ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Acetone and Butanol Production by *Clostridium Species* from sugar cane juice

Muralidhar TS[1*], Sushma S Murthy[2], Sripal Kothari[1], Vinit Shah[1], Meghana Prasad[1]
Muralidhar TS[1*]

[1]Post Graduate Department of Biotechnology, R and D Center, Dayananda Sagar College of Biological Sciences, Kumarswamy Layout, Bangalore-560078, India.
[2]Post Graduate Department of Microbiology, R and D Center, Dayananda Sagar College of Biological Sciences, Kumarswamy Layout, Bangalore-560078, India.

ABSTRACT

Among various renewable and sustainable energy sources being explored, biobutanol has been recognized as one of the promising alternatives for biofuels due to its attractive physical and chemical properties. Compared to ethanol, butanol offers many advantages as a substitute for gasoline because of higher energy content and higher hydrophobicity. Typically 1-butanol is produced by Clostridium in mixed-product fermentation. To facilitate strain improvement for specificity and productivity, various inventions have been reported for the biological production of butanol, maintaining its competitiveness in efficiency, economy, and production scale. Therefore, there is an increasing interest in developing microbial fermentation processes for production of biobutanol as this will allow for the use of a wide range of raw-materials, including **sugar cane**, corn, and biomass. Present work focuses on production of **Acetone and Butanol** by fermentation process. The selected organisms were *E. coli, Clostridium acetobutylicum & Clostridium sporogenes* strains and were grown on sugar cane extract and allowed for fermentation for 92-98 hrs. From 250 ml of sugar cane extract through ABE fermentation process the maximum yield production of acetone (0.85%), butanol (0.41%) and ethanol (0%) were recorded and identified by GC.

Keywords: Microbial biofuels, Butanol, ABE, *E. coli, Clostridium sporogenes & Clostridium acetobutylicum.*

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Petroleum and natural gases are currently the main energy source used in the world, but the amount of viable fossil fuels are slowly depleting. Also, research is being done to produce more environmentally friendly fuels to combat greenhouse gas emissions produced from petroleum. Scientists have turned to biofuels as an effective alternative to fossil fuels. Until now, bioethanol has been the primary biofuel, because it is economically favorable to produce and easy to manufacture. It is also a renewable fuel that is made from agricultural feedstock. However, biobutanol is proving to be much more advantageous than bioethanol.

Compared to bioethanol, biobutanol has more energy per gallon, thus more miles pergallon. Biobutanol has 110,000 BTUs per gallon, while bioethanol only has 84,000 BTUs per gallon. Butanol can also be blended with gasoline at much higher levels than bioethanol without any necessary engine alterations, because its physical attributes are more similar to gasoline. It has lower vapor pressure, which makes it safer to store, and handle. Because biobutanol is a better biofuel than ethanol, this project has designed a process to create this solvent. This new plant will hopefully replace ethanol plants, so it was mirrored as closely as possible to the ethanol process. The economic feasibility of the plant was investigated, and the cost was compared to that for producing ethanol. [4].

Traditionally, an Acetone-Butanol-Ethanol (ABE) process is used for biobutanol production. This one stage batch process uses a Clostridium strand (generally *C. beijerinckii* or *C. acetobutylicum*) to produce a mixture of butanol, acetone and ethanol. First, fermentation produces a mixture of butyric, lactic and acetic acid. Later, the culture pH drops and butanol, acetone and ethanol are produced in a 6:3:1 by mass ratio respectively. The main problems with this process are the low conversion of glucose to biobutanol, and the large amount of undesired solvents produced. Also, the process is very complicated and difficult to control, so its use has dramatically declined since the 1950s.

Now, butanol is mostly produced via petrochemical routes, which is not eco-friendly. This, however, is not an environmentally conscious method of making butanol [2]. David Ramey of Butyl Fuel, LLC, has created a process that uses two-stage anaerobic fermentation to produce green butanol with higher specificity and efficiency than the ABE process. Up until now, there have only been laboratory-scaled productions of Butanol using this process.

MATERIALS AND METHODS

Strains and culture maintenance

The strains of *Clostridium acetobutylicum, Clostridium sporogenes* and *Escherichia coli* were obtained from NCIM, National Chemical Laboratory (NCL) Pune.

