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Production and Optimization Techniques of Bioethanol from Withered Flowers of *Allamanda schottii* L. by Activated Dry Yeast

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Production of bioethanol from renewable carbohydrates materials has become worldwide interest. Floral wastes are easily, freely and abundantly available bio resource which has distinct advantages such as high fermentable sugars and zero investment. In this paper the production of ethanol from golden trumpet flower (*Allamanda schottii L)* by fermentation using yeast *Saccharomyces Cerevisiea* was studied. The optimization of various parameters for the production of ethanol and emission testing with the blend of petrol and ethanol has been studied. The flower of this study allamamda contains 65% total sugars. The yeast strain *Saccharomyces Cerevisiea* was purchased from local super market as baker's yeast. The ethanol obtained was maximum 18.75 ml for 100 ml slurry composition by optimizing various parameters like slurry composition as 1: 8, pH as 5.5, inoculum age as 72 hours, inoculum level as 3.75 g 100 ml^{-1}, temperature at 35p C and fermentation period as 5 days. Maximum production of ethanol is obtained by the addition of urea. This method can be tried for large scale ethanol production from *Allamanda Schottii* flowers.

Keywords: *Allamanda Schottii*, ethanol, *Saccharomyces Cerevisiea,* fermentation.

The tremendous amounts fossil fuel usage poses pollution threat to the atmosphere. In near future the industries may depend upon bio fuels as an alternative source to petroleum based fuel. The best fuel which can supplement petrol is ethanol.Bio ethanol is most widely used used bio fuel for transportation(Balat, 2010). Ethanol is a clean fuel which can be obtained from renewable energy source and can play important role to solve the oil shortage to occur in near future. (Zhang and Feng, 2012). Demand for ethanol occurred world wide due to drastic in crease in population and industrialization. Ethanol produced from corn and sugarcane were not able to meet the world's demand since they are consumed as food and feed (Khan and Dwivedi, 2013). Ethanol produced from cereal crops will pose threat on food prices and food security (Seag *et al*., 2013).

In order to cut down the production costs of fuel the source should be available freely and abundantly in nature. In the developing countries like India a balance between energy and food security is possible only if ethanol production is from non-food crops and biomass (Ragaukas *et al*., 2006; Lin and Tanaka, 2006)

Keenan and ASCE discussed the potential for bio mass utilization as a source of fuel, petro chemicals and petroleum sparing substances. The following plant families Euphorbiaceae, Asclepiadaceae, Apocyanaceae, Urticaceae ,Concvolvulasceae, Sapotaceae, were studied for their suitability as petro crops by various workers(Dipul kalita, 2008). At present world wide researchers show their interest in finding various bioresources for ethanol production..

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The world energy crisis can be solved in producing hanol by fermentation (Ward O.P. and Singh A. 2006). The floriculture industry of India is largest among other countries in the world. The sugars present in flowers can be easily converted in to ethanol. The production of ethanol from mahua flower (*Madhuca indica*) through submerged fermentation (smf) was studied recently by D.S.N.Benarji *et al* (2010) using *Scharacomyces Cerevisiae* -3090.

Madhuca latifolia flowers are very rich in fermentable sugars (28.1-36.3g kgL^{-1}) . The batch fermentation of fresh and 12 month stored flowers yields 193 and 148g kg^{-1} by using free cells and 205 and 152g kg^{-1} by using immobilized cells of *Saccharomyces cerevisiae* was studied by Swain *et al* (2007)

Sujit *et al* (2009) found Madhuka latifolia L. flowers in solid state fermentation by *Saccharomyces cerevisiae* can yield maximum ethanol after 72 hours of fermentation (225.0±4.0 g kg^{-1} flower) with optimized parameters as moisture level 70%, pH 6.0 and temperature 30p C.

