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Research Article

BIO ETHANOL PRODUCTION USING BANANA PSEUDO STEM BY FUNGAL CONSORTIUM

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ABSTRACT

The present study was carried out with the production of ethanol by *Aspergillus niger*, *Rhizopus oryzae*, *Saccharomyces cerevisiae* and baker's yeast using banana pseudostem as a substrate. Ethanol production was optimized by different pH and temperature under fermentation process. Maximum amount of ethanol concentration of 3.82±0.4 g/100 ml was produced at pH 5 and temperature 30°C. Maximum amount of ethanol produced by *Aspergillus niger*, *Rhizopus oryzae* and *Saccharomyces cerevisiae* inoculated media was analyzed by HPLC. From the results it is evident that the aminoacid present in samples were Serine, Histidine, Lysine, Aspartic acid, Glutamic acid, and Tryptophan. Since these compounds were found to be present in the sample, it might to be responsible for the ethanol production. It was observed that pretreated banana pseudostem can be economically utilized as a cheaper substrate for ethanol production.

INTRODUCTION

Ethanol is a straight chain alcohol and its molecular formula is C_2H_6OH. It empirical formula is C_2H_6O. Ethanol or ethyl alcohol (C_2H_6OH) is a clear colourless liquid, it is biodegradable, low in toxicity. The world ethanol production has reached about 51000 million liter, being the USA and Brazil the first producers and India stands fourth among the top fuel ethanol producers. World production of banana is estimated at 48.9 millions tones, out of which 10.4 millions tones is contributed by India. India, where 4.796×10^5 ha of banana is cultivated, farmers discard banana waste into rivers, lake and road, causing serious problems.

An environmental friendly solution and alternative economic use for this agricultural residue is needed. The main residuals of banana crops are leaves and pseudo stem both containing high levels of lignocelluloses. Thus avoid the environmental problem due to the decomposition of waste, it usable to make energy from banana waste as biofuel production source. Biofuels may be classified under the categories of first or second generation biofuels. First generation of biofuels are generally made from carbohydrates, lipids and oils or agro industrial wastes using conventional technologies. Second generation biofuels are generally derived from lingo cellulosic biomass including cellulosic plant biomass such as stalks, stems, wood. Many second generation biofuels such as biohydrogen, biomethanol and mixed alcohol are under development (Naik *et al*., 2010). Various microorganisms are used for ethanol production namely *Aspergillus niger*, *Rhizopus oryzae* and *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Collection of soil sample: The soil samples were collected from paddy field and stored in a sterile polythene bags. Then the soil was transferred to the laboratories.

Serial dilution technique: Serial dilution was performed by using the collected soil sample to isolate the fungal population (Ronald Atlas, 1998).

Isolation of fungi: Fungal population present in the soil sample were determined by plating technique (Warcup, 1950).

Conidial population: The number of colonies determined by Colony Forming Unit (CFU) with dilution factors.

Identification of fungi: *Aspergillus niger* and *Rhizopus oryzae* were identified by lacto phenol cotton blue technique (Gillman, 1957).

Yeast inoculums preparation: Dry Baker yeast purchased from Department Store. Yeast was grown on Yeast Extract Peptone Dextrose agar plate at 30ºC for 48 hours. A loopful of the yeast

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colony was transferred from agar plate into 100 ml of 5 % YEPD broth and incubated at room temperature on a shaker at130 rpm for 48 hrs (Scholar and Benedikte, 1999).

Sample collection: Banana pseudostem were collected from Vadamazhai Manakkadu, Vedaraniyam (T.K), Nagappatinam (D.T), Tamil Nadu, South India.

Moisture Content: The known weight of sample was placed in a petridish and dried in a Hot air oven to 60-65°C for 36 hrs. The moisture content, expressed as percentage was calculated by using following formula.

$$\text{Moisture \%} = \frac{\text{Wet weight of sample} - \text{Dry weight of sample}}{\text{Wet weight of sample}} \times 100$$

Treatment methods

Physical treatment method: The Banana pseudostem was cut in to the four slices, 30 cm length and crushed to remove its natural liquor and then sent for pretreatment. The crushed pieces were dried in a tray dryer and milled in a Solab knife mill until attaining particles smaller than 30 mesh size.

