent [$K_2Cr_2O_7 + H_2SO_4$] test. 2ml of $K_2Cr_2O_7$ (2%), 1 ml of conc. H_2SO_4 was added to 1ml of fermented sample. Ethanol oxidizes to acetic acid with potassium dichromate in the presence of sulphuric acid and gives blue green color (Bowden et al., 1946).

(b) Quantitative estimation of bioethanol was done by specific gravity method. Specific gravity refers to the density of any liquid (Pharmacopoeia of India, 1985). Twenty five millilitres fermented sample was mixed with distilled water (make up the volume 150 ml) and this mixture was distilled on distillation unit. After distillation ethanol percentage was calculated by specific gravity method (Yadav, 2003). Percentage in v/v was obtained from the standard table correlating percentage volume of ethanol with specific gravity at 25 °C. Each step was repeated three times. All the values are mean \pm standard error, values differ significantly at 5% as analyzed by Duncan multiple Range Test by SPSS.

$$\rho = \frac{W3 - W1}{W2 - W1} \times \text{Density of water at } t \text{ } ^\circ\text{C}$$

Where ρ = specific gravity, W1 = weight of empty specific gravity bottle, W2 = weight of empty bottle + distilled water, W3 = weight of empty bottle + fermented liquid

Effect of incubation period on bioethanol production

Incubation period is an important parameter which affects the process of fermentation. To study the effect of incubation period on bioethanol production the sample was incubated and distilled on every 24, 48, 72 and 96 hrs of fermentation.

Effect of temperature on bioethanol production

Temperature is one of the most important factor affecting production either by enhancing or inhibiting

the process. The study of temperature effects on the production was evaluated at 31 °C, 34 °C, 37 °C, 40 °C and 43 °C.

Effects of pH on bioethanol production

pH has a marked effect on the enzymatic activity thus influencing the production. The effect of pH was studied by ranging the initial pH from 3, 4, 5, and 6 to evaluate the effect on the bioethanol production process.

Effect of nutrients on bioethanol production

Nutrients are the most important constituents for the growth of microorganisms. The effect of different nutrients was studied using nitrogen in the form of urea, phosphorus in the form of potassium dihydrogen phosphate (KH_2PO_4) and sulphur in the form of ammonium sulphate (NH_4SO_4). All nutrients were prepared 1% stock solution (1 g in 100 ml of distilled water) and inoculated in autoclaved samples with *Z. mobilis* MTCC92 inoculum.

Results and Discussions

Effects of incubation period on bioethanol production

In the present study bioethanol was produced from *Shorea robusta* seeds by *Z. mobilis* MTCC92. On the study of effects of incubation time, it was observed that on 24 hrs, the production was $6.8 \pm 0.5\%$, $8.6 \pm 0.4\%$ on 48 hrs, $10.0 \pm 0.0\%$ on 72 hrs and $7.8 \pm 0.1\%$ on 96 hrs (Figure 1). The results showed that the maximum bioethanol was produced on 72 hrs of incubation period. Yoswathana et al. (2010) found bioethanol production from rice straw that was maximum at 72 hrs of fermentation.

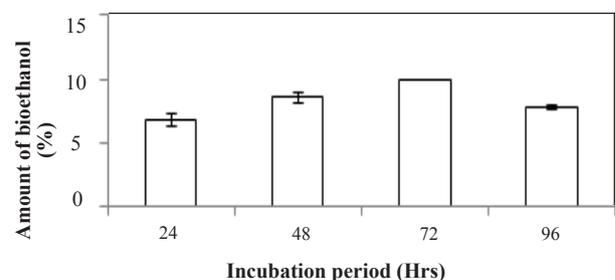


Figure 1 Amount of bioethanol at different incubation period.

