

## Study of *Coprinus cinereus* with the aid of *Carica papaya* (Peel) as a substrate for the production of higher yield Bioethanol by RNA Interference

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### Abstract

Over the last few years, the undesirable impacts of fossil fuel on the environment and resulting global warming, progressive demand for energy, inevitable reduction of the world's energy supply, and the unstable oil market (such as the energy crisis of the 1970s) have renewed the interest of society in searching for substitute fuels. Ethanol as a neat fuel or uniform in the blended form with gasoline has a long history as automotive fuel. Microorganisms play an important role in production of ethanol from renewable resources and thus, selection of suitable strain is essential for separate process. Adding onto this for the production of bioethanol microorganisms play a key role and till date number of species are being used for the process to be carried but still there are some of the species that are yet to be explored, one of this comes under the phylum Basidiomycota as *Coprinus cinereus*. for the higher production of bioethanol gene silencing technology was used so as to focus onto one of desired pathway for the project since once RNA interference was applied the microbe was subjected to the only pathway which was required and the utilization of carbohydrate in some other pathway was blocked.

### Introduction

At the beginning of the 20th century, many industrial materials such as dyes, solvents, and synthetic fibers were prepared from trees and agricultural crops. By the late 1960s, however, many of these bio-based chemical products were swapped by petroleum byproducts which could be produced at lower cost [1, 2]. Bioethanol is being widely investigated as a renewable fuel source because in several respects it is superior to gasoline fuel. Ethanol delivers energy that is renewable and less carbon concentrated than oil [3]. Production of fuel ethanol from biomass seem like to be an interesting alternative to traditional fossil fuel, which can be applied as a sole fuel in cars with loyal engines or in fuel blends. Ethanol is presently produced from sugars, starches and cellulosic materials. Cellulosic resources obtained from wood and agricultural residuals, municipal solid wastes and energy crops signify the most abundant global source of biomass [4].

Ethanol as a neat fuel or uniform in the blended form with gasoline has a long history as automotive fuel.

As long as global demand for energy continues to grow, oil prices are not likely to decrease. Consequently, the demand for a renewable and environmentally benign fuel will rise. Microorganisms play an important role in production of ethanol from renewable resources and thus, selection of suitable strain is essential for separate process. Ethanol production is much more challenging and difficult when lignocellulosic and/or cellulosic materials are to be used as raw materials. However, the most frequently used microbe has been yeast and among the yeasts, *S. cerevisiae* which can tolerate ethanol concentration as high as ca. 20% of fermentation medium is the favored strain [5]. *Coprinus cinereus* S. F. Gray is a coprophilous heterothallic basidiomycete of the Agaricaceae, which grows on horse dung as the natural Substrate. *C. cinereus* is commonly known as an ink cap [6]. Adding onto this for the production of bioethanol micro-organisms play a key role and till date number of species are being used for the process to be carried but still there are some of the species that are yet to be explored, one of this comes under the phylum basidiomycota as *Coprinus cinereus*. The major reason to go for this strain was that it lacks the production of lactic acid by lactate dehydrogenase and it can very well grow in the laboratory conditions as its life cycle was short.

### Method and Materials

#### FUNGIpath TOOL

FUNGIpath [7] has been designed as a user-friendly website. FUNGIpath further allows the conservation of pathways among different fungi to be checked and visualized. This can be done at the level of a particular step (corresponding to a defined EC number) either in a pathway or by considering all the steps of a complete pathway. FUNGIpath appears to be a useful and innovative tool for helping to resolve some artifact pathway holes. FUNGIpath appears to be a reliable tool for the analysis of fungal metabolism.

#### *Coprinus cinereus* (422) Strain

*Coprinus cinereus* was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC).

#### Fungal Strain and Growth Media Preparation

Fungus *Coprinus cinereus* was used in all the experiments (MTCC 422)

**Culture Conditions**

The fungus grew and formed spores on potato dextrose agar plates at 25° C. After 24 hours some signs of growth could be observed on the surface of agar plate and after 4-5 days incubation of the fungi and its spores was spread on the surface of the PDA plates [8].

**Substrate**

The substrate *Carica papaya* was used in fermentation. The peel part of the papaya was used. Peel of the papaya was dried under sundry conditions and was used for the process of fermentation.

**Hydrolysis of cellulose**

In the hydrolysis step, fruit extract in the form of sundried condition was boiled that resulted the polymers breakdown into fermentable glucose monomers.

**Acid hydrolysis**

Acid hydrolysis can be carried out with either concentrated or dilute acid, with distinct differences between the methods. The concentrated acid procedure operates at a low temperature of around 30–40°C, usually utilizing H<sub>2</sub>SO<sub>4</sub> because of its low cost, but HCl and HF have also been used [4, 9].

**Fermentation**

**Microorganism**

Ethanol production is much more challenging and hard when lignocelluloses and/or cellulosic materials are to be used as raw materials. Therefore, capability of consuming both pentose and hexose sugars, high tolerance against substrate, ethanol as well as inhibiting compounds, high ethanol yield and least nutrient requirements are the essential features of an ideal microorganism [10].

**Fermentation strategies during the set-up**

Sugar such as glucose is directly metabolized by the yeast cells through the glycolysis pathway to gain energy for biosynthesis. Under anaerobic conditions, the overall reactions produce two moles of ethanol and CO<sub>2</sub> per mole of glucose consumed [11].

**Incubation**

The incubation was carried out for 144 h in rotary shaker with an agitation rate of 110-150 rpm and 25 °C.

**RNA interference**

It is a process that is characterized by small interfering RNAs mediating the silencing of genes at the DNA or RNA level [12]. Two sequences were used one was sense and another was antisense. Length of this small interfering RNA was 21, and its G/C percent was 47%. It has been customized this siRNA to target Pyruvate dehydrogenase gene. The pack was provided with 5N moles, amount of siRNA in lyophilized form and 1 ml nuclease free water. The shelf life of this siRNA is 1 year when stored at -20° C in a non-frost free freezer.

**Table 1:- Details of siRNA**

Codes	Sequence	Scale	of 5' end	3'	Purity
		Syn		end	
SENSE	GGGCCUCAUGGACAAGUUUTT	80N	#	#	DSL
ANTISENSE	AAACUUGUCCAUGAGGCCCTT	80N	FLOUROSENSE	#	DSL

**Analytical Methods**

**Sugar analysis**

Some sugars are capable of reducing silver ions to free silver, and copper (II) ions to copper (I) ions, under prescribed conditions. Such sugars are called reducing sugars. The Benedict, Fehling tests depend on the formation of copper (I) oxide precipitate to indicate a positive reaction [13].