Clostridium acetobutylicum & Clostridium sporogenes NCIM 2337 and NCIM 2559 were grown anaerobically at 37°C for 24 h and maintained at 37°C in Cooked Meat Media (RCM, Hi Media). *Escherichia coli* NCIM 5051 was grown at 37°C for 24 h and maintained at 4°C in nutrient broth and Nutrient agar plates (Hi Media).

Sugarcane extract clarification

Juice was extracted from sugar cane it was filtered with double cheese cloth and 250ml of juice was transferred to air tight screw cap 500ml glass bottles. The bottles were autoclaved at 15psi for 10 minutes. After autoclave the bottles were taken to LAF and aseptically cultures were inoculated.

Fermentation technique

250ml of sterile sugarcane medium were aseptically inoculated with 5ml of *Clostridium acetobutylicum*, *Clostridium sporogenes* and *Escherichia coli* separately and were maintained anaerobically by using Anaero Gas Pack (HI MEDIA) at 37^0C for 24 hours. The initial pH of the medium 5.3 was not regulated throughout the fermentation process. Hydrogen gas was spurged for maintaining anerobic condition. The fermentation process was then carried out at 37^0C for 96 hours. The butanol was recovered from the fermented media by distillation. All the trails were carried out in triplicates.

Analytical methods

Fermented media was subjected to centrifugation process for 10 mins at 3000rpm and supernatant was collected. Total sugar in the medium was quantified by Di-nitro salicylic acid assay method. The solvent was recovered by Distillation Process. Analysis of solvent production was made by drop and raise in pH of the fermentation medium, the pH graphs reveal the fermentation history. Acetone, Ethanol and Butanol was determined by Gas Chromatography at Azyme Biosciences, Bangalore, India.

RESULTS

The results obtained from fermentation showed that C. *Acetobutylicum* NCIM 2337 and *Clostridium sporogenes* NCIM 2559 are capable of utilizing products of lingo cellulosic biomass hydrolysates efficiently for the production of ABE.

C. *Acetobutylicum* NCIM 2337 is capable of utilizing mixed sugars for ABE production. After 98 h of fermentation, 0.26% of butanol and 0.85% of acetone was produced.

Clostridium sporogenes NCIM 2559 is capable of utilizing mixed sugars for ABE production. After 98 h of fermentation, 0.41%of butanol and 0.8%of acetone was produced.

ISSN: 0975-8585

Escherichia coli NCIM 5051 was our test organism is facultative anaerobic bacteria. It showed the negative results for the ABE fermentation and very less amount of acetone was produced which was below detection level. The results are given in Tables 1 and 2 and further details are showed in the Figures 1, 2,3,4,5 and 6.

Table 1: comparison of fermentation results and conditions

Sl no	parameters	Microbes used		
		***E. coli NCIM:*2337**	***Clostridium acetobutylicum NCIM* : 2559**	***Clostridium sporogenes NCIM : 5051***
		Parent strain	**Parent strain**	**Parent strain**
1	Fermentation time	92-98 hours	92-98 hours	92-98 hours
3	Total reducing sugar g/L	66.0	66.0	66.0
4	% of Butanol produced	Nil	0.26	0.41
5	% of Acetone produced	BDL	0.85	0.8
6	% of Ethanol produced	Nil	Nil	*Nil*
7	Initial pH	5.30	5.30	*5.30*
8	Final pH	4.75	5.11	5.17

TABLE 2: Results of GC in terms of percentage of solvents

Sample particulars	Acetone	Butanol	Ethanol
Clostridium acetobutylicum	0.85%	0.26%	Nil
Clostridium sporogenes	0.8%	0.41%	Nil
Escherichia coli	Bellow detection level	Nil	Nil