In the present study the substrate chosen for ethanol extraction was withered waste flowers. Thorough study was made on the Allamanda species as a source for ethanol production by S*accharomyces cerevisiae.* Extensive research was done on screening and selecting the plant species of *Allamanda schotti* (family Apocyanaceae) as substrate for the production of ethanol. The micro organism *Saccharomyces cerevisiae* is efficient in converting hexose sugars in to ethanol and carbon di oxide (Potphode Arati and Agarwal Seema, 2015). The application of activated dry yeast for production of ethanol quickens fermentation and prevents any contamination which can occur due to bacteria. (Daoqiong *et al*, 2013).

Allamanda schottii is abundantly found as hedge crop in tropical countries. Hence these flowers can be used to produce ethanol through fermentation which may be an economic advantage in the Indian context. This study also paves way for the production of ethanol from nonfood crop i.e. second generation bio fuels. *Allamanda schottii* has rich source of carbohydrate 69%. The physio chemical properties of *Allamanda schottii* were studied and the analysis revealed that allamanda contains total sugars 60-65%, protein 2.40 %, ash 1.4% and moisture content 79.40%.

MATERIAL AND METHODS

Substrate

Flowers of *Allamanda schotti* collected from APEI campus, G.B.Nagar, kalavai, Vellore district, Tamil Nadu during the month of March-August and authenticated at department of floriculture, Adhiparasakthi Horticultural College, G.B.Nagar, kalavai. The withered flowers were collected by engaging women labourer and manually cleaned by them. The flowers were then shade dried The yeast .*Saccharomyces cerevisiae* was purchased from local super market as commercial activated dry yeast (ADY) and it was used for fermentation.

Sterilization

The withered flowers of Allamanda were manually collected and cleaned. The allamanda flowers are sterilized in an autoclave at a pressure of about 10 lb / $inch^2$. The sterilization was carried out for 25 minutes. For quick drying the flowers were kept in a hot air oven (HASTHAS) at 65p C for 5-6 hours.The dried flower was powdered in Willey mill (SECOR, 220 Volts, 0.37 Kw, 0.5 Hp, rpm 1440). The flower powder was sealed in polythene cover and used for analysis.

Estimation of sugar

The anthrone method was followed to find out the sugar content in the allamanda flowers and it was estimated to be 60-65%.

Medium for seed culture

Yeast *Saccharomyces Cerevisiae* was obtained from local super market in the form of activated dry yeast. Yeast *Saccharomyces cerevisiae* was maintained on the yeast extract on potato dextrose broth at pH 5.5 (2.4g in 100ml distilled water). The inoculated medium was incubated for 3 days at 35°C. The inoculum size was assessed by serial dilution and plating technique and the samples were adjusted to cell concentration of 10^9/ml of sample using sterilized medium.

Ethanol Estimation

Potassium di chromate oxidation method and Spectro photometric method was followed to estimate ethanol. The ethanol production was tested by LCMS (Liquid Chromatography Mass Spectrometer).

Experimental

About 10 g of allamanda flower powder

was mixed with 80 ml distilled water and made into a slurry. The slurry was thoroughly mixed in an autoclave at 120°C for 25 minutes and then the contents were cooled to room temperature. The slurry was pretreated with H_2SO_4 at a concentration of 0.05N and NaOH 0.5% alkalinity. The yeast strain *Saccharomyces Cerevisiae* were prepared and added at room temperature (30±2°C) to the suspension. The slurry was centrifuged at 1000 rpm for 15min. The slurry was kept at room temperature for fermentation. Optimization of various parameters like slurry composition as 1: 8, pH as 5.5, inoculum age as 72 hours, inoculum level as 3.75 g 100 ml-1, temperature at 35p C and urea 0.05g L-1were maintained in the The fermentation medium to get best results. The process was done in triplicates. The ethanol obtained was maximum 18.75 ml for 100 ml slurry composition.