Effect of temperature on bioethanol production

Temperature is one of the most important environmental factors affecting microbial activity (Torija et al., 2003). Temperature showed a varied effect on the production of bioethanol. The production of bioethanol at different temperatures was $9.8 \pm .0\%$ at $31\text{ }^{\circ}\text{C}$, $8.3 \pm .3\%$ at $34\text{ }^{\circ}\text{C}$, $10.0 \pm .5\%$ at $37\text{ }^{\circ}\text{C}$, $9.0 \pm .0\%$ at $40\text{ }^{\circ}\text{C}$ and $5.5 \pm .4\%$ at $43\text{ }^{\circ}\text{C}$ (Figure 2). The maximum production was recorded at $37\text{ }^{\circ}\text{C}$. Lee et al. (1997) studied the biological conversion of lignocellulosic biomass to ethanol and found that the recombinant strain utilizing the *Z. mobilis* genes produced the maximum ethanol at $37\text{ }^{\circ}\text{C}$. Tiwari et al. (2012) studied production of bioethanol from jatropa Oil Cake and found $37\text{ }^{\circ}\text{C}$ was optimum for bioethanol production. Tiwari et al. (2010) studied the effect of temperature variation in the bioethanol production process and found the $40\text{ }^{\circ}\text{C}$ optimum for the process. Tofighi et al. (2014) found similar results with a novel autochthonous thermo-tolerant yeast isolated from wastewater with the optimum temperature over $35\text{ }^{\circ}\text{C}$.

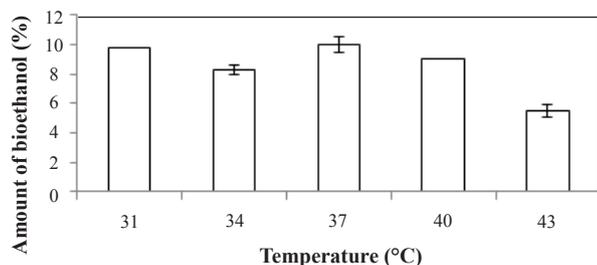


Figure 2 Amount of bioethanol at different temperature.

Effect of pH on bioethanol production

During study of the pH (3, 4, 5, 6), it was found that the production was $9.6 \pm 0.0\%$ at pH 3, $9.8 \pm 0.1\%$ at pH 4, $8.3 \pm 0.3\%$ at pH 5 and $7.2 \pm 0.3\%$ at pH 6 (Figure 3). Maximum bioethanol production was obtained at pH 4 and further bioethanol production starts decreasing by further increasing in pH. (Periyasamy et al., 2009) obtained the highest ethanol production at pH 4 from substrate molasses using *Saccharomyces cerevisiae*. Asli (2010) obtained maximum bioethanol production at pH 4.5 using *Saccharomyces cerevisiae* SCI. (Tahir et al., 2010) studied the bioethanol production from molasses and

found the maximum production at pH 4.5.

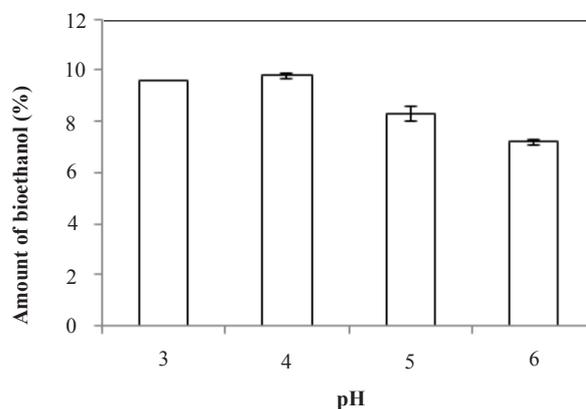


Figure 3 Amount of bioethanol at different pH

Effect of nutrients on bioethanol production

The observed production was $4.7 \pm 0.4\%$ with urea as a nitrogen source, $8.6 \pm 0.1\%$ with KH_2PO_4 as phosphorus source and $8.6 \pm 0.5\%$ with NH_4SO_4 as a sulphur source. It was observed that there was a decrease in the amount of production because the sugar present is chiefly utilized as a source of carbon and energy and hence the lignocellulosic hydrolysates need limited nutrients for the production process. The lignocellulosic biomass have low amount of nitrogen (Jorgensen, 2009). The previous work reports that, the amount nitrogen present slows the process of fermentation (Jones and Ingledew, 1994) and this lowered the amount of fermentable sugars and decreased the concentration of ethanol produced. The similar result was observed by (Laopaiboon et al., 2009) that the addition of $(\text{NH}_4)_2\text{SO}_4$ during fermentation resulted in a lower ethanol production as compared to the cultivations without nitrogen source. They concluded that addition of ammonium produced by-products that utilize the carbon sources resulting in the decrease in bioethanol production.