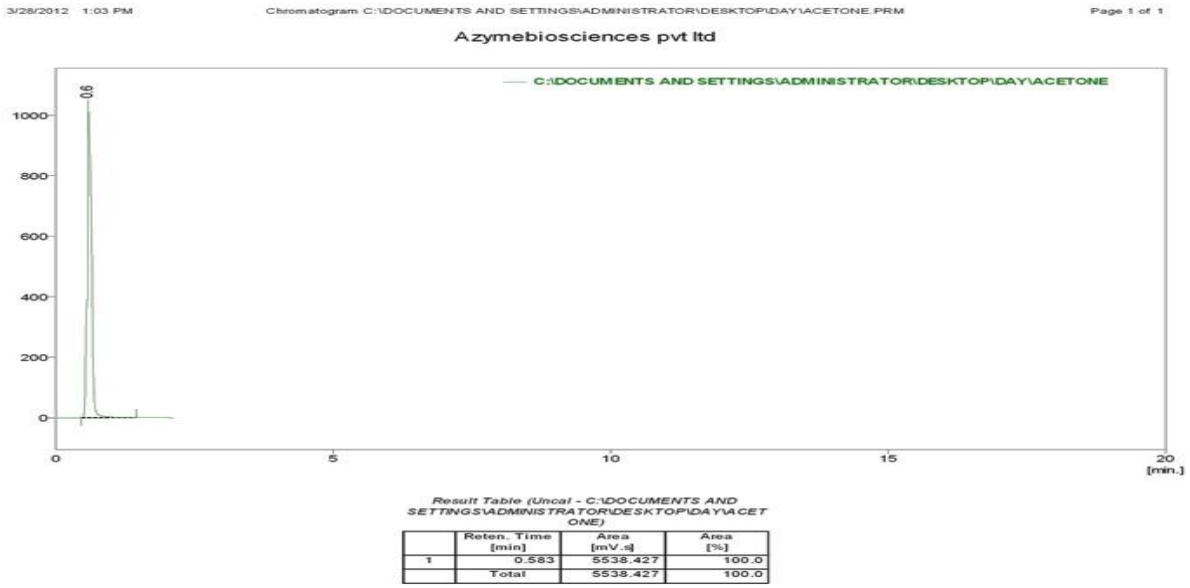

Figure 1: GC of Standard acetone

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