Distillation

The fermented broth was removed after 5 days and the contents were analyzed for ethanol. The filtration was done with whatman no 1 filter paper to collect the supernatant from the fermented slurry. The simple distillation unit consists of a round bottom flask, a condenser and a distillate. The filtered sample was transferred to round bottom flask heated by heating mantle (ILECO- 300 watt- Capacity 1000ml). Ethanol was separated at a temperature of 78.5°C (boiling point of ethanol). The vapours of ethanol was condensed by the circulating water around the condenser and received in the distillate. Moisture free ethanol was obtained by keeping the distillate under refrigerated condition. The water present in the sample freezed at 0°C and 99.50% pure ethanol (freezing point -117°C) was collected and stored.

Potassium dichromate method was adopted to estimate ethanol followed by confirmation with LCMS (Liquid Chromatograph Mass Spectroscope). The distillate contained 1.5 ml of ethanol. The volatile and semi volatile compounds in the distillate can be found using the above analytical technique. LCMS was done at Shiva Analyticals (India) Private Limited, Bangalore (NABL (ISO / IEC 17025: 2005) Accredited & ISO 9001: 2008 Certified Laboratory)

RESULTS AND DISCUSSION

The appearance of blue green colour in Jones reagent test shows that the fermented sample has ethanol (Tripti Agarwal *et al*. 2013). Oxidation of ethanol to acetic acid with an excess of potassium dichromate in the presence of sulphuric acid showed off a blue green colour (Brooks *et al* 2008).

Quantitative estimation of ethanol as volume of ethanol per volume of fermented liquid from Allamanda flowers was found to be 15 ml/100 ml slurry, i.e 18.75ml 100 gm^{-1} of dry flower. Behera *et al*., 2010 studied fermentation of mahula flowers produced ethanol (154.5gm kg^{-1} flowers) by immobilized cells of *Saccharomyces cerevisiae* in calcium alginate beads. After 96 hrs fermentation Swain *et al*., (2007) studied

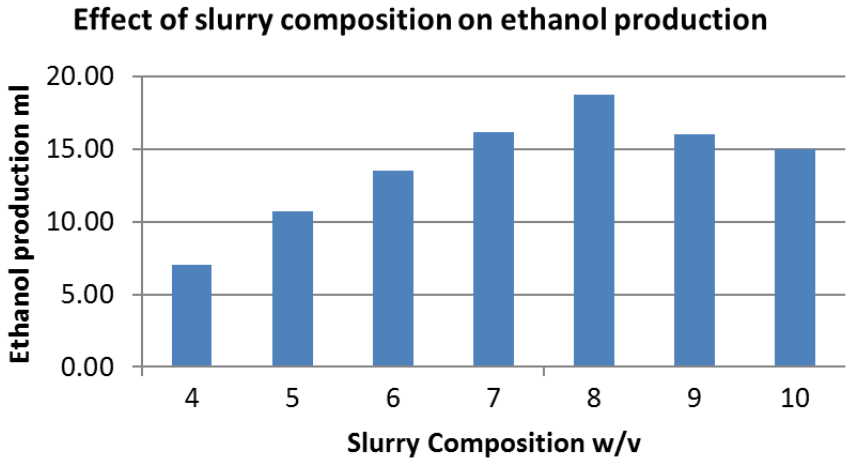

Fig. 1. Effect of slurry composition on ethanol production

fermentation of Madhuca latifolia L. using free cells of yeast *Saccharomyces cerevisiae* produced ethanol 193 and 148gm kg^{-1} from fresh and 12 month mahula flowers. Recently Mandal *et al*., studied fermentation of mahulla flowers madhuca latifolia L. using *Saccharomyces cerevisiae* -3040 gives maximum yield of ethanol (38ml @1.5 kg L^{-1}) ratio of slurry for 48 hrs. Arati *et al*., (2015) studied submerged fermentation of fresh rangoon creeper (Quisqualis indica) flowers with *Saccharomyces cerevisiae* yield ethanol 1.41gm% with fermentation efficiency of 36.29%. Geetha *et al*., (2013) studied the potential of degrained sunflower head waste as substrate for ethanol production and found that ethanol yield was maximum in the treatment with acid hydrolysis(20.528gm L^{-1}).