**Fehling**

**3 Benedict**

Benedict's test determines whether a monosaccharide or disaccharide is a reducing sugar, and hence similar in purpose to the Tollen's and Fehling's test. This makes use of a single solution of copper (II) citrate which does not deteriorate as

quickly on standing. The color ranges from green to yellow to orange to brick-red depending on the amount of reducing sugar in the sample; with a sample containing 1% glucose, the precipitate is usually brick-red. Benedict's test will give a color change for any mono- or disaccharide containing a hemiacetal or hemiketal group. Since sucrose or table sugar does not contain these groups, it will not give a positive test [13].

**Anthrone**

Anthrone dissolved in sulphuric acid may be used for the quantitative determination of different carbohydrates. Quantitative determination is only possible when the identity of sugar components is

known because color development varies with different sugars.

The test is commonly used for reducing sugars but is known to be not specific for aldehydes. For example, fructose gives a positive test with Fehling's solution [13]. A positive test was indicated by a green suspension and a red precipitate. The test is sensitive enough that even 1 mg of glucose will produce the characteristic red color of the compound [13]

**Ethanol Determination**

**1 Dichromate test:**

This method uses a redox titration to find the concentration of ethanol in an aqueous solution. The ethanol is oxidized to ethanoic acid by reacting it with an excess of potassium dichromate in acid. The amount of unreacted dichromate is then determined by adding potassium iodide solution which is also oxidized by the potassium dichromate forming iodine [14].

**2. Gas Chromatography [15]**

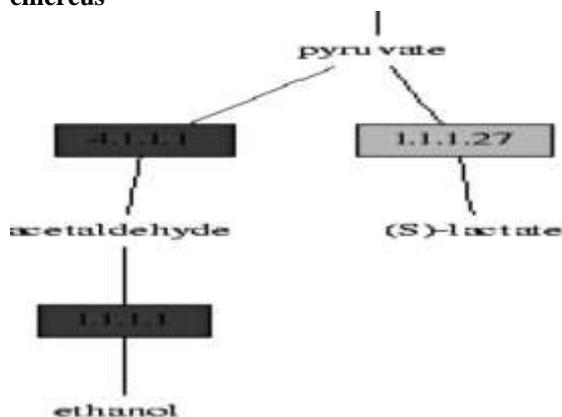
Gas chromatography used to detect ethanol.

**RESULTS AND DISCUSSION**

**Strain Selection by Computational Approach**

**1 FUNGIpath**

**Fig 1: Ethanol producing pathway of Coprinus cinereus**



Strain selection was done using bioinformatics tools FUNGIpath V3.0. FUNGIpath was used, which was

an online tool that is used to study the metabolic prospective of fungal species in brief. In the FUNGIpath, SUCROSE DEGRADATION TO ETHANOL AND LACTATE (Anaerobic) pathway was used in all species of fungi.

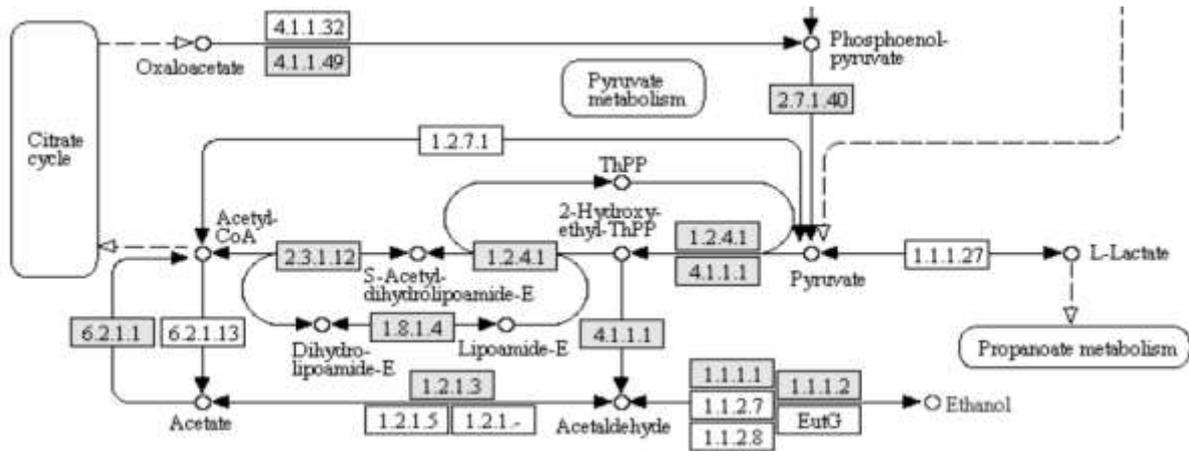
**The organism was chosen due to the following reasons:**

1. Enzyme commission no. 1. 1. 1. 1(Alcohol Dehydrogenase) was present and 1. 1. 1. 27(L-Lactate Dehydrogenase) was absent. As it is a well-known fact that alcohol dehydrogenase is being used in fungus for ethanol production and lactate dehydrogenase is being used for the lactate fermentation. So if enzyme lactate dehydrogenase is absent in the organism, the organism may produce maximum concentration of ethanol with the maximum utilization of reducing sugar. This concept was used for the production of normal as well as higher yield of bioethanol.
2. This strain is a novel strain, and has not been used previously in any other concept for the production of bioethanol.
3. This strain is not a pathogenic strain and can be utilized in general laboratory levels.
4. This strain is a model organism for the genetic engineering purposes these days.

**2 KEGG (Kyoto Encyclopedia of Genes and Genomes) [16]**

The KEGG pathway was used for the Glycolysis / Gluconeogenesis pathway analysis of Coprinus cinereus and find analysis that strain will produce the ethanol or not and also that the strain does not produce the lactate. And second analysis was that if enzyme 1. 2. 4. 1 (Pyruvate Dehydrogenase) stop its function in turn it may be possible that maximum concentration of sugar can be utilize by the enzyme 1. 1. 1. 1 (Alcohol Dehydrogenase) to produce by higher yield of ethanol. For inhibiting the function of the gene encoding enzyme siRNA designing tool from EUROFINS was used and siRNA sequence can inhibit the function of 1. 2. 4. 1 enzyme. Than that enzyme will not use much concentration of carbohydrates that it might be using earlier.