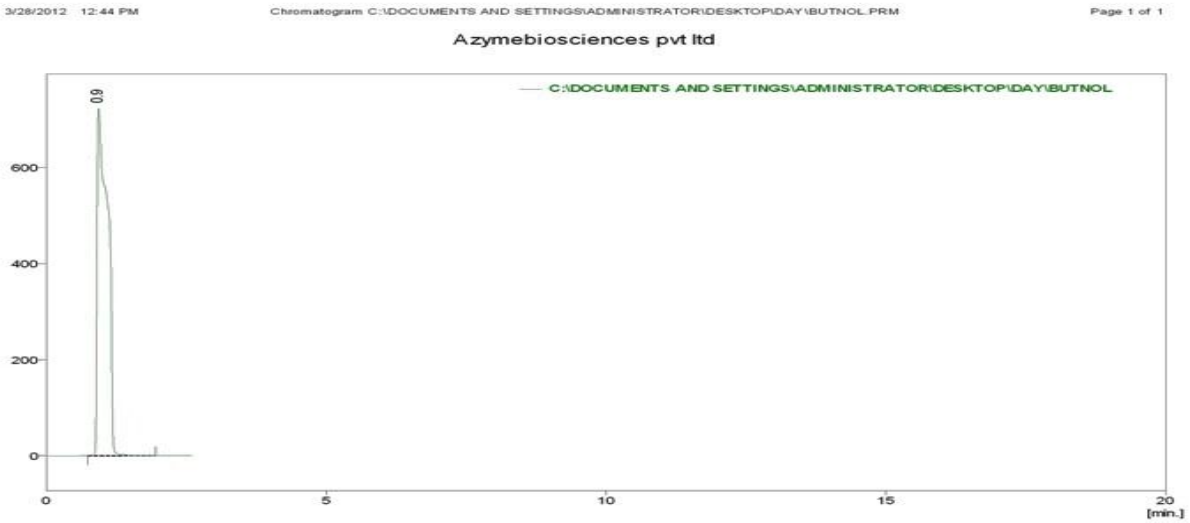
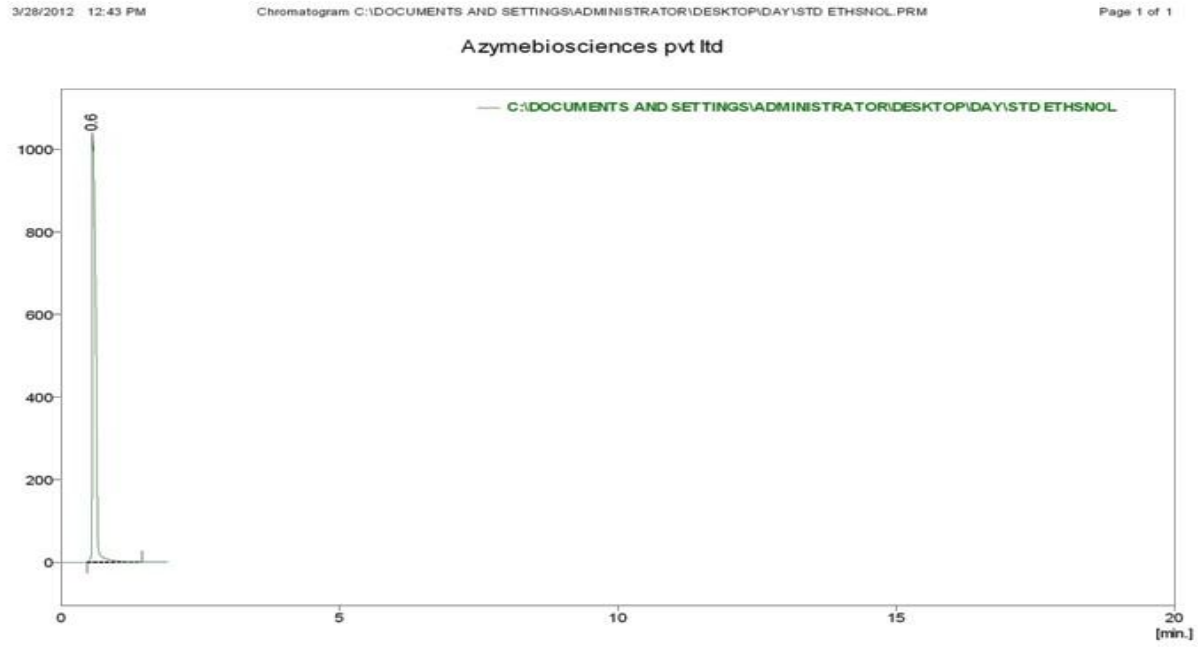


Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\DAY\BUTNOL)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	0.930	9448.563	100.0
	Total	9448.563	100.0

Figure 2 GC of standard butanol



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\DAY\STD ETHSNOL)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	0.563	4640.870	100.0
	Total	4640.870	100.0

Figure 3 GC of standard ethanol

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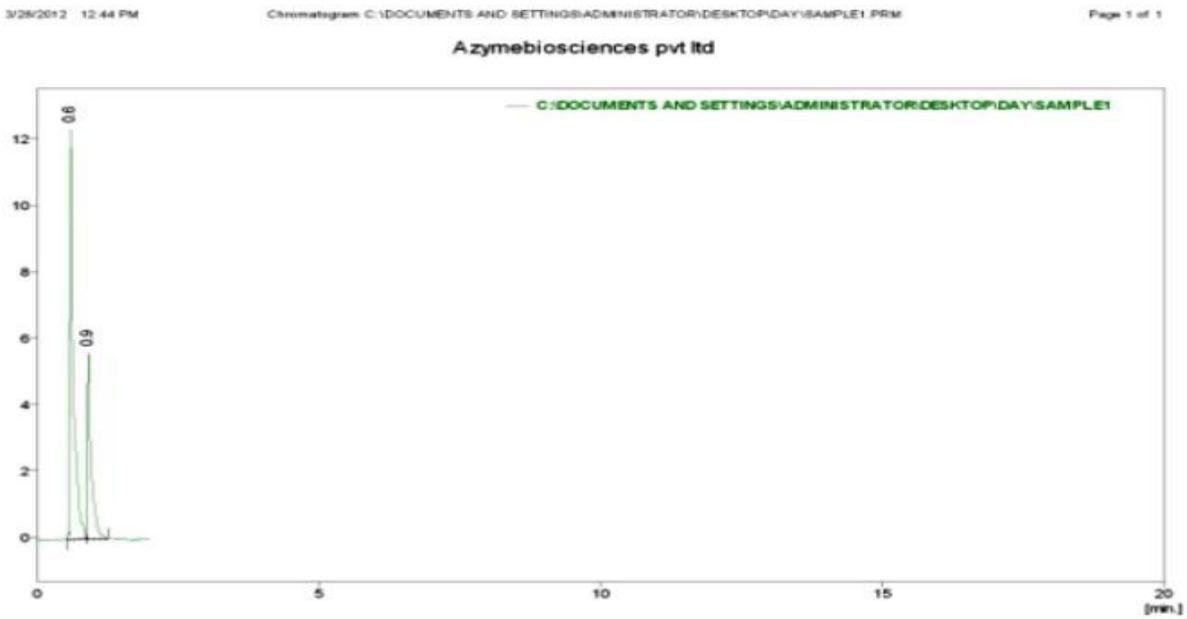
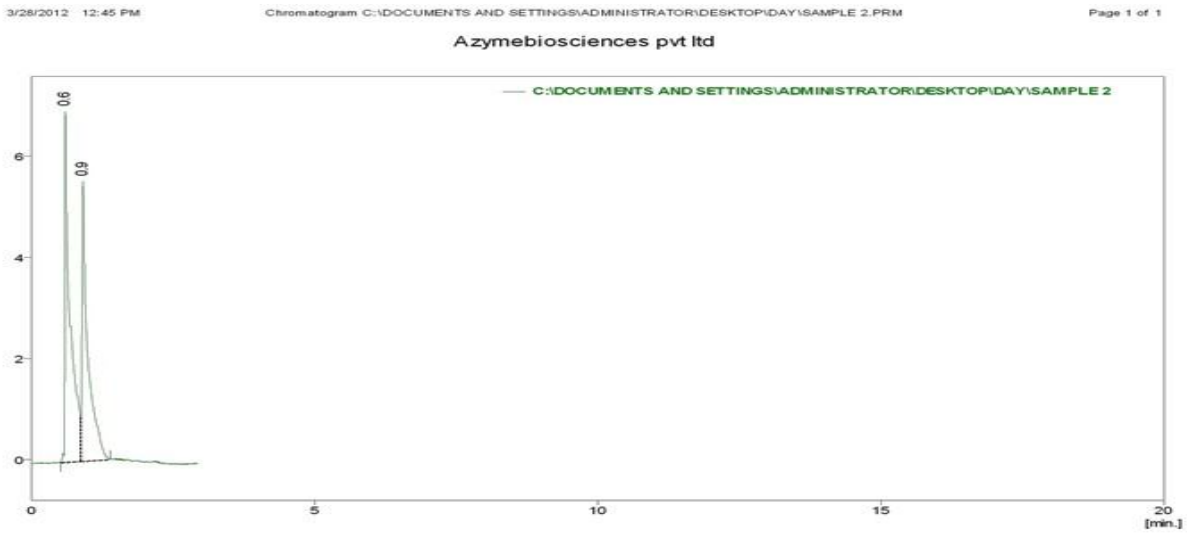


Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\DAY\SAMPLE1)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	0.580	47.113	65.7
2	0.897	24.542	34.3
	Total	71.654	100.0

Figure 4 GC of distilled extract of fermented medium from *Clostridium acetobutylicum*



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\DAY\SAMPLE 2)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	0.593	44.358	53.0
2	0.907	39.341	47.0
	Total	83.699	100.0

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Figure 5 GC of distilled extract of fermented medium from *Clostridium sporogenes*