Conclusions

Lignocellulosic materials represent abundant feedstocks for bioethanol production. It was observed that Sal seeds might serve as a good source of bioethanol production and *Z. mobilis* was the suitable bacteria for the production with this feedstock. For the bioethanol production from Sal seeds the

different parameters of incubation time, temperature, pH and nutrients were important aspects for bioethanol production. It was concluded that the incubation time of 72 hours, temperature 37 °C and pH 4 and supplementation of phosphorus and sulphur nutrients supported bioethanol production to some extent.

Acknowledgment

Authors are thankful to Head, School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur Chhattisgarh State, India, for his valuable guidance, support and facilities provided during the research work.

References

- Asli, M. S. (2010) A study on some efficient parameters in batch fermentation of ethanol using *Saccharomyces cerevisiae* SCI extracted from fermented saihe sardast pomace. *African Journal of Biotechnology* 9(20): 2906-2912.
- Balat, M. and Balat, H. (2009) Recent trends in global production and utilization of bio-ethanol fuel. *Applied Energy* 86(11): 2273-2282.
- Beliya, E., Tiwari S., Jadhav, S. K., and Tiwari, K. L. (2013) De-oiled rice bran as a source of bioethanol. *Energy Exploration and Exploitation* 31(7): 771-782.
- Behera, S., Mohanty, R. C. and Ray, R. C. (2010) Ethanol fermentation of mahula (*Madhuca latifolia*) flowers using free and immobilized bacteria *Zymomonas mobilis* MTCC 92, *Biologia* 65(3): 416-421.
- Bowden, K., Heliron, I. M., Jones, E. R. H., and Weedon., B. C. L. (1946) Researches on Acetylenic compounds Part 1. The preparation of acetylenic ketones and oxidation of acetylenic carbinols and glycols. *Journal of the Chemical Society*: 39-45.
- Demirbas, A. (2005) Bioethanol from cellulosic materials: A renewable motor fuel from biomass. *Energy Sources* 27: 327-337.
- Dumsday, G. J., Jones, K., Stanleyand., G. A., and Pamment, N. B. (1997) Recombinant organisms for ethanol production from hemicellulosic hydrolysates, a review progress. *Australian Biotechnology* 7: 285-295.
- Fogarty, J. and McCally, M. (2010) Health and safety risks of carbon capture and storage. *JAMA* 303: 67-69. Gunasekaran, P., Krunakaran, T., Kamini, N. R., and Mukundan, A. (1990) Current status and prospects of an ethanol producer *Zymomonas mobilis*. *Indian Journal of Microbiology* 30:107-133.
- Hagerdal, H. B., Gale, M., Gorwa-Grauslund, M. F., Liden, G. and Zacchi, G. (2006) Bioethanol: The fuel of tomorrow from the residues of today. *Trend Biotechnology* 24(12): 549-556.
- Inderwildi, O. R. and King, D. A. (2009) Quo Vadis biofuel. *Energy and Environmental Science* 2: 343-346.
- Jones, A. M. and Ingledew, W. M (1994) Fuel alcohol production: Appraisal of nitrogenous yeast foods for very high gravity wheat mash fermentation. *Process Biochemistry* 29: 483-488.
- Jorgensen, H. (2009) Effect of nutrients on fermentation of pretreated wheat straw at very high dry matter content by *Saccharomyces cerevisiae*. *Applied Biochemistry and Biotechnology* 153: 44-57.
- Laopaiboon, L., Nuanpeng, S., Srinophakun, P., and Klanrit, P. (2009) Ethanol production from sweet sorghum juice using very high gravity technology: Effects of carbon and nitrogen supplementations. *Bioresource Technology* 100(1): 4176-4182.
- Lee, J. (1997) Biological conversion of lignocellulosic biomass to ethanol. *Journal of Biotechnology* 56: 1-24.
- Nejadkoorki, F., Nicholson, K., Lake, I., and Davies, T. (2008) An approach for modeling CO₂ emissions from road traffic in urban areas. *Science of the Total Environment* 406: 269-278.
- Olsson, L. and Higerdal, B. H. (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme and Microbial Technology* 18: 312-331.
- Pandey, A., Tiwari, S., Tiwari, K. L. and Jadhav, S. K. (2013) Bioconversion of lignocellulosic azolla into bioethanol. *Journal of Applied Phytotechnology in Environmental Sanitation* 2(2): 59-64.
- Periyasamy, S., Venkatachalam, S., Ramaswamy, S., and Srinivasam, V. (2009) Production of bioethanol from sugar molasses using *Saccharomyces cerevisiae*. *Modern Applied Science* 3(8): 32-37.
- Pharmacopoeia of India. (1985) *The Indian Pharmacopoeia*, 3rd ed., pp. 113-115. Controller of Publications, Government of India, Delhi.
- Pimentel, D. and Patzek, T. W. (2005) Ethanol production using corn, switchgrass, and wood; biodiesel production using soybean and sunflower. *Natural Resources Research* 14(1): 65-76.
- Rogers, P. L., Lee, K. J., and Tribe, D. E. (1980) High productivity ethanol fermentations with *Zymomonas mobilis*. *Process Biochemistry* 15: 7-11.
- Saigal, D. (1993) Yeast strain development for ethanol production. *Indian Journal of Microbiology*, 33 :159-168.