Chemical pretreatment methods: Two type of chemical pretreatments were evaluated both at 120°C/15 min namely Acid hydrolysis (NaOH 3%) and Alkaline hydrolysis (H_2SO_4 2%). Dilute sulfuric acid was prepared with a concentration range 0%, 0.5%, 1.0% up to 5.0% the flasks were added with 5g of processed banana pseudo stem and dilute sulfuric acid autoclaved at 121°C for 30 minutes. The flasks containing the pre-treated banana pseudo stem were then neutralized by washing with distillled water (Balat, 2008). Dilute sodium hydroxide was prepared with a concentration range of 0%, 0.5%, 1.0% up to 5.0% The flasks were added with 5g of processed banana pseudostem and dilute sodium hydroxide autoclaved at 121°C for 30 minutes. The flasks containing the pre-treated banana pseudostem were then neutralized by washing with distillled water (Pandey, 2000).

Fermentation media: 5 gm treated substrate were utilized for ethanol production using *Aspergillus niger*, *Rhizopus oryzae* and *Saccharomyces cerevisiae* by fermentation. The media containing following composition. The composition of the media was (g/ 100 ml): Glucose - 20; Yeast extract - 3; $(NH_4)_2SO_4$ -0.5; K_2HPO_4 - 0.5 ; $CaCl_2.2H_2O$ - 0.1 ; $MgSo_4.7H_2O$ - 0.1g. The fermentation media containing following composition

A. Banana pseudo stem + Aspergillus niger+ Rhizopus oryzae + Saccharomyces cerevisiae
B. Banana pseudo stem + Aspergillus niger + Saccharomyces cerevisiae
C. Banana pseudo stem + Rhizopus oryzae + Saccharomyces cerevisiae
D. Banana pseudo stem + Saccharomyces cerevisiae
E. Banana pseudo stem + Aspergillus niger + Rhizopus oryzae + Baker yeast
F. Banana pseudo stem+ Aspergillus niger + Baker yeast
G. Banana pseudo stem + Rhizopus oryzae +Baker yeast
H. Banana pseudo stem + Baker yeast
I. Control

100 ml of the fermentation medium in 250 ml of Erlenmeyer flasks was used for experimentation purpose and the flasks were sterilized at 121 °C at 15 psi for 20 minutes. All fermentations were performed in a incubator for 72 hours (Szczodrak and Fiedurek, 1996).

Confirmation and Estimation of ethanol: Iodoform test was used to confirm the production of ethanol. Estimation of ethanol was performed by AOAC method (AOAC, 1990).

Optimization of ethanol production

Effect of pH and temperature: The pH of the sterilized broth was set as 3,4 and 5 and temperature was set as 20°C, 25°C and 30°C. A loopful of *Aspergillus niger*, *Rhizopus oryzae*, *Saccharomyces cerevisiae* and baker yeast were inoculated into the flask. The flasks were incubated for 3 days (Neves, 2007).

Determination of Residual sugars: The residual sugars was determined using Dinitrosalicyclic (DNS) method (Miller, 1959).

Cell Biomass: The sample was estimated by centrifuged the known volume of sample in a pre dried and pre weighed for 20 minutes. After resuspension in 2 ml of distilled water and further centrifugation, the cell mass dried. The dried cell mass calculated by reweighing the tube (Sadasivam and Manickam, 1992).

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Statistical analysis: The results were obtained in the present investigation were subjected to statistical analysis like Mean X and Standard Deviation (SD) (Gupta, 2009).

Mean

The mean for the data was calculating using given formula

$$\text{Mean} \quad X = \frac{\Sigma X}{N}$$

Standard deviation

The Standard deviation calculated by the formula

$$(SD) = \sqrt{\frac{\sum (x - \overline{x})^2}{N-1}}$$

RESULTS AND DISCUSSION

Isolation and identification of fungi: The soil samples were collected from paddy field. The soil samples were prepared to serial dilution. The PDA agar plate was prepared. 10^{-3} - 10^{-5} dilution were inoculated into the Potato Dextrose Agar medium by spread plate technique. After incubation period, the plates were observed and identified the fungal colonies *Aspergillus niger*, *Rhizopus oryzae* by Wet Mount technique.

Conidial population: The number of colonies present in 1gm of the soil samples were determined by Colony Forming Unit (CFU). The no of colonies were 17×10^3 with dilution factors.

Estimation of moisture content and Reducing sugar: The moisture content in the substrate was estimated under hot air oven, calculated as per standard estimation procedures. The value of total moisture was found to be 35.5%. Banana pseudo stem contain total residual sugar was 0.5± 0.3g/100ml. After the pretreatment of the banana pseudo stem contain total residual sugar was 4.3± 0.2g/100ml.