**Fig 2: Glycolysis pathway of Coprinus cinereus from KEGG database**



### 3 siRNA DESIGN

The siRNA sequence design was designed by Invitrogen siRNA tool. *Coprinus cinereus* strain enzyme sequence of pyruvate dehydrogenase was taken from KEGG. NCBI-Gene ID: 6013789

Table 2:- siRNA details

Codes	Sequence	Scale of Syn	5' Mods	3' Mods	Purity
SENSE	GGGCCUCAUGGACAAGUUTT	80N	#	#	DSL
ANTISENSE	AAACUUGUCCAUGAGGCCCTT	80N	FLORESCENCE	#	DSL

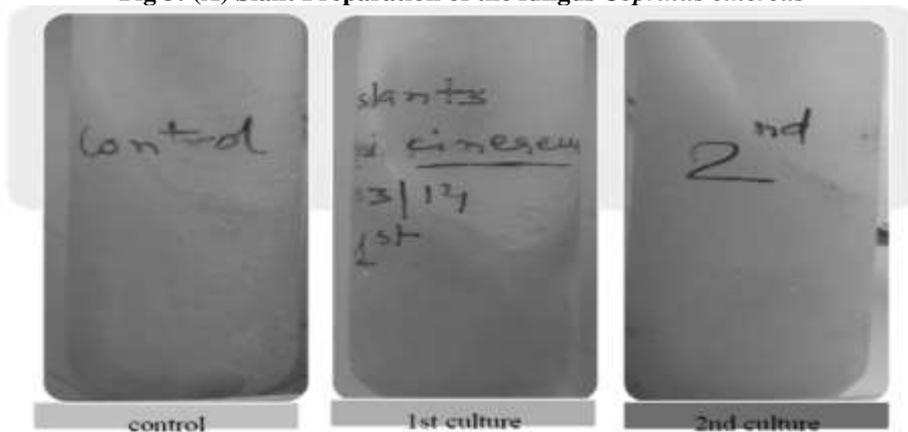
The antisense was design with 47% of GC content so it can easily make strong bond. It was ordered with florescence sequence at 5' prime because with the help of that can analysis that siRNA will be transfected or not.

### 2 Culturing

Filamentous fungal strain *Coprinus cinereus* (MTCC 422) was used in all the experimental set-ups. After 24 hours some signs of growth could be observe on the surface of agar plate and after 4-5 days incubation the fungus and its colonies were observed on the surface of the PDA plates.

In the earlier studies it was shown that *Saccharomyces cerevisiae* was incubated on orbital shaker at 30o C and 150 rpm for 22h which was perpetually similar to *Coprinus cinereus* [17] in which its growth was taken in account.

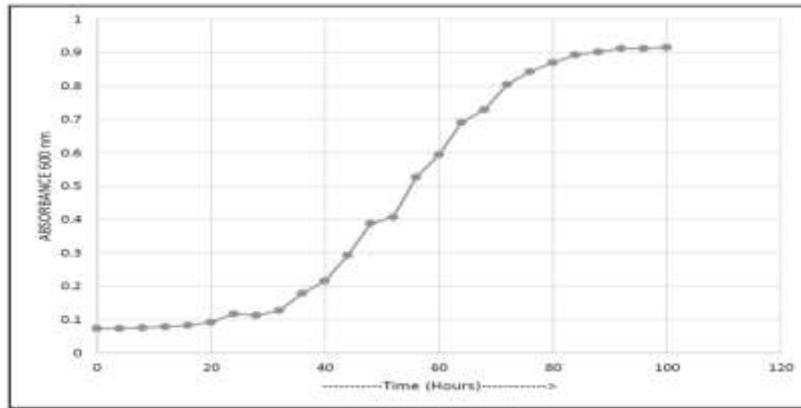
Fig 3: (A) Slant Preparation of the fungus *Coprinus cinereus*



### 3. Growth Curve

Microorganism *Coprinus cinereus* was cultured at 25°C in Potato Dextrose Broth and analysis of the growth of organism was done.

Fig 4:- Growth curve of fungal strain *Coprinus cinereus*



Growth Kinetics of the organism was observed for 4 days, that is for 96 hours. Looking over to the graph it was observed that till 24 hours the organism was adapting itself to the environment provided, after which it entered in to the exponential phase, the phase of maximum growth that lasted for 72 hours, after the exponential phase the organism entered into the stationary phase.

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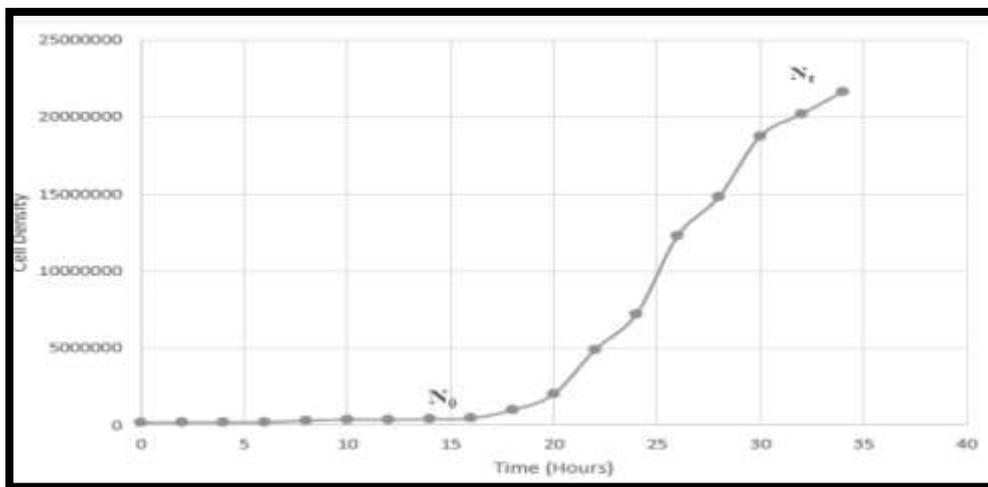
**4. Specific Growth Rate**

It can be defined as growth per mass of cells. The units of a specific growth rate are: Mass/(mass

cells\*time). Specific growth rate of the organism came out to be 0. 2136 per hour, indicating the fact that the optimum condition for the organism was 25°C and pH 5. 5. This growth rate was considered as the optimum growth rate for the organism that will be taken as the control growth rate.

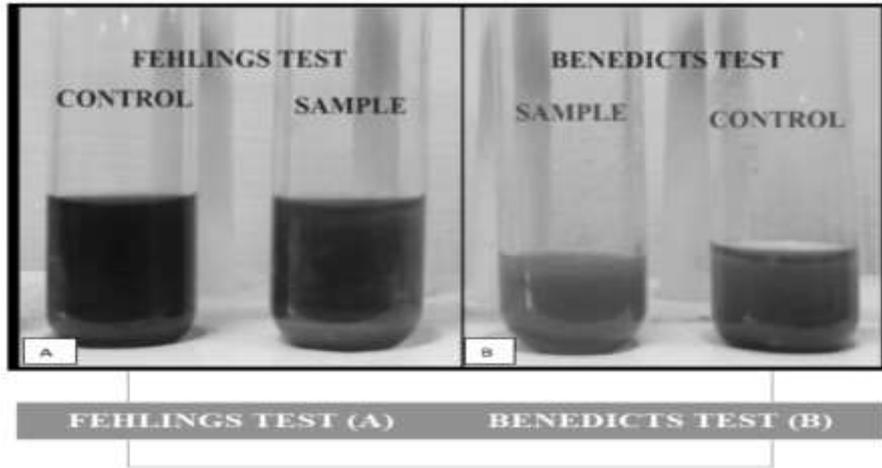
After the calculation of the specific growth rate, organism’s generation time and doubling time was calculated. Generation time of the organism came out to be 0. 3081 and doubling time came out to be 3. 24 hours.