- Saini, J. K., Saini R., Tewari, L. (2015). Lignocellulosic agriculture wastes as biomass feedstocks for second generation bioethanol production: concepts and recent developments. *3Biotech*.5:337-353.
- Tahir, A., Aftab M., and Farasat T., (2010). Effect of culture conditions on ethanol production by locally isolated *Saccharomyces cerevisiae* Bio-07. *Journal of Applied Pharmacy*, 3(2): 72-78.
- Tewfik, S. R. (2004). Biomass utilization facilities and biomass processing technologies. *Energy Education Science Technology*, 14: 1–19.
- Thauer, R. K, Jungermann, K., and Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacterial Review* 41(1): 100-180.
- Tiwari, K. L, Jadhav, S. K., and Tiwari, S. (2010) The effect of temperature variation in the bioethanol production process. *Bioprocess Journal* 9(1): 53-54.
- Tiwari, S., Jadhav, S. K., and Tiwari, K. L. (2012) Production of bioethanol from “Jatropha Oil Cake. *Researcher* 4(7): 7-10.
- Tiwari, S., Jadhav, S. K., and Tiwari, K. L. (2013) Comparative study of bioethanol production from different carbohydrate sources. *Researcher* 5(12): 219-221.
- Tiwari, S., Jadhav, S. K, Sharma, M., and Tiwari, K. L. (2014) Fermentation of waste fruit for bioethanol production. *Asian Journal of Biological Science* 7: 30-34.
- Tiwari, S., Jadhav, S. K., and Tiwari, K. L. (2015) Bioethanol production from rice bran with optimization of parameters by *Bacillus cereus* McR-3. *International Journal of Environmental Science Technology* 12: 3819-3826.
- Tofighi, A., Assadi, M. and Karizi, Z. (2014) Bioethanol production by a novel autochthonous thermo-tolerant yeast isolated from waste water. *Journal of Environmental Health Science Engineering*. 12:107.
- Torija, M. J., Rozes, N., Poblet, M., Guillamon, J. M., and Mas, A. (2003) Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 80: 47–53.
- Yadav, J. B. (2003) *Advanced practical physical chemistry*, Goel publishing house unit of Krishna Prakashan media, Meerut, pp. 52-56.
- Yoshwathana, N., Phuriphapat, P., Treyawutthiwat, P., and Eshtiaghi, M. (2010) Bioethanol production from rice straw. *Energy Research Journal* 1: 26-31.