Fermentation: 5 gm treated substrate was utilized for ethanol production using *Aspergillus niger*, *Rhizopus oryzae*, *Saccharomyces cerevisiae* and Baker yeast in the fermentation.

Confirmation and Estimation of ethanol: Ethanol production in all the media confirmed by the produced cloudy formation and yellow precipitate and antiseptic smell in iodoform test. Ethanol was estimated by standard distillation method (AOAC, 1974).

Optimization of ethanol production

Effect of pH and temperature: As shown in Table.1 fermentation media prepared and adjusted in different pH (3,4,5) and different temperature (20ºC, 25ºC and 30ºC). *Aspergillus niger*, *Rhizopus oryzae* and *Saccharomyces cerevisiae*, (**A)** inoculated fermentation medium was prepared and adjusted in different pH (3,4,5) and different temperature (20ºC, 25ºC and 30ºC) . The maximum amount of (3.82 ± 0.4 g/100 ml) ethanol was produced at pH 5 and temperature 30ºC, when compared to other media. Low amount of (2.02 ± 0.2 g/100 ml) ethanol as produced in pH 3 and temperature 20ºC in Baker yeast **(H)** alone inoculated fermentation medium.

Estimation of ethanol, reducing sugars and cell biomass: As shown in Table.2 *Aspergillus niger*, *Rhizopus oryzae*, *Saccharomyces cerevisiae* **(A)** inoculated fermentation medium produced 3.82 ± 0.4 g/100 ml. The total residual sugar was 2.65 ± 0.3g/100ml and the cell biomass was 1.34 ± 0.2 g/100ml when compared with other media. Baker yeast (**H**) inoculated fermentation medium produced 2.11 ± 0.3 g/100 ml of ethanol was produced and the total residual sugar was 2.04± 0.1 g/100 ml and the cell biomass was 1.12 ± 0.2 g/100ml.

Table- 1 Effect of pH and temperature on ethanol production

S.No	Treatment	pH and temperature		
		3 20 ºC	4 25 ºC	5 30 ºC
1	Banana pseudostem + *A.niger* + *R.oryzae* + *S.cerevisiae* (A)	3.52±0.7	3.72±0.5	3.82±0.4
2	Banana pseudostem +*A.niger*+ *S.cerevisiae* (B)	2.43±0.5	2.64±0.3	2.80±0.3
3	Banana pseudostem +*R.oryzae* + *S.cerevisiae* (C)	2.36±0.3	2.44±0.4	2.52±0.1
4	Banana pseudostem + *S.cerevisiae* (D)	2.24±0.1	2.36±0.1	2.43±0.2
5	Banana pseudostem+ *A.niger* + *R.oryzae* + Baker yeast (E)	2.31±0.6	2.34±0.5	2.36±0.5
6	Banana pseudostem + *A.niger* + Baker yeast (F)	2.15±0.4	2.18±0.	2.24±0.3
7	Banana pseudostem+ *R.oryzae* + Baker yeast (G)	2.8±0.5	2.12±0.1	2.14±0.4
8	Banana pseudostem+ Baker yeast (H)	2.2±0.2	2.8±0.2	2.11±0.3
	Control (Untreated Banana pseudostem) (I)	1.4±0.1	1.9 ±0.4	1.6 ± 0.1

Values are triplicates, mean ± standard deviation

Table-2 Estimation of ethanol, reducing sugars and cell biomass

S.No	Treatment	Total Residual Sugars (g /100 ml)	Ethanol (g/100ml)	Cell Biomass (g / 100ml)
1	Banana pseudostem+ *A.niger* + *R.oryzae* + *S.cerevisiae*(A)	2.65±0.3	3.82±0.4	1.34± 0.2
2	Banana pseudostem +*A.niger* +*S.cerevisiae*(B)	2.52± 0.1	2.80±0.3	1.26± 0.2
3	Banana pseudostem +*R.oryzae* +*S.cerevisiae*(C)	2.33± 0.2	2.52±0.1	1.21± 0.1
4	Banana pseudostem + *S.cerevisiae*(D)	2.22±0.1	2.43±0.2	1.12± 0.2
5	Banana pseudostem*A.niger* +*R.oryzae* + Baker yeast (E)	2.25± 0.3	2.36±0.5	1.24± 0.4
6	Banana pseudostem+ *A.niger* + Baker yeast (F)	2.12± 0.2	2.24±0.3	1.16± 0.1
7	Banana pseudostem+ *R.oryzae* + Bakeryeast (G)	2.08 ± 0.3	2.14±0.4	1.14± 0.3
8	Banana pseudostem+ Baker yeast(H)	2.04± 0.1	2.11±0.3	1.10± 0.2
9	Control (I) (Untreated Banana pseudostem)	1.08± 0.2	1.06 ± 0.1	1.03± 0.1