**Fig 5:- specific growth rate of Fungal strain Coprinus cinereus**



**5. Identification Tests for Carbohydrates**

**Fig 6:- Identification tests for carbohydrates by Fehling’s test and Benedicts test**



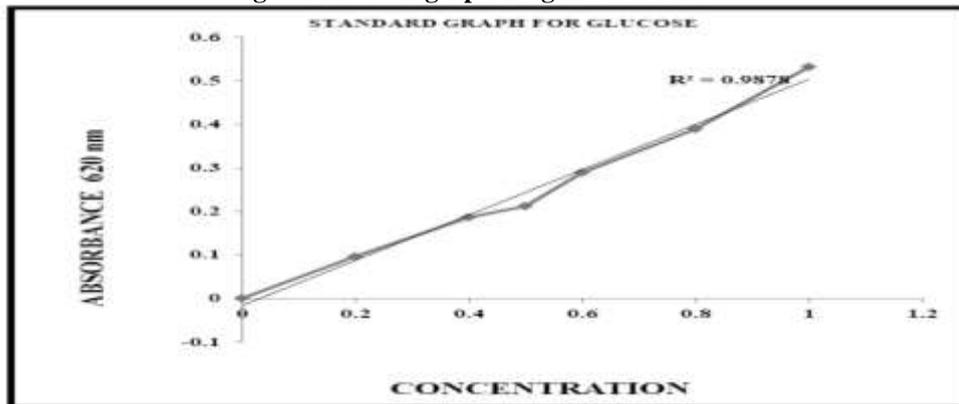
After the rigorous warming, the samples gave the brick red color that confirmed the samples having reducing sugars.

**6. Confirmatory Test for the Analysis of Carbohydrates**

1 Anthrone Test (Papaya peel (0.1 g))

Absorbance of the diluted sample of the papaya peel extract that was obtained as a hydrolyzates came out to be 0.021 at 620 nm on addition of anthrone to the sample solution. This solution whose optical density came out to be 0.021 was treated as an unknown sample whose concentration was measured on known to unknown basis.

**Fig 7:- Standard graph for glucose estimation**



On calculation, the concentration of unknown sample came out to be 0.049 mg/ml. Amount of carbohydrate present in 100 mg (0.1 g) of the sample obtained was 0.98 mg. Therefore, on calculating the values of glucose for 3 g, 5 g and 7 g were 0.3 g, 0.5 g and 0.7 g respectively.

Correspondingly, to validate the quantity of glucose in PDB, anthrone test was carried out, and the absorbance of the PDB came out to be 0.004 at 620 nm for 0.1 g of the sample. On calculation, it was observed that for 0.1 g, 2.24 mg of glucose was there.

**7. Applying Statistics**

1 MINITAB 14

Minitab is statistical analysis software. It was chosen for analysis and learning about statistics as well as statistical research during the project undertaken.

**Table 3:- Statistical analysis at different label by Minitab software**

S. No.	C. V. (T)	Temp.	C. V. (RS)	RS	C. V. (pH)	pH	SGR	S. D.
1	1	25	1	3	1	4.5	0.2828	0.002205
2	1	25	1	3	2	5.5	0.2406	0.003144
3	1	25	1	3	3	6.5	0.3736	0.002205
4	1	25	2	5	1	4.5	0.2817	0.002613
5	1	25	2	5	2	5.5	0.2611	0.001796
6	1	25	2	5	3	6.5	0.2646	0.003511

7	1	25	3	7	1	4.5	0.2658	0.003716
8	1	25	3	7	2	5.5	0.2386	0.002654
9	1	25	3	7	3	6.5	0.2489	0.002776
10	2	28	1	3	1	4.5	0.2740	0.002164
11	2	28	1	3	2	5.5	0.2600	0.003186
12	2	28	1	3	3	6.5	0.3455	0.003103
13	2	28	2	5	1	4.5	0.2744	0.003511
14	2	28	2	5	2	5.5	0.2634	0.003511
15	2	28	2	5	3	6.5	0.2402	0.002817
16	2	28	3	7	1	4.5	0.2228	0.029115
17	2	28	3	7	2	5.5	0.2693	0.00196
18	2	28	3	7	3	6.5	0.2497	0.000816
19	3	32	1	3	1	4.5	0.2320	0.025845
20	3	32	1	3	2	5.5	0.2556	0.003021
21	3	32	1	3	3	6.5	0.2657	0.002939
22	3	32	2	5	1	4.5	0.2377	0.003389
23	3	32	2	5	2	5.5	0.2479	0.001837
24	3	32	2	5	3	6.5	0.2406	0.046587
25	3	32	3	7	1	4.5	0.2243	0.021781
26	3	32	3	7	2	5.5	0.2338	0.000898
27	3	32	3	7	3	6.5	0.2839	0.002572

C. V. = Coded Values by the statistical tool.

Temp. = Temperature

RS = Reducing Sugar

SGR = Specific Growth Rate

S. D. = Standard Deviation

Fig 8:- Fungal strain *Coprinus cinereus* had shown fluctuated growth in 27 samples (calculated mean values).