The effect of slurry composition

The effect of slurry composition on the production of ethanol using *Saccharomyces Cerevisiae* was carried out by varying slurry composition of 1:4 to 1:10 ratios (w/v). The various parameters such as inoculum age 72 hours, inoculum level 3.75g/100ml, agitation 100 rpm, urea 0.5g w/v, pH 5.5 at room temperature of 35p C was maintained.

It can be seen from fig 1 the slurry composition 1:8 showed the maximum yield of ethanol (15ml/10g). The fermentation time was observed as 5 days and it can be seen from fig, that the production of ethanol was decreased with increase in slurry composition. It was confirmed that the substrate Allamnada flower powder slurry of 1:8 is an optimum slurry composition for

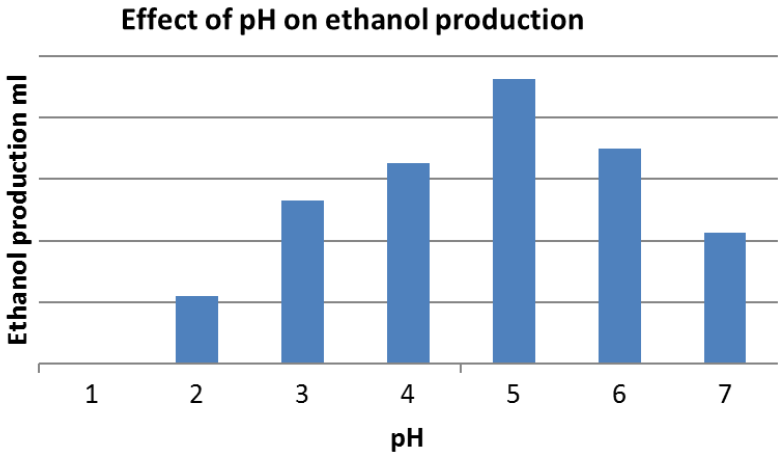

Fig. 2. Effect of pH on ethanol production

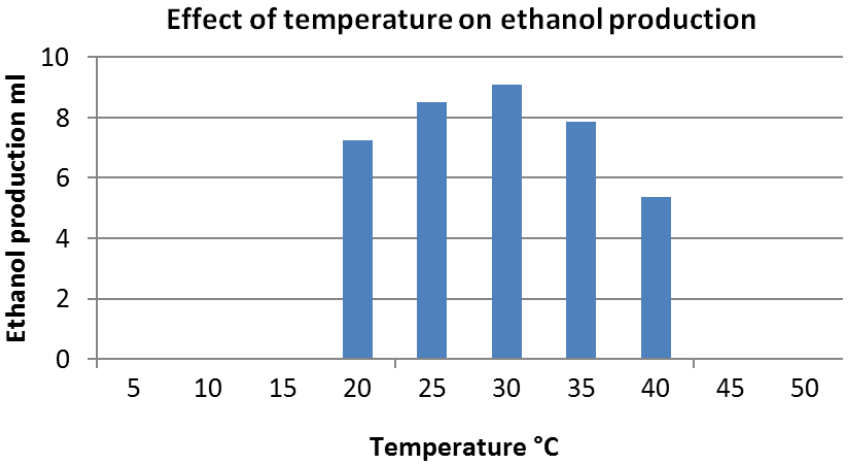

Fig. 3. Effect of temperature

maximum ethanol production. Taking this in view, slurry composition1:8 were taken as optimum and the effect of pH, Temperature, Inoculum level, inoculum age, agitation, addition of urea were studied. (Banerji D.S.N. *et al*, 2010)

Effect of pH

It has been seen from the fig 2 that the rate of ethanol production was maximum at pH of 5.5. This is due to the fact that proteins function in an environment that reflects this pH (Berg, 2007). A of pH 2 had the lowest carbon dioxide production presumably because the low pH encourages the production of acid instead of alcohol (Jennings, 1995).The value of pH during fermentation period changed from 5.5 to 4.5. The change in pH was adjusted by the addition of NaOH and H_2SO_4 at every 5 hours.