Values are triplicates, mean ± standard deviation

HPLC (High Performance Liquid Chromatography: HPLC method is one of the most fast and reliable method for identification of amino acids. The chromatographic separation of amino acids such as Serine, Histidine, Lysine, Aspartic acid, Glutamic acid and Tryptophan. High amount of ethanol produced in the fermentation media **A)** *Aspergillus niger*, *Rhizopus oryzae* and *Saccharomyces cerevisiae* inoculated fermentation medium analysed for the aminoacid based on the Retention time (Rt) showed Serine (Rt-2.528), Histidine (Rt-3.206), Lysine (Rt-6.375) and Aspartic acid (Rt-9.927). **B)** *Aspergillus niger* and *S.cerevisiae* inoculated fermentation medium analyzed for the aminoacid based on the Retention time (Rt) They were Aspartic acid (Rt-93.441), Glutamic acid (Rt-8.602), Aspartic acid (Rt-9.47 and Tryptophan. (Rt-10.36). *Rhizopus oryzae* and *Saccharomyces cerevisiae* inoculated fermentation medium analyzed for the aminoacid based on the Retention time (Rt). Serine (Rt-2.469), Histidine (Rt- 2.985), and Aspartic acid (Rt-9.852) present **D)** *S.cerevisiae* inoculated fermentation medium analyzed for the aminoacid based on the Retention time (Rt) i.e Histidine (Rt-3.990) The obtained value was compared. Snegal *et al*., 2014 focuses on exploitation of banana pseudostem as a sources for bioethanol production from the sugars released due to different chemical and biological pretreatments.

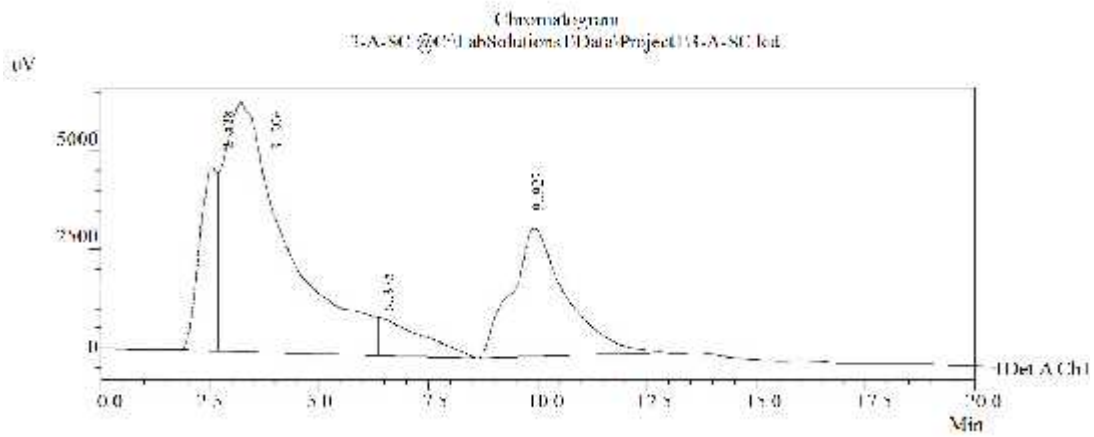
Two fungal strains *Aspergillus ellipticus* and *Aspergillus fumigatus* reported to producing cellulolytic enzymes were used under co culture fermentation on banana pseudostem to degrade hololcellulose and facilitate maximum release of reducing sugars. The hydrolysate obtained after alkali and microbial treatments was fermented by *Sacchromyces cerevisiae* NCIM 3570 to produce ethanol. Fermentation of cellulosic hydrolysate (4.1g %) gave maximum ethanol (17.1g/L) with yield (84 %) and productivity (0.024.g %/ h) after 72 h. In present study showed that *Clostridium thermocellum* DSM1313 for the hydrolysis of pretreated sugarcane bagasse and banana pseudostem for ethanol production Ethanol titter of 26.27 and 9.12 mM and release 4.770± 0.107 and 3.281 ± 0.109 g/L of reducing sugars from sugarcane bagasse and banana pseudostem respectively were observed in flask level studies (Nisha *et al*., 2016).

Figure 1 Report (Report Editor) Status:Temporary.Joseph's college (Autonomous), Thiruchirapalli-2.