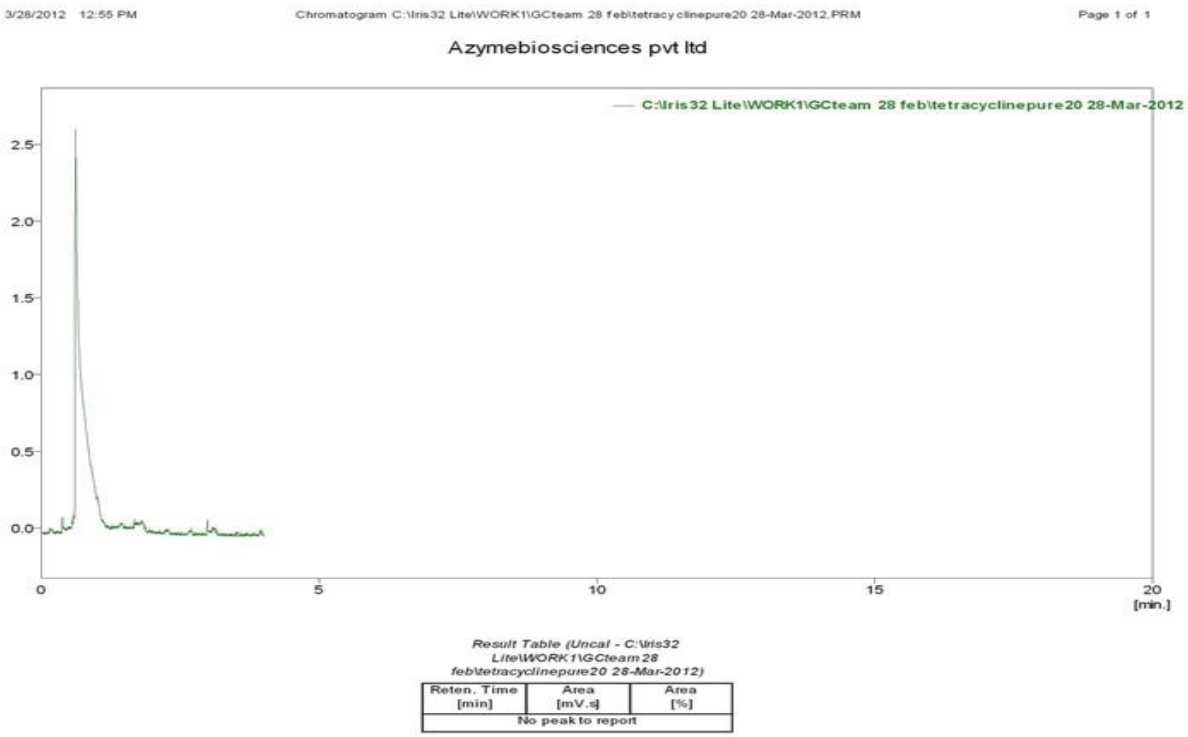

Figure 6 GC of distilled extract of fermented medium from *Escherichia coli*

DISCUSSION

The changes in the pH of the batch fermentation media, approximately after 48 hrs, indicated 4.7 and 4.81 in *Clostridium acetobutylicum and Clostridium sporogenes* inoculated medium respectively which indicates that microorganisms were active. At 96 hours, gradually pH started to increase to 5.11 and 5.17 respectively which confirmed the presence of ABE. As mentioned before, ABE fermentation undergoes two phases. In the first phase, microorganisms produce acids and as a response, pH decreases. In the second phase, acids are transformed to acetone, butanol and ethanol, which drive the pH back to less acidic state. Graph 1and 2 show the changes of pH during fermentation process which proves that it has undergone both phases actively.

The change in pH of the batch fermentation of E.coli, after approximately 48 hrs, indicated 4.95 which further declined rapidly to 4.75 till the end of the process. That confirms negligible amount of ABE production (Graph 3).