Effect of temperature

The previously reported studies show that fermentation temperature is an important parameter which affects the production of ethanol. It was found that maximum ethanol yield was obtained when the fermentation medium was 30 - 32°C. The further increase in temperature affects microbial growth and decreases the production of ethanol. (Fig 3)

Effect of inoculum age

Inoculum age was investigated to determine the potentiality of the yeast *Saccharomyces Cerevisiae*. The process of fermentation was carried out for a period of 6 days and ethanol yield was calculated after every 24 hours. It was found that the optimum yield of 8.23 ml was found after 72 hours. The fermentation was

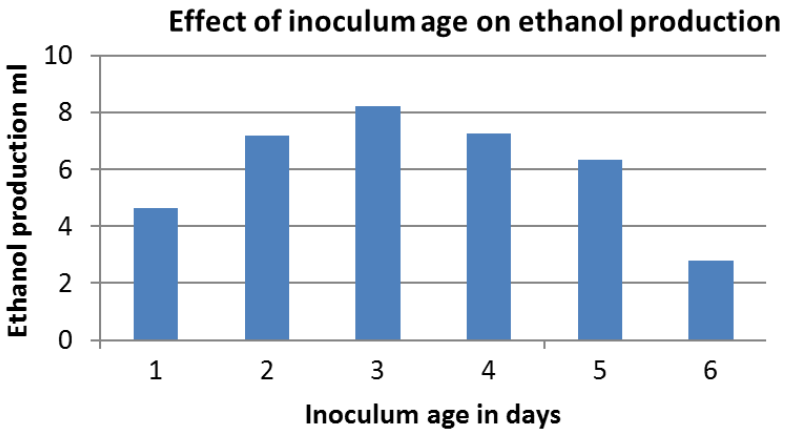

Fig. 4. Effect of inoculum age

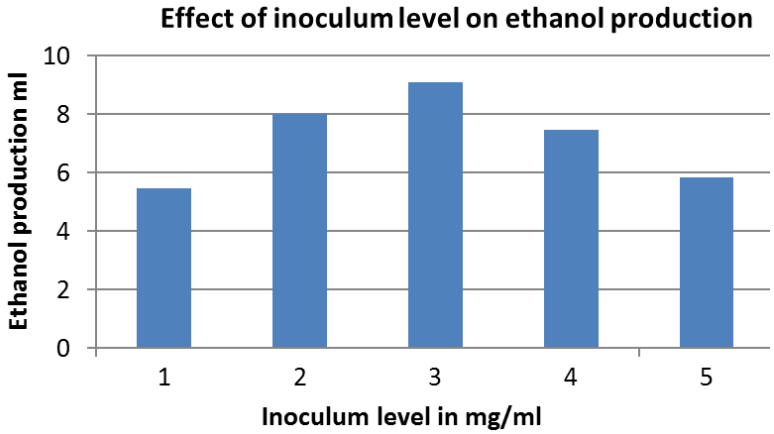

Fig. 5. Effect of inoculum level

carried out for all six days but it was noted that the ethanol production dropped drastically to 2.80 ml after 6th day. The results are reported in fig 4

Effect of inoculum level

The inoculum level was studied to determine te percentage of inoculums in the media for optimum fermentation. The range selected was from 1 mg to 5mg . It was found that 3 mg of inoculum was effective yield of ethanol as 9.1ml. The results obtained are reported in fig 5.

