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BIOETHANOL PRODUCTION FROM NEEM LEAVES BY *Bacillus firmus*

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ABSTRACT

This study was investigated with the aim of evaluating the biofuel production from neem leaves *(Azadirachta indica).*There are many sources used for the production of ethanol but we tried to find out a new source for ethanol production from plant leaves. Bioethanol mainly produced from biological methods involving fermentation of cellulosic biomass. The bulk amount of neem leaves were hydrolyzed by *Bacillus firmus* and the sugar content was analysed by Anthrone method. The hydrolysed filtrate samples were sterilised and inoculated with the same microbes in different concentration for 5 days. After incubation, the broths were distillated to obtain ethanol. The quality of ethanol was determined by acidified $K_2Cr_2O_7$ and other tests also. The results showed that the significantly increase in the level of ethanol in 1.5 ml/30g flask and decreased further. Hence, the study proved that the neem leaves could be used as an alternative to fossil fuel. So the waste sources can serve as an efficient substrate for ethanol production.

KEY WORDS: Enzymatic hydrolysis, *Bacillus firmus*, Anthrone reagent, Cellulase, *Azadirachta indica.*

INTRODUCTION

Bioethanol is one such fuel which exhibits several advantages over conventional fossil fuel (Kumar et al., 2009; He et al., 2014). This includes its renewable nature, easy storage, higher oxygen content, higher octane number. The fact that it is free of sulphur and it contributes less to global warming and air pollution (Junchen, 2012). In early days, bioethanol production is derived from edible sources like starch, sugar crops, fruit juices, etc. These above said crops can grow renewably in almost all climates around the world with lower emission of green house gases (Brooks, 2008; Rabah, 2011). Balat et al., (2008) suggested that the production of bioethanol from non edible raw materials and also as good alternative to other fuels. Heinrich (2005) was reported that the neem tree has contained 23% carbohydrate yield higher ethanol than the edible sources. The goal of this study is to increase the bioethanol production from dry leaves of neem and it can be transformed to sugar in a short period using *Bacillus firmus* which produced high activity of cellulase against the cellulose (Hari Krishna, 1997). The evolution of new biofuel production technologies could help to alleviate the use of organic content of municipal solid waste as a transportation fuel feedstock and associated with reduce waste disposal.

MATERIALS AND METHODS

Collection of samples

The leaves of *Azadirachta indica* and soil samples were collected from our campus and cleaned the leaf surface with distilled water.

Isolation and characterisation of bacterial isolates

The *Bacillus firmus* was isolated from the soil samples by plating method. The isolate was characterized by biochemical tests such as IMViC, Carbohydrate fermentation, Catalase, Oxidase, Urease and Nitrate. The culture was maintained for further experimental works.

Inoculum preparation

A flask containing 100ml of nutrient agar was inoculated with the *Bacillus firmus* and incubated for 24 hours in an aerobic condition.

Enzymatic hydrolysis

Powdered form of neem leaves were added with distilled water in the ratio of 1:10. Then the flasks were wrapped with aluminium foil and sterilised. Each flask

was inoculated with different concentration of cell suspension (1ml, 1.5ml, 2ml) and incubated for 5 days at 37^0 C.

Sugar concentration determination

A series of glucose standard tubes were prepared and their readings noted at 620nm. Then the hydrolysate filtrate samples were treated with 4ml of anthrone reagent and kept in a boiling water bath for 10 minutes to develop dark blue colour. The absorbance of each test tube was measured at 620nm. The concentration of standard and filtrate samples were plotted (Akpinar, 2009; Rabah, 2011).

Conversion of bioethanol

In this analysis, the hydrolysed filtrate samples were used as fermentation medium inoculated with *Bacillus* and there was no addition of supplements for 5 days. After that, the broth obtained was distilled to get pure ethanol (Oyeleke and Jibrin, 2009).

