Comparative study on Bioethanol production from Neem (*Azadirachta indica*) leaves using *Saccharomyces* spp. and *Bacillus* spp.

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ABSTRACT

The present study was aimed to investigate the potential of Neem tree leaves (*Azadirachta indica*) in bioethanol production by using *Saccharomyces spp.* and *Bacillus spp.*as fermenting organisms. Dried powdered leaves of neem tree (*Azadirachta indica*) were hydrolyzed using different concentrations of 5%, 10%, 15% and 20% sulfuric acid and also 1%, 2%, 5% and 10% sodium hydroxide. The hydrolyzed samples were then fermented using *Saccharomyces spp.* and *Bacillus spp*. After fermentation the broths formed were distillated to obtain ethanol. Acidified potassium dichromate was used to determine the bioethanol produced. Further, the quantification of produced bioethanol was carried out using UV spectrophotometer and the bioethanol produced was compared to check whether *Saccharomyces spp.* or *Bacillus spp.* produced more e ethanol. It was found that *Saccharomyces spp.* was producing more ethanol i.e. 6% when neem leaves were treated with 1% NaOH.

Keywords: *Azadirachta indica*, Bio-ethanol, *Saccharomyces spp.*, *Bacillus spp.*, acid hydrolysis, alkaline hydrolysis.

INTRODUCTION

The hydroxy derivatives of aliphatic hydrocarbons (compounds having their carbon atoms in chains and not in the form of rings) are called alcohols. Alcohol is among the most common organic compounds. They are used as sweeteners and in making perfumes, are valuable intermediates in the synthesis of other compounds and are among the most abundantly produced organic chemicals in industries. Perhaps the two best-known alcohols are ethanol and methanol (Puttaswamy *et al.*, 2016). Ethanol is miscible with water and is a good general purpose solvent. It is found in paints, tinctures, markers, and personal care products such as mouthwashes, perfumes and deodorants. However, polysaccharides precipitate from aqueous solution in the presence of alcohol, and ethanol precipitation is used for this reason in the purification of DNA and RNA. Also there are several advantages of ethanol amongst few are listed as follows, exhaust gases of ethanol are much cleaner, it burns more cleanly, the use of ethanol-blended fuels such as E85 (85% ethanol and 15% gasoline) can

reduce the net emissions of greenhouse gases by as much as 37.1%, which is a significant amount, any plant can be used for production of bioethanol; it only has to contain sugar and starch. The best choice is sugar cane, but potatoes, barley, wheat etc. can also be used, it is carbon neutral i.e. the carbon dioxide released in the bioethanol production process is the same amount as the one the crops previously absorbed during photosynthesis, ethanol is considered a renewable energy resource (Jaisamut *et al.,* 2013) because it is primarily the result of conversion of the sun's energy into usable energy, it benefits energy security as it shifts the need for some foreign-produced oil to domestically-produced energy sources. It reduces greenhouse gases, the fuel spills are more easily biodegraded or diluted to nontoxic concentrations. [advantages-of-bioethanol.html]. As ethanol can be used as fuel, disinfectant, in beverages, in pharmaceutical industries, etc. there is an increase in the demand of ethanol around the world (Behera *et al.,* 2013). Bioethanol seems to be an alternative fossil fuel as it is renewable energy source, also nontoxic, clean burning, biodegradable, etc.(Muhammad *et al.,* 2016; Mutreja *et al.,* 2011; Yaliwal *et al.,* 2015). Thus, the aim of this paper was to produce bioethanol from neem leaves by using yeast or bacteria and compare the potential of the yeast and bacteria for alcohol production by using neem leaves as raw material.

MATERIALS AND METHODS

Sample collection: commercially available dry neem powder was used for fermentation (Xin- Qing Zhao *et al.,* 2012).

Screening of *Bacillus spp.*: 7 soil samples were collected from different areas and were named accordingly (Gopinath *et al.,* 2015; Dey *et al.,* 2016). Further, 1 g soil sample was added in 5 ml of sterile saline and was given a heat shock treatment at 80°C for 10 mins. Total of four Gram positive short rods with spores *were* selected and were maintained on nutrient agar slants. Then the isolates were checked for production of different enzymes like cellulase, lipase, amylase and gelatinase on different media like cellulose agar, tributyrin agar, starch agar and gelatin agar. The *Bacillus* isolate showing high enzyme production on agar was selected for fermentation.

***Saccharomyces spp.*:** commercially available dry yeast was used (Muhammad *et al.,* 2014). The yeast was activated in sterile Sabouraud's broth by vortexing. The activated yeast was than maintained on Sabouraud's slant for further use.