Effect of nitrogen

Most microbes utilize nitrogen to

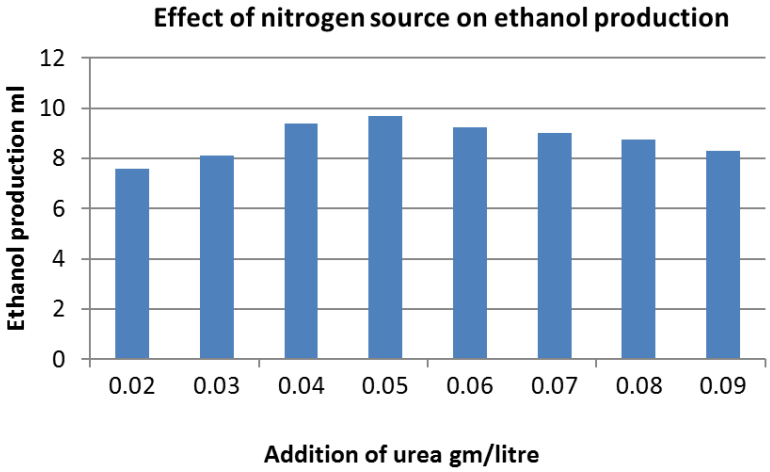

Fig. 6. Effect of nitrogen source

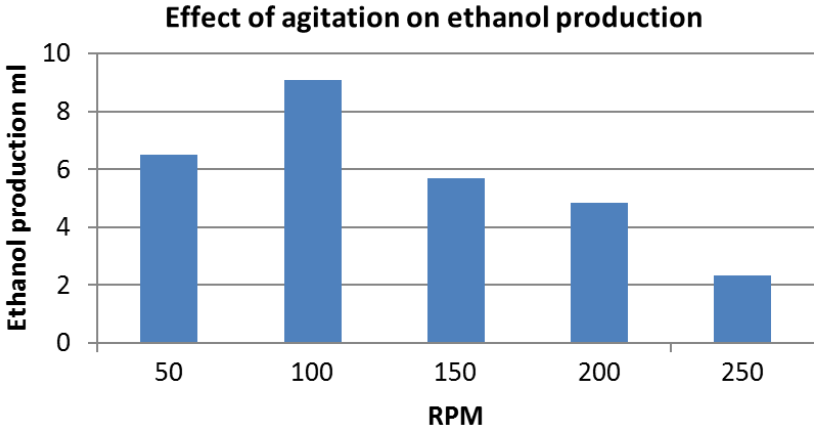

Fig. 7. Effect of agitation

Fig. 8. Withered flower

Fig. 9. Shade dried flower

metabolize nitrogenous substances for the growth and their activity, effect of nitrogen source (Beltran *et al*., 2007). Urea was added to the slurry so that yeast S*accharomyces Cerevisiae* can be metabolized the nitrogenous substances for their growth and activity. It was observed that maximum production was obtained at 0.05g/L concentration of urea. From the Fig.6 it can be concluded that the maximum production was obtained at .05 gm/litre concentration of urea and it has been seen that the ethanol production increased from .02gm/litre to .05 gm/litre, and further increase in urea decreases the ethanol production. The addition of urea had a significant effect on the rate of production of ethanol and it can be concluded that addition of 0.05gm / litre urea is desirable for maximum ethanol production.

Effect of agitation

Agitation at optimum rpm plays an important role in utilizing maximum sugars for the growth of *Saccharomyces cerevisiae* and for ethanol production through fermentation. By maintaining the optimized parameters, agitation was studied. In this experiment the rpm was varied from50 to 250 rpm with an increment of 50 rpm. It is seen from the fig.7 the ethanol production increased with increase in agitation up to 100 rpm, but further increase in rpm shows the decrease in ethanol yield and hence 100 rpm was found as optimum agitation for further studies.

The changes in various parameters such as slurry composition, pH, Temperature, inoculum age, inoculums level, addition of chelating agents during the course of fermentation of allamada flower slurry were studied. The confirmation of presence of ethanol in the distillate was done by LCMS. The LC analysis confirms the presence of ethanol in the distillate (Fig.11 and Fig.12) and result for MS shows that along with Ethanol,