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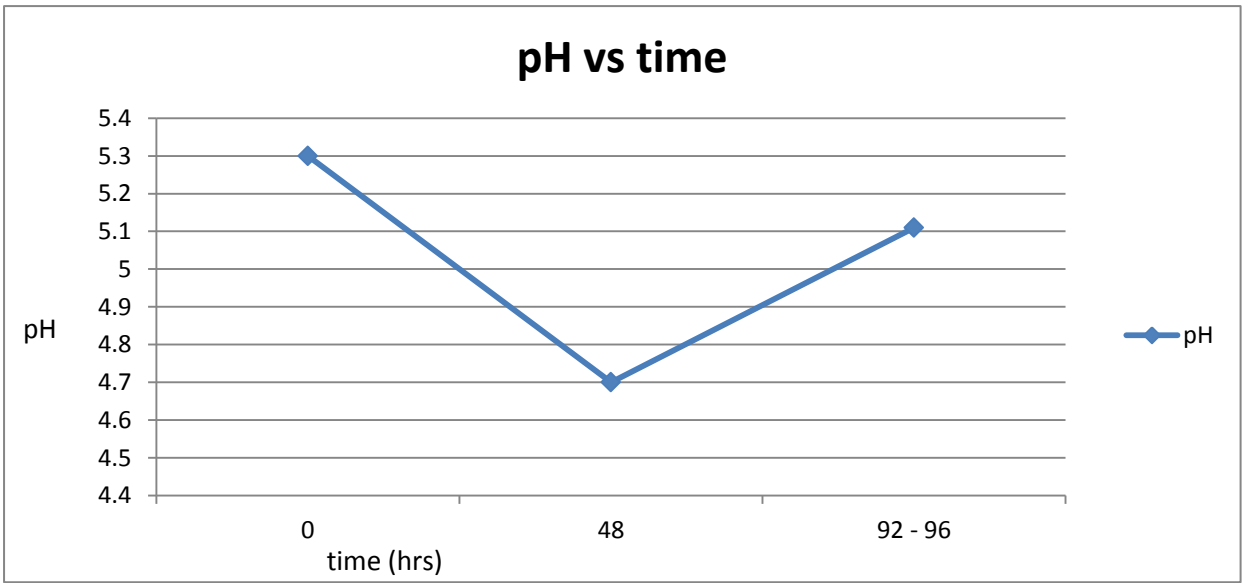
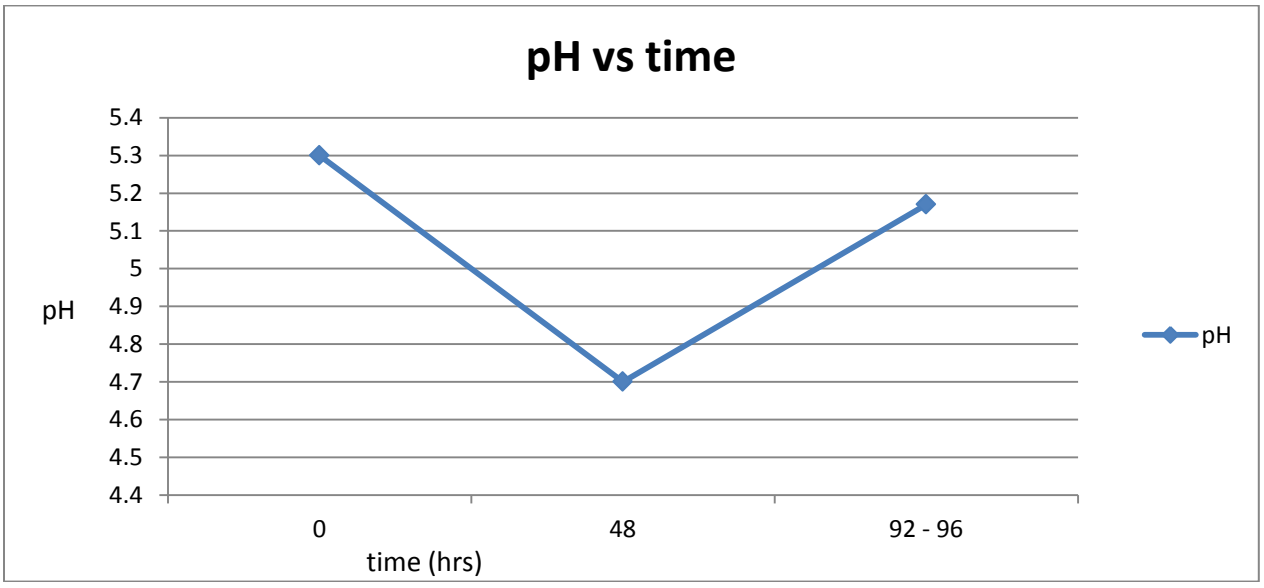
Graph 1: pH vs Fermentation Time of *Clostridium acetobutylicum*