Pretreatment Neem leaves powder with H_2SO_4 and NaOH pretreatment, sugar estimation and fermentation: 5 g of neem leaves powder was treated with 50 ml. of different concentration of H_2SO_4 i.e. 5%, 10%, 15% and 20%, likewise different concentration of NaOH i.e. 1%, 2%, 5% and 10% was used and further the sugar concentration was estimated using DNSA method.

Standard used was 1000mcg/ml of dextrose and absorbance was taken calorimetrically at 530 nm. Then each filtrate was inoculated with 1ml of *Bacillus* and *Saccharomyces* culture suspension and was incubated at 37°C for 5 days. After that distillation of each filtrate was performed at 73°C for 1 hr.

Qualitative test: 2 ml of 0.4M $K_2Cr_2O_7$ and 2ml of H_2SO_4 added in 1 ml of distillate, heated for 10 mins in boiling water bath and checked for colour change from yellow to green (Singh *et al.*, 2015).

Quantitative test: 50%ethanol was used as standard and the range used was 0.2 – 1.0 mg/ml. 2ml of 0.4M K2Cr2O7, 3.0 ml of distilled water and 2.0 ml of H2SO4 was added in each tube. U.V. spectrometer was used at 540 nm for absorbance. In the same way test sample was also estimated.

RESULT AND DISCUSSION

Screening and isolation of *Bacillus spp.*: Soil samples was collected from different locations at different time and were named as Tulsi soil 1 – TS1, Tulsi soil 2 – TS2, Curry soil – CS, Valerian soil – VS, Shivaji park garden soil – SPG, Sugarcane soil – SS and Garden soil – Garden. Well isolated colonies were selected and Gram staining was carried out. The colony characteristics of different selected isolates are given in Table 1.

Table 1: Colony characteristics of selected colonies on Nutrient agar plates

Sample	Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram characteristic
TS1-2	6mm	Circular	White	Entire	Flat	Opaque	Butyrous	Gram positive rods
TS2-1	4mm	Circular	White	Entire	Flat	Opaque	Butyrous	Gram positive rods
SS	4mm	Circular	White	Entire	Flat	Opaque	Butyrous	Gram positive rods
Garden	5mm	Circular	White	Entire	Flat	Opaque	Butyrous	Gram positive rods

Later, endospore staining of the selected colonies was done and all were found to be spore formers than the cultures were checked for its enzyme activity on different media like Gelatin agar for gelatinase, Starch agar for amylase, Tributyrine agar for lipase and Cellulose agar for cellulase enzyme production. The enzymatic activities of different isolates are given in Table 2.

Table 2: Enzyme activiies enzymatic activities of different isolates

Enzyme	Culture	Zone in mm
Amylase	Garden	10
	SS	-
	TS1-2	6
	TS2-1	-
Gelatinase	Garden	-
	SS	-
	TS1-2	-
	TS2-1	-
Lipase	Garden	-
	SS	4
	TS1-2	6.5
	TS2-1	8.5
Cellulase	Garden	5
	SS	-
	TS1-2	3.5
	TS2-1	-

As the garden soil culture was showing maximum enzyme activity it was used for carrying out further studies. The dry yeast was activated and Gram staining of the culture was performed and it was found to be Gram positive oval shaped.

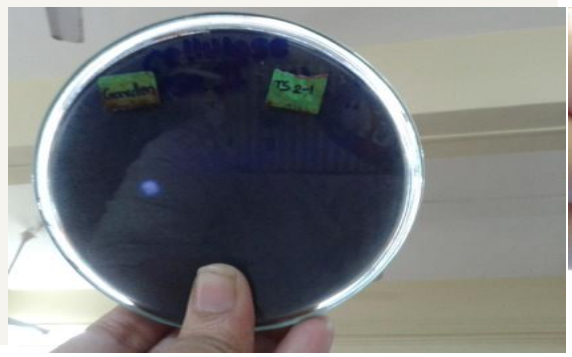

Fig 1: Zone of clearance on cellulose agar

Fig 2: Zone of clearance on starch agar

Fig 3: *Saccharomyces spp.*fermentation after 5 days incubation

Quantitative estimation of ethanol: Qualitatively the ethanol was estimated using potassium dichromate. Ethanol was found to be present in all test samples. Quantitatively ethanol was estimated using potassium dichromate method. UV spectrophotometer was used at 540 nm.