Fig. 10. Oven dried flower

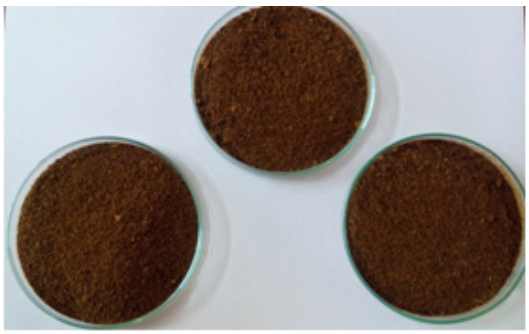

Fig. 11. Dry flower powder

Fig. 12. Flower slurry

Fig. 13. Distillation unit

Fig. 14. Willey mill

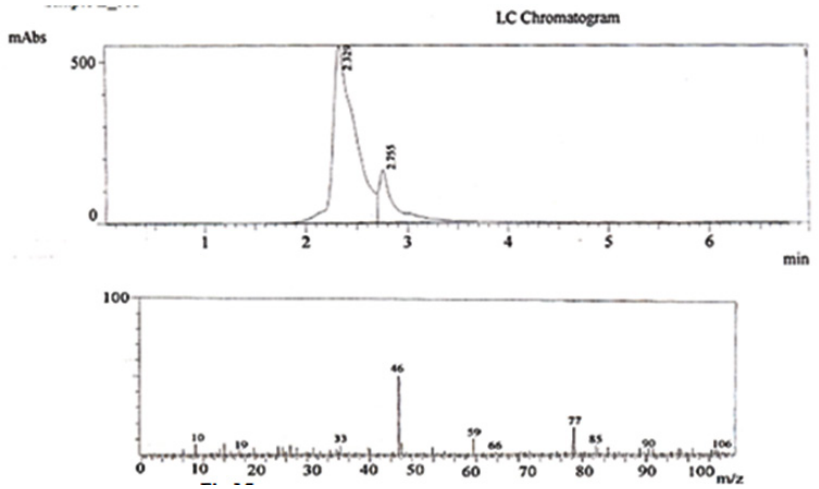

Fig. 15. LCMS reports showing the presence of ethanol

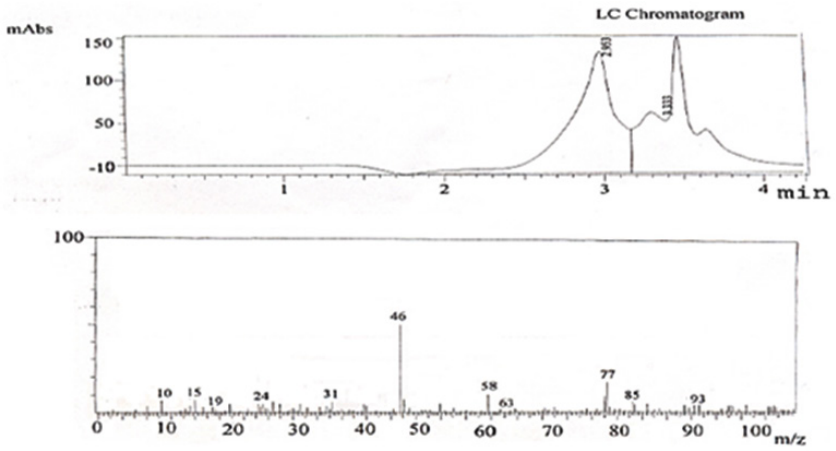

Fig. 16. LCMS reports showing the presence of ethanol

Dimethyl ether, Propanol, Iso butane, Methyl nitrate, Hexane, Acetaldoxime, Malononitrile are also present in the distillate.

CONCLUSION

Bio ethanol is a viable transportation fuel which can replace petrol and extend its availability for a longer run. The bio ethanol produced from non food crop provides energy security to satisfy the energy needs of growing population. Withered waste flowers, a low cost substrate available freely and abundantly in nature could be a best alternative to produce bio ethanol than producing it from food crops. The withered waste flowers of Allamanda is a potential unutilized agriculture residue contains considerable amount of which can form an ideal medium for ethanol production. Therefore the substrate was evaluated for ethanol production by optimizing various parameters such as slurry composition, pH, Temperature, Inoculum level, Inoculum age, Fermentation time and nutrient supplementation.

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