HPLC Analysis Report

Sample Information

Acquired by	: Admin
Sample Name	: 3-A-SC
Sample ID	:
Vail#	:
Injection Volume	: 20 uL
Data Filename	: 3-A-SC.lcd
Method Filename	: SJC-Law-Method.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 22-07-2016 PM 02:34:22
Data Processed	: 22-07-2016 PM 02:54:25



1 Det.A Ch1 / 254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %	Amino acid
1	2.528	127803	4665	11.402	30.660	Serine
2	3.206	644608	6318	57.510	41.522	Histidine
3	6.375	65476	979	5.842	6.437	Lysine
4	9.927	282983	3253	25.247	21.381	Aspartic acid
Total		1120870	15216	100.000	100.000	

Figure 2 Report (Report Editor) Status: Temporary. Joseph's college (Autonomous), Thiruchirapalli-2

HPLC Analysis Report

Sample Information
Acquired by: Admin
Sample Name : 4-A-R-BY
Sample ID :
Vail# :
Injection Volume : 20 uL
Data Filename : 4-A-R-BY.lcd
Method Filename : SJC-Law-Method.lcm
Batch Filename :
Report Filename : Default.lcr
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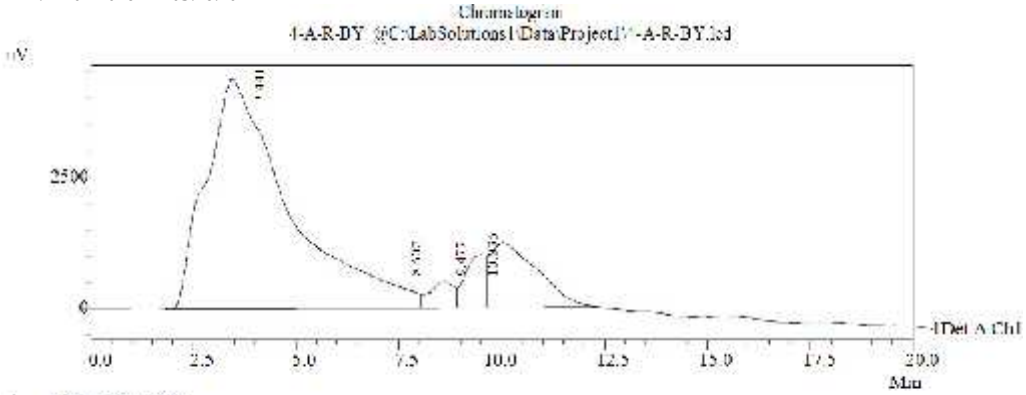
Data Processed : 22-07-2016 PM 03:16:20



Peak Table

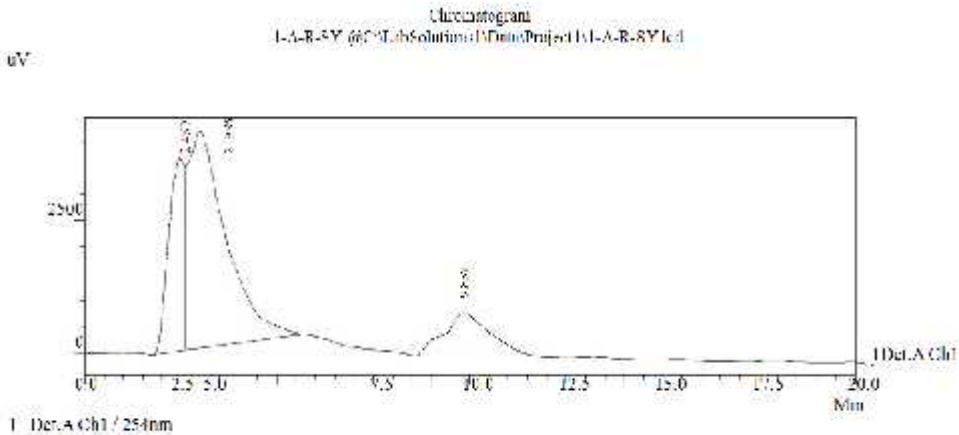
Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %	Aminoacid
1	3.441	620283	4393	80.026	61.355	Aspartic acid
2	8.602	20862	515	2.691	7.188	Glutamic acid
3	9.477	35618	1012	4.595	14.135	Aspartic acid
4	10.036	98342	1240	12.688	17.322	Tryptophane
Total		775105	7160	100.000	100.000	

Figure 3 Report (Report Editor) Status: Temporary. Joseph's college (Autonomous), Thiruchirapalli-2.