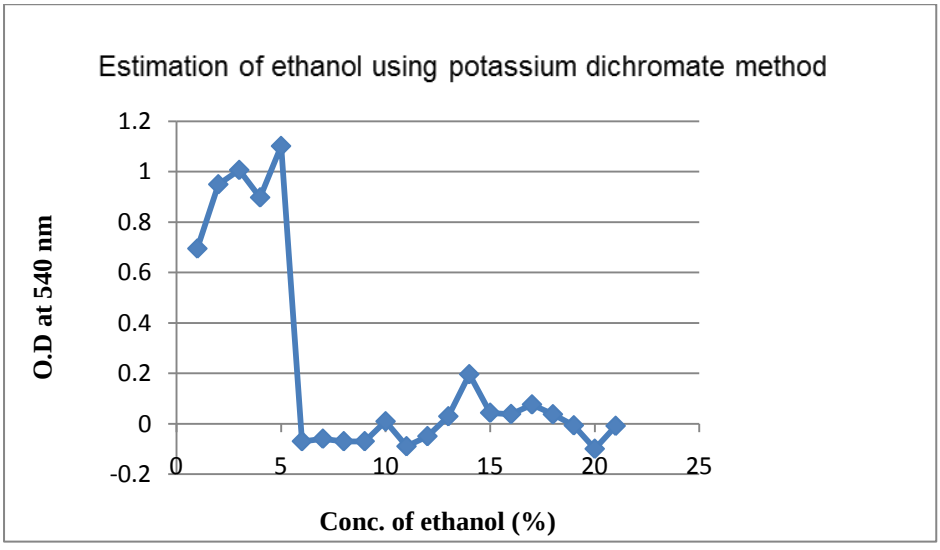

Fig 5: Ethanol estimation by using dichromate method

Table 4: Estimation of ethanol using potassium dichromate method

Conc. of ethanol (%)	Stock (ml)	Diluent – D/W (ml)	Total vol.(ml)	$K_2Cr_2O_7$ (ml)	D/W (ml)	Conc. H_2SO_4 (ml)	O.D. at 540 nm	Conc of Bioethanol (%)
Blank	-	1.0	1.0	2.0	3.0	2.0	-	-
10	0.2	0.8	1.0	2.0	3.0	2.0	0.695	10
20	0.4	0.6	1.0	2.0	3.0	2.0	0.950	20
30	0.6	0.4	1.0	2.0	3.0	2.0	1.007	30
40	0.8	0.2	1.0	2.0	3.0	2.0	0.898	40
50	1.0	-	1.0	2.0	3.0	2.0	1.102	50
Test-H_2SO_4 5%-1	1.0	-	1.0	2.0	3.0	2.0	-0.07	-
5%-2	1.0		1.0	2.0	3.0	2.0	-0.06	-
10%-1	1.0	-	1.0	2.0	3.0	2.0	-0.07	-
10%-2	1.0	-	1.0	2.0	3.0	2.0	-0.07	-
15%-1	1.0	-	1.0	2.0	3.0	2.0	0.009	1
15%-2	1.0	-	1.0	2.0	3.0	2.0	-0.09	-
20%-1	1.0	-	1.0	2.0	3.0	2.0	-0.05	-
20%-2	1.0	-	1.0	2.0	3.0	2.0	0.029	1
Test-NaOH	-							
1%-1	1.0	-	1.0	2.0	3.0	2.0	0.196	6
1%-2	1.0	-	1.0	2.0	3.0	2.0	0.043	2
2%-1	1.0	-	1.0	2.0	3.0	2.0	0.038	1
2%-2	1.0	-	1.0	2.0	3.0	2.0	0.076	3
5%-1	1.0	-	1.0	2.0	3.0	2.0	0.037	1
5%-2	1.0	-	1.0	2.0	3.0	2.0	-0.007	-
10%-1	1.0	-	1.0	2.0	3.0	2.0	-0.10	-
10%-2	1.0	-	1.0	2.0	3.0	2.0	-0.009	-

CONCLUSION

Comparison of yield was done on production of ethanol by fermenting agent *Saccharomyces spp.* and *Bacillus spp.* With H_2SO_4treatment *Saccharomyces spp.* could produce 1% ethanol at 15% conc. of $H_2SO_4$and *Bacillus spp.* also produced 1% ethanol at 20% conc. of H_2SO_4. With NaOH treatment *Saccharomyces spp.* produced 6% ethanol at 1% conc. of NaOH, 1% ethanol at 2% & 5% conc. of NaOH. *Bacillus spp.*produced 2% ethanol and 3% ethanol at 1% and 2% conc. of NaOH respectively. *Saccharomyces spp.* produced more ethanol i.e. 6% when treated with 1% NaOH which could then be used for large scale production of ethanol.

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