Analytical method

Ethanol and reducing sugar content in the hydrolysates were determined by acidified potassium dichromate. The content of the tube was changed to green colour that indicates the presence of ethanol in distillate and absence of green colour in bottom settled filtrate samples. The quality of ethanol was determined by adding potassium dichromate and the absorbance of each concentration was measured at 580nm.

RESULT AND DISCUSSION

In this study, the biochemical characteristics of *Bacillus firmus* was checked by starch hydrolysis, IMViC tests. Here, *Bacillus* showed negative result for starch, indole, VP, citrate and no acid gas production from glucose. The remaining tests were positive result.

Estimation of sugar

The standard graph has been plotted by using glucose as working standard solution (100ug/ml) for estimating the reducing sugar level. The values have shown in table 1 and figure1. The sugar content of hydrolysed neem leaves have been calculated by comparing their optical density values with the standard graph. The neem leaves were hydrolysed with 1.5ml inoculum suspension release the highest reducing sugar value (1.81) followed by 2ml (1.53) and 1ml (1.18). No significant variation was observed using different concentration of *Bacillus firmus.* Among the three extract, the results showed that the glucose level was high in 1.5ml of inoculum containing flask.

Table 1: Estimation of sugar by Anthrone method

Volume of standard glucose (ml)	Volume of distilled water (ml)	Volume of Anthrone reagent (ml)	Absorbance at 620nm
0	3	4 ml	0
0.3	2.7	4 ml	0.59
0.6	2.4	4 ml	1.23
0.9	2.1	4 ml	1.65
1.0	2	4 ml	1.70
1.2	1.8	4 ml	1.82

Table 1.1: Estimation of sugar in neem leaves by Anthrone method after fermentation

Volume of standard glucose (ml)	Volume of distilled water (ml)	Volume of Anthrone reagent (ml)	Absorbance at 620nm
(1.0) 3ml	-	4 ml	1.18
(1.5) 3ml	-	4 ml	1.81
(2.0) 3ml	-	4 ml	1.53

Confirmation test

The presence of ethanol in all the flasks were performed and confirmed by Litmus and Lucas tests. In Litmus test there was no colour change and precipitation like coagulation formed in Lucas test.

Qualitative and quantitative test for ethanol

The concentration of ethanol was determined by potassium dichromate method. The highest yield of ethanol was reported in 1.5ml inoculum suspension containing flask (1.81nm). According to the value of colorimetry reading, 2ml and 1ml flasks were reported as low ethanol producer than 1.5ml.

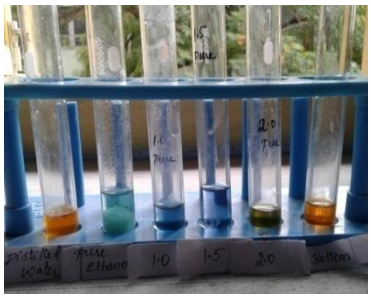

Table 2: Quality test for ethanol production

Volume of standard glucose (ml)	Volume of distilled water (ml)	Volume of $K_2Cr_2O_7$ reagent (ml)	Absorbance at 580nm
0.3	2.7	2 ml	0.16
0.6	2.4	2 ml	0.18
0.9	2.1	2 ml	0.20
1.2	1.8	2 ml	0.31
1.5	1.5	2 ml	0.32
2.0	1.0	2 ml	0.36

Table 2.1: Quality test for ethanol production in different inoculum suspension

Volume of unknown samples (ml)	Volume of distilled water (ml)	Volume of $K_2Cr_2O_7$ reagent (ml)	Absorbance at 580nm
(1.0) 3ml	-	2 ml	0.03
(1.5) 3ml	-	2 ml	0.43
(2.0) 3ml	-	2 ml	0.23

CONCLUSION

The population of human being is increasing in every year, hence the demand for energy source increases. The production of ethanol from these neem leaves can be further improved by various technologies. From the experimental results, the neem leaves showed high alcohol yield at low amount of sugar. After distillation, the parameters were considered for the production of ethanol. According to the results, *Bacillus firmus* act as a good microorganism for better production of alcohol.

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