Graph 2: pH vs Fermentation Time of *Clostridium sporogenes*



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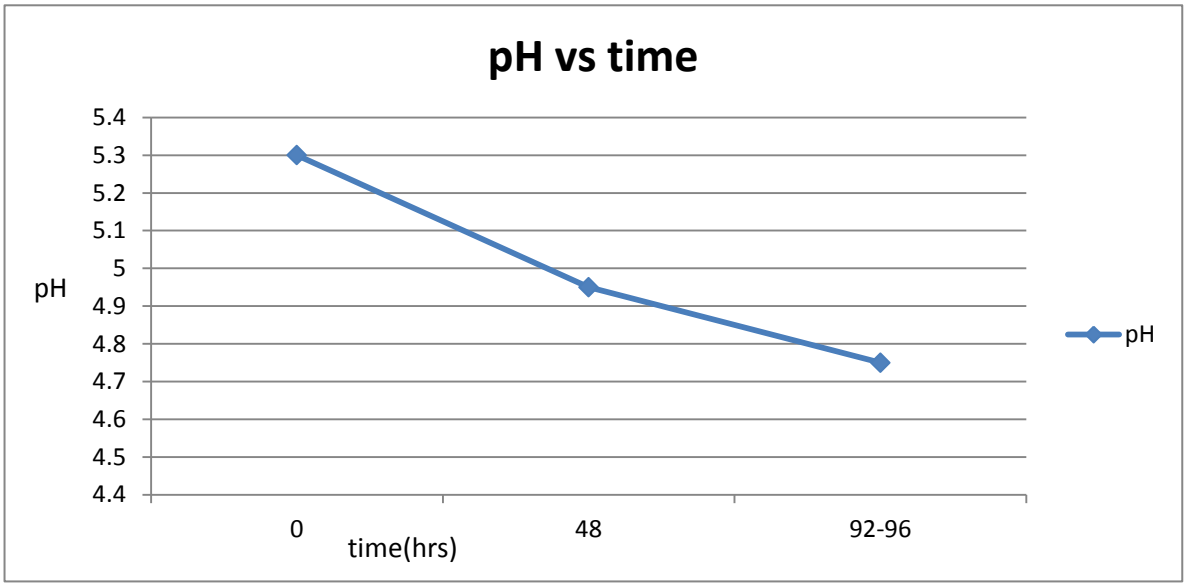
Graph 3: pH vs Fermentation Time of *Escherichia coli*



Clostridium acetobutylicum NCIM 2337 and *Clostridium sporogenes* NCIM 2559 are capable of utilizing Cane sugar substrates for ABE production. After 98 h of fermentation, both butanol and acetone were produced with an increased yield; it could be beneficial for commercial application in near future.

A Butanol tolerant parental strain of Clostridium acetobutylicum & Clostridium sporogenes along with Eshcherichia coli were studied and maximum Butanol yield was achieved in Clostridium sporogenes.

Butanol production mainly dependent on the microorganisms used in the process, as well the suitable culture media to grow. The conventional substrates for ABE fermentation are starch or molasses. Especially in developing country like India where there is lot of biomass available and which goes waste, can be utilized for production of Butanol and other important solvents which can act as our future Biofuel and can cut down the cost of production.

The resulting genetically engineering strain has an ethanol production rate similar to yeast [7], and its ethanol tolerance has been increased by selection on enrichment media [8]. A number of organisms, such as *Clostridium acetobutylicum*, produce butanol from sugars via the ABE (acetone, butanol, and ethanol) fermentation and can be manipulated to produce mostly butanol.

Cellulose biomass has the potential to contribute to meeting the demand for liquid fuel. Degrading lignocellulose are a handful of model organisms such as the fungus *Trichoderma resei* and the bacterium *Clostridium thermocellum* [1]. Most fermenting organisms such as *S.cerevisiae* cannot tolerate ethanol concentrations exceeding 25% (v/v) [9].

ISSN: 0975-8585

In this present project, work proposed on the synthesis of next-generation biofuels products, such as higher-chain alcohols, alkanes, and molecules with structures and activities closer to that of petroleum and diesel. Butanol, for example, can either be used in conventional engines in pure form or mixed with petroleum, can be transported by existing pipelines, and has higher energy content than petroleum [2].

Using sugarcane juice as Carbon source for *Clostridium acetobutylicum* and *Clostridium sporogenes,* delivered Butanol production of 0.26% and 0.41% respectively after five days of fermentation. The pH of the solution, after autoclaving and inoculation, was 5.30, which initially decreased, as the sugars were utilized and acids were produced. After five days of fermentation process, pH increased, the presence of compound was identified and quantified using Gas Chromatography.

CONCLUSION

In this present Project work revealed that the fermentation process using these strains of bacteria and sugarcane juice as a medium, was successful. Microorganisms produced good yield in the Carbon source provided, at a lesser cost and utility of biomass showed the growth trend expected for batch fermentation. Since Butanol has the primary expected product of this fermentation, was successfully produced with a maximum yield of 0.41% in *Clostridium sporogenes,* hence it could be beneficial for commercial application in near future.

ACKNOWLEDGEMENT

Dr Premchandra Sagar –Vice Chairman and CEO, DSI Bangalore.
Dr P.S Rao -Vice President R&D, Life sciences and Engineering DSI Bangalore.
Ms Shobha.K.Jyanna HOD PG Department Of Microbiology DSI Bangalore .

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