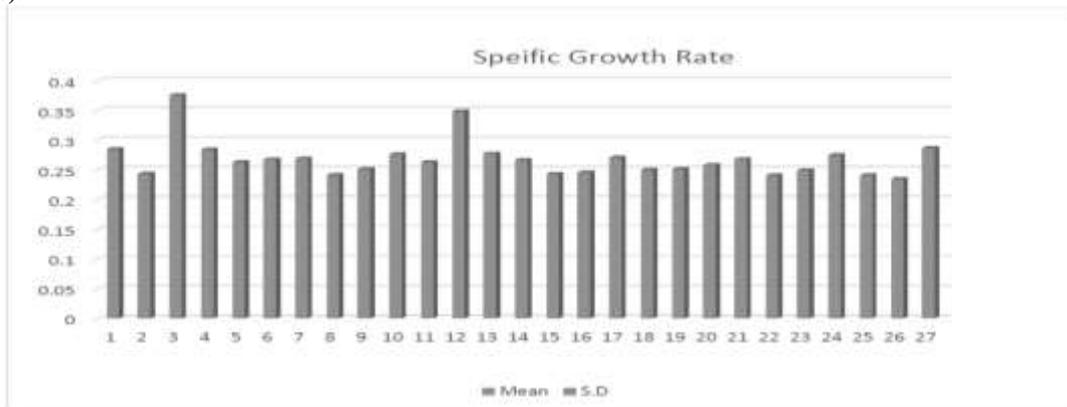
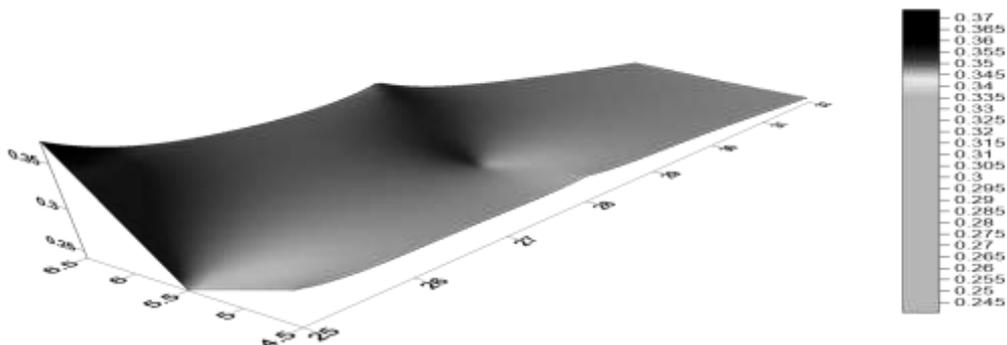
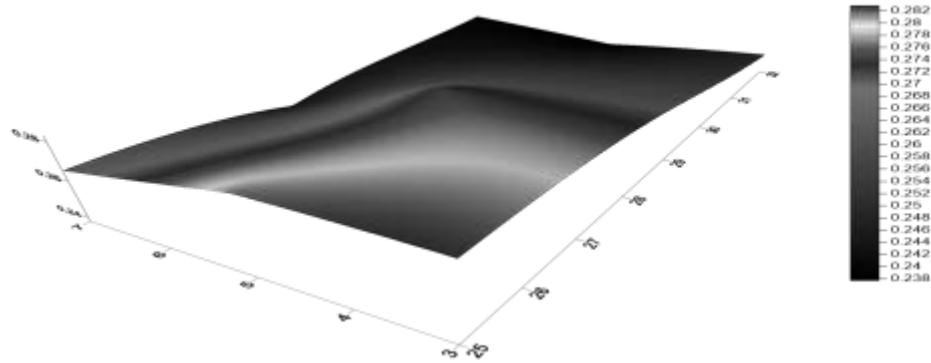


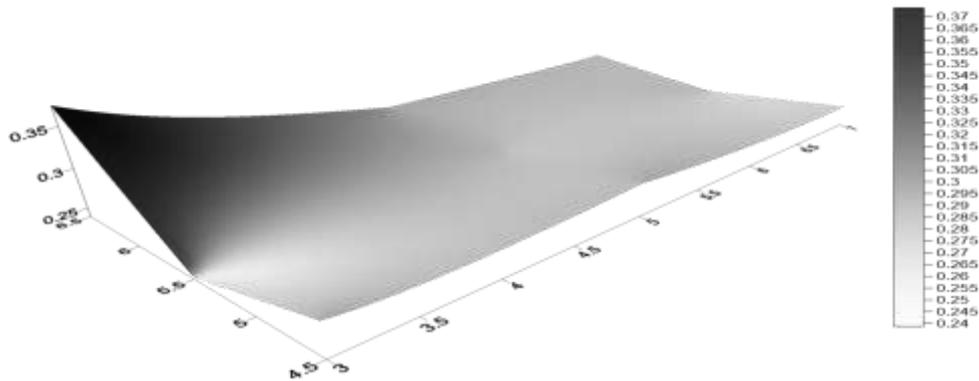
Fig 9:-Mean values of the parameters Vs. Specific growth rate of the organism *Coprinus cinereus*  
Temp Vs. pH Vs. Specific growth rate



Temperature Vs. Substrate Vs. Specific growth rate



Substrate, pH, Specific growth rate



The graph was plotted to represent the fluctuations in the data obtained after the calculations of the specific growth rate. The plotted graph represented that at 25°C, pH 6.5 and at substrate concentration 3 g. Specific growth rate came out to be highest, due to which this set-up was taken further for the shake flask fermentation method and for RNA interference aided fermentation.

Table 4:-Analysis of variance (ANOVA)