HPLC Analysis Report

Acquired by : Admin
Sample Name : 1-A-R-SY
Sample ID :
Vail# :
Injection Volume : 20 uL
Data Filename : 1-A-R-SY.lcd
Method Filename : SJC-Law-Method.lcm
Batch Filename :
Report Filename : Default.lcr
Date Acquired : 22-07-2016 PM 03:18:17
Data Processed : 22-07-2016 PM 03:38:20



Detector A Ch1 254nm

Peak Table

Peak#	Ret. Time	Area	Height	Area %	Height %	Amino acid
1	2.469	104449	3633	23.019	42.573	Serine
2	2.985	281228	4098	61.979	48.024	Histidine
3	9.852	68068	802	15.001	9.403	Aspartic acid
Total		453745	8533	100.000	100.000	

Figure 4 Report (Report Editor) Status: Temporary. Joseph's college (Autonomous), Thiruchirapalli-2.

HPLC Analysis Report

Sample Information
Acquired by : Admin
Sample Name : 2-A-BY
Sample ID :
Vail# :
Injection Volume : 20 uL
Data Filename : 2-A-BY.lcd
Method Filename : SJC-Law-Method.lcm
Batch Filename :
Report Filename : Default.lcr
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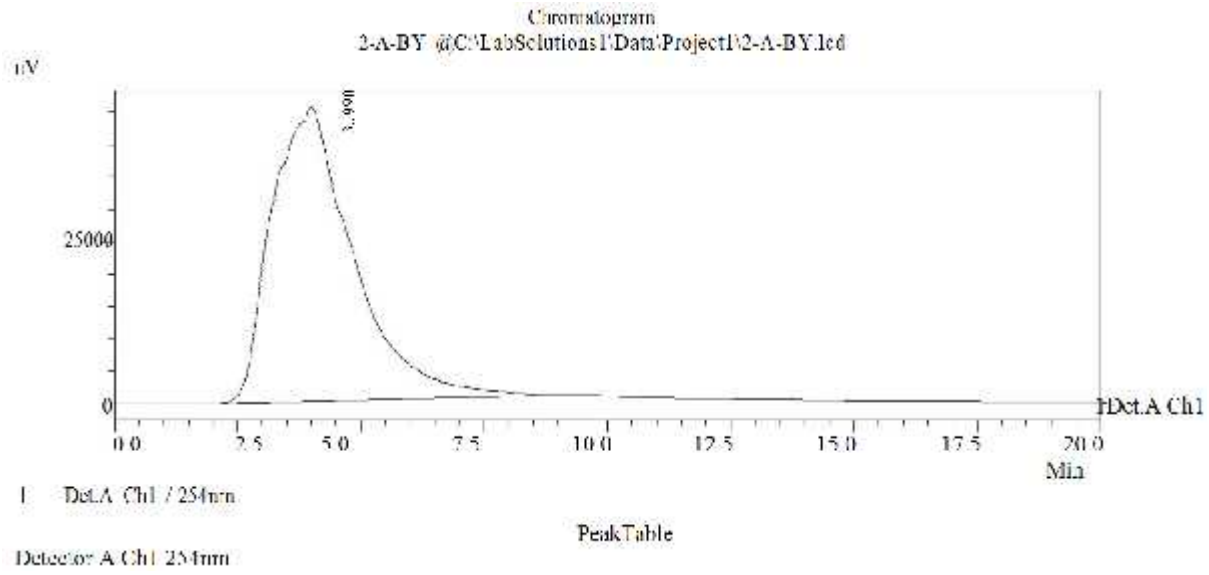
Data Processed : 25-07-2016 PM 01:18:31



Peak#	Ret. Time	Area	Height	Area %	Height %	Aminoacid
1	3.990	5261272	45459	100.000	100.000	Histidine
Total		5261272	45459	100.000	100.000	

CONCLUSION

Agricultural waste is renewable, less costly and available in nature. Most agricultutal waste contains starch, lignocelluloses, hemicelluloses and sugar that are sufficient for fermentation. Banana pseudostem could be used to produce bioethanol effectively. It can be concluded that, produced bioethanol from banana biomass was of good quality and can be used in the engine for transporation purpose with producing less emission. In addition that, it can be used as recycling process for waste management. Lignocellulolytic microorganisms, especially fungi, have attracted a great deal of interest as biomass degraders for large scale application due to their ability to produce large amounts of extracellular lignolytic enzymes.

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