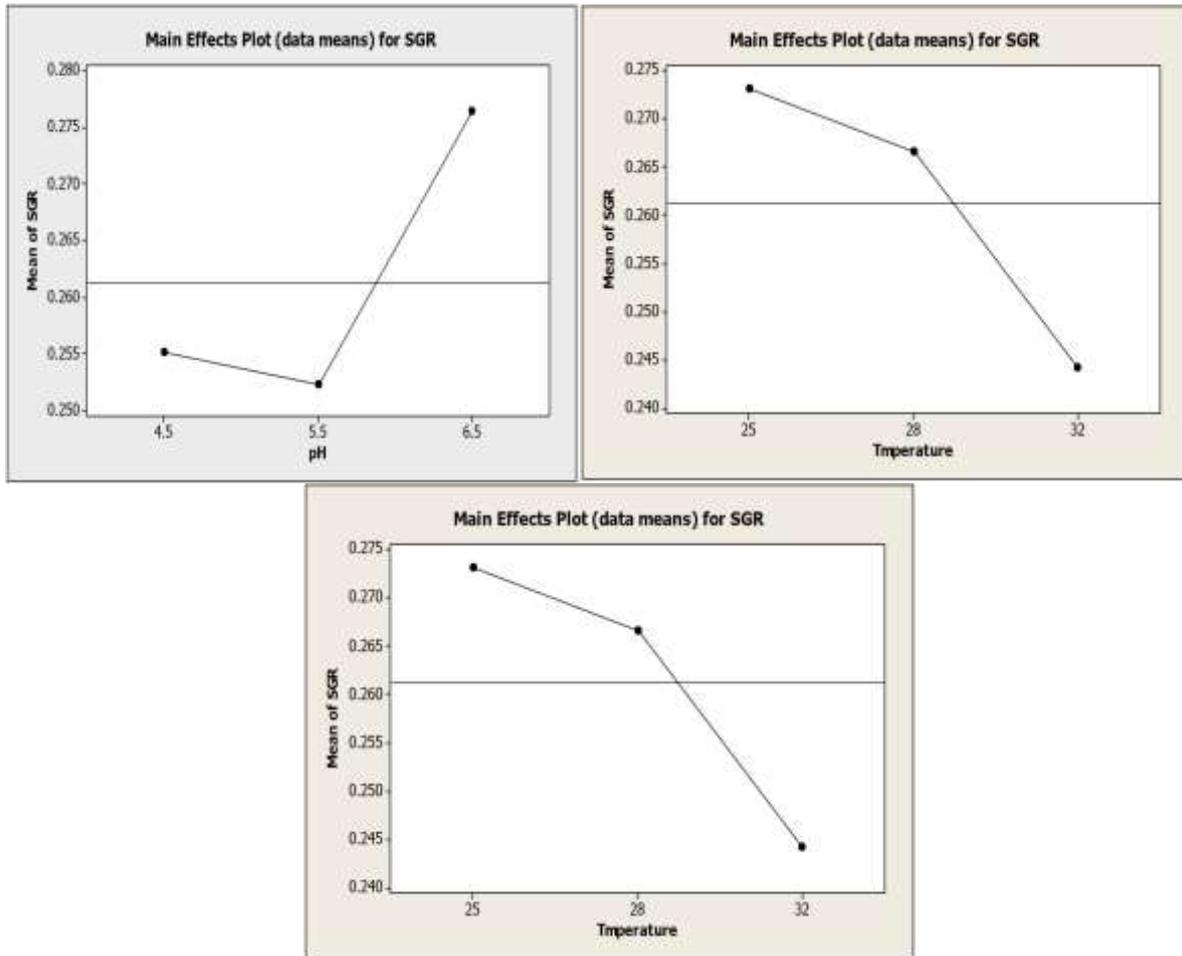
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Temperature	2	0.0124810	0.0124810	0.0062405	20.93	0.000
Ph	2	0.0094581	0.0094581	0.0047291	15.86	0.000
Reducing Sugar	2	0.0127676	0.0127676	0.0063838	21.42	0.000
Temperature*pH	4	0.0062240	0.0062240	0.0015560	5.22	0.001
Temperature*Reducing Sugar	4	0.0081143	0.0081143	0.0020286	6.81	0.000
pH*Reducing Sugar	4	0.0181607	0.0181607	0.0045402	15.23	0.000
Temperature*pH*Reducing Sugar	8	0.0205806	0.0205806	0.0025726	8.63	0.000
Error	54	0.0160973	0.0160973	0.0002981		
Total	80	0.1038836				

S = 0.0172655      R-Sq = 84.50%      R-Sq(adj) = 77.04%

SS-Sum of Squares; DF- Degrees of Freedom; MS- Mean Square

2. Plots for Main Effects on Specific Growth Rate of the Organism

Fig 10: Plot indicating the effect of Ph, Temperature and reducing sugar on the specific growth rate of *Coprinus cinereus*



On concluding, main effect, plots indicated that at pH 6.5, Temperature 25° C and at lowest concentration of reducing sugar that is at 3 g of papaya peel extract, maximum specific growth rate of the organism was observed. This might be due to the reason that the organism is likely to be comfortable with all its metabolic pathways at the conditions described above.

Table 5:- Effective data points result.

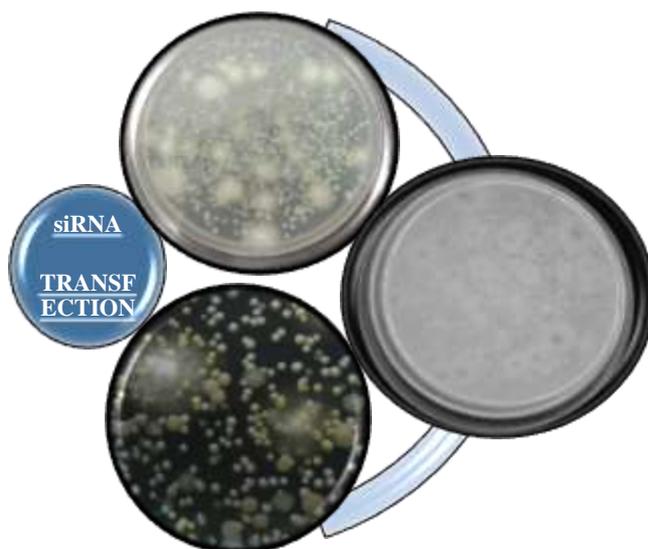
T	RS	pH	SGR
25	3	6.5	0.3736

This was the best conditions at which the organism was capable to show the maximum specific growth rate. On calculation, this specific growth rate gave the closest doubling time 1.8 hours which was very close to the result obtained from literature (1 hour) [18].

### 8. siRNA TRANSFECTION

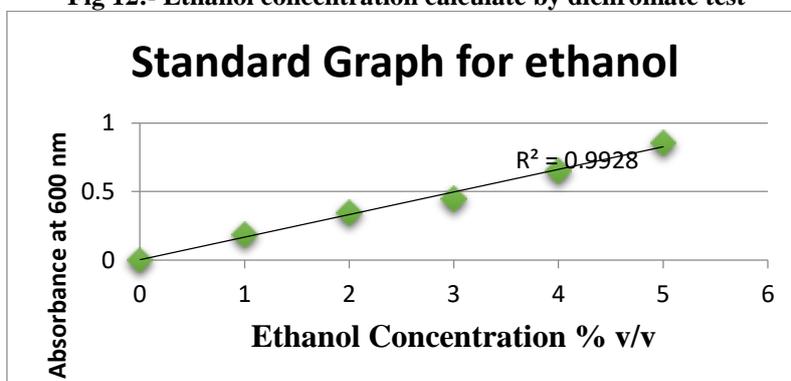
Protocol for transfection was followed and after the incubation period for 3 days the growth of the organism was observed. Since the siRNA that was transfected into the organism contained a fluorescence probe at 5' prime end of the strand, the colonies were expected to carry the same probe. This was observed under UV trans-illuminator, and was concluded that not all colonies got transfected, some of the colonies grown were observed as wild and some were transfected that shown the glowing effect under the trans-illuminator.

Fig 11: siRNA transfected culture of *Coprinus cinereus*



**9. Dichromate Test for the Detection of Ethanol**

**Fig 12:- Ethanol concentration calculate by dichromate test**



At first strength of the ethanol for standard curve was calculated using volume and strength relation. This relation was subjected to the conversion of volume of ethanol (0. 2, 0. 4, 0. 5, 0. 6, 0. 8 and 1. 0) ml to percentage of ethanol (1 %, 2 %, 3 %, 4 % and 5 %). These percentage values (% v/v) were then to be converted to the ethanol concentration in grams/ 100 ml.

As per the data recorded; ethanol concentration after fermentation with PDB gave fluctuating results as was expected, ethanol production with the control media as PDB and wild strain gave 1. 22 % (v/v) i. e. 0. 96 g/ 100 ml.

Correspondingly, with PDB + Papaya peel + wild strain gave 1. 8 % (v/v) i. e. 1. 4 g/ 100 ml ethanol concentrations.

Further, on calculating the concentration of ethanol utilizing PDB along with papaya peel and transfected strain gave 2. 8 % (v/v) ethanol concentration i. e. 2. 23 g/100 ml.

These observations gave the present study that ethanol production increased with the transfected organism. This RNA interference technique proved its potential for the production of bioethanol at a much higher yield as compared to the wild type strain.

**Fig 13- Chromatogram for standard ethanol (HPLC grade)**

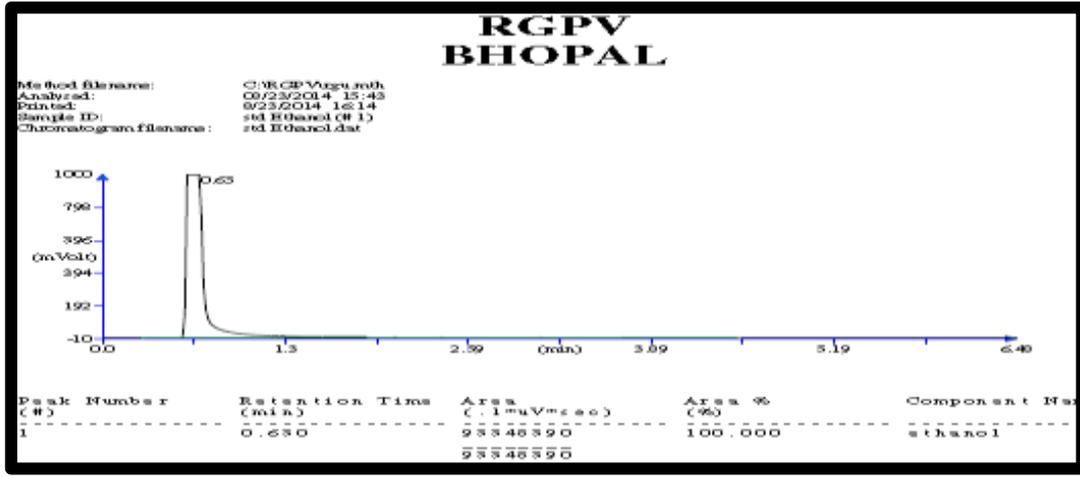


Fig 14- Chromatogram for wild strain in Papaya peel along with Potato Dextrose Broth

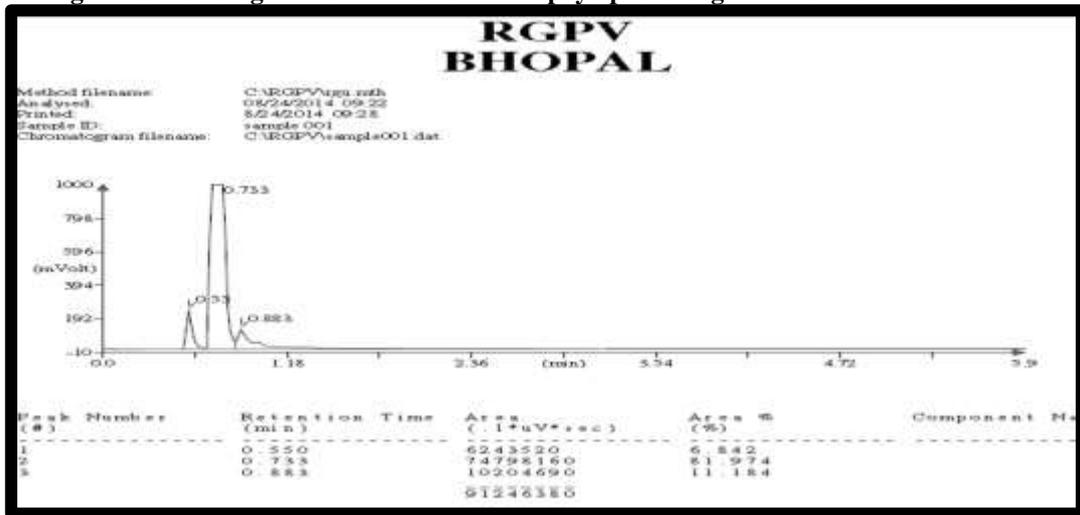


Fig 15- Chromatogram for transfected strain in Papaya peel along with Potato Dextrose Broth

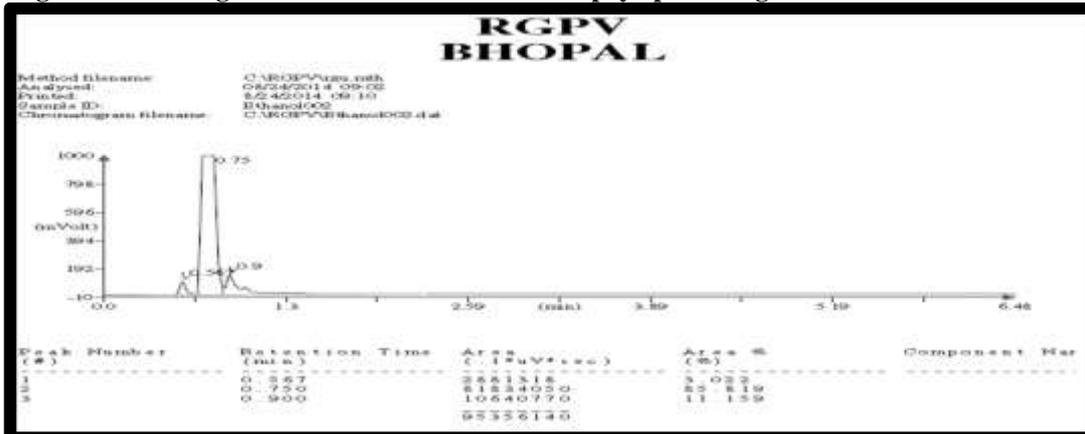


Table 6 - Gas Chromatography sample retention time

S.No.	SAMPLE DETAIL	RETENTION TIME (minutes)
1.	Standard Ethanol	0.650
2.	Ethyl acetate	0.617
3.	n-Butanol	0.983

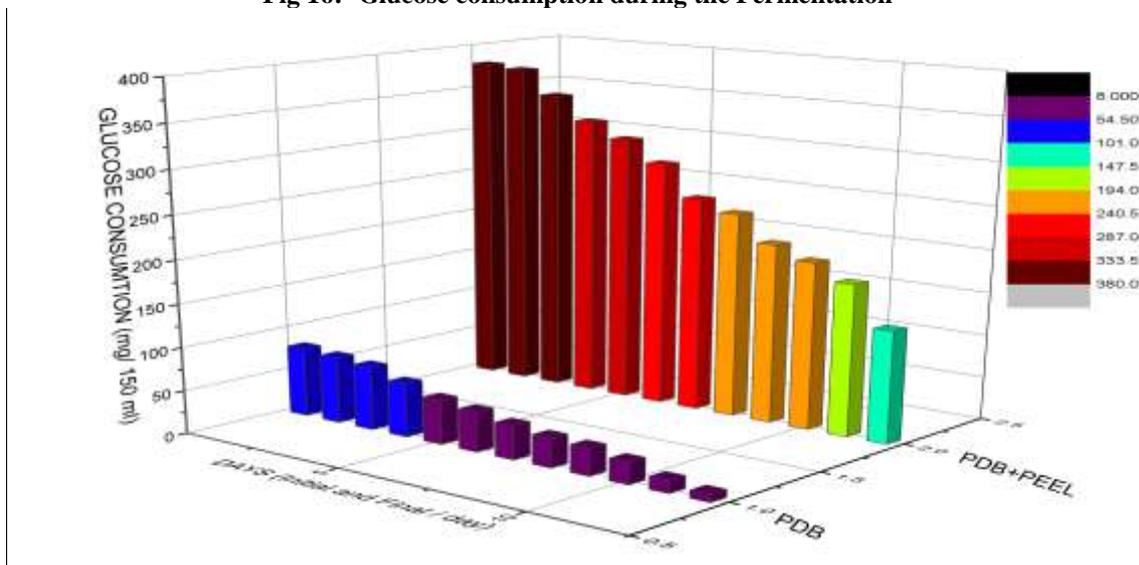
4.	Control	0.683
5.	Sample (Wild)	0.733
6.	Sample (Transfected)	0.750

Analysis of the gas chromatograms for the set-ups were carried out to determine the efficiency of the product recovered. The retention time for gas chromatography was considered for the analysis of ethanol in the fermentation media. For standard ethanol it came out to be 0.65 minutes additionally, for the control media it came out to be 0.68 minutes where as for the wild strain in media supplemented with papaya peel it came out to be 0.733 minutes. Considerable, increase in the separation time as well as area was observed in the transfected strain in which the retention time came out to be 0.750 minutes. This increase in retention time will in turn depict the increase in the area percentage of product retrieved.

This further gave the study a perspective that RNA interference technology aided in the field of bioprocess engineering for the successful increment in the yield of bioethanol.

### 10. Glucose Consumption during the Process of Fermentation

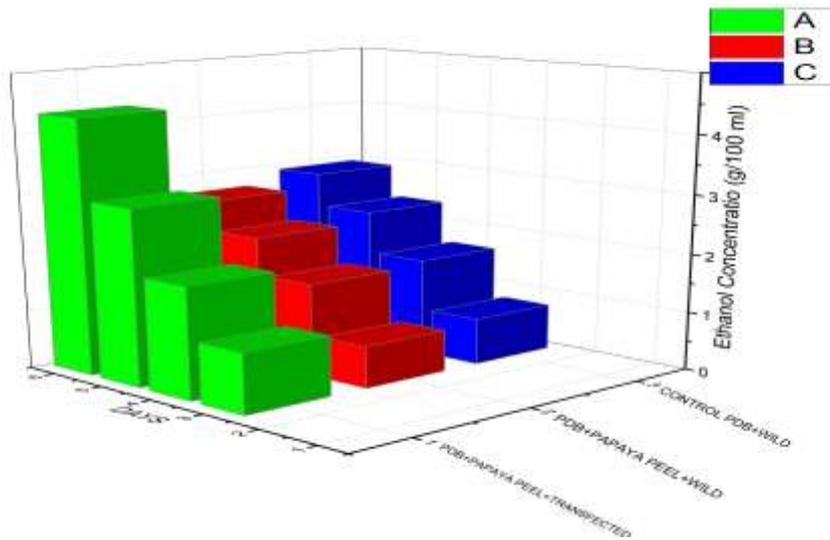
**Fig 16:- Glucose consumption during the Fermentation**



Looking over the graph plotted, glucose consumption was found to be decreasing on day to day basis. It was found to be 80 mg for PDB alone and for PDB along with the peel it came out to be 379 mg. With the extension of duration for fermentation these values tremendously decreased and reached at a comparatively less amount at the verge of the exponential phase. But as the values were decreasing, it did not gave the validation that the organism was producing equivalent amount of ethanol since the organism might be utilizing the carbohydrate content for its growth and respiration process.

### 11. Ethanol Production during Fermentation

**Fig 17:- Ethanol production during fermentation**



Pondering over the graph it was observed that with the utilization of PDB, the organism did produced ethanol in an acceptable manner. Ethanol production as per the graph plotted came out to be 0.96g with the wild type strain with the utilization of PDB alone, similarly with PDB + Peel ethanol production with wild type strain came out to be 1.4g/100 ml. Further, to test the consistency of ethanol produced with the transfected strain, it came out to be 2.23g/100 ml. This gave the study the potentiality of RNA interference mechanism for the fungal strain *Coprinopsis cinereus*.

These all values gave the study a specific route to add an organism to the field of bioprocess technology that can show its potential if taken on large scale (industrial level).

### Conclusions

On a global scale, synthetic ethanol accounts for about 3-4% of entire production while the rest is produced from fermentation of biomass – mainly sugar crops, e. g. cane and beet, and of grains (mainly corn) [5]. Ethanol as a neat fuel or uniform in the blended form with gasoline has a long history as automotive fuel.

Once the ethanol production was done the wild strain was transformed with the aid of gene silencing technology. This study gave a direction to the objective that wild strain produced 1.4g/100 ml bioethanol in media containing papaya peel and with the transfected strain a higher yield of bioethanol that is 2.23g/100 ml.

Effective data points for the attainment of higher yield of bioethanol were shown at Temperature 25, pH 6.5 and substrate concentration 3g both strains in wild as well as transfected. The retention time for gas chromatography was considered for the analysis of ethanol in the fermentation media. For standard ethanol it came out to be 0.65 minutes additionally, for the control media it came out to be 0.68 minutes where as for the wild strain in media supplemented with papaya peel it came out to be 0.733 minutes. Considerable, increase in the separation time as well as area was observed in the transfected strain in which the retention time came out to be 0.750

minutes. This increase in retention time will in turn depict the increase in the area percentage of product retrieved. This on the whole suggested that the transfection of the wild strain came out to be effective in the production of bioethanol with the utilization of papaya peel as a substrate along with the synthetic media.

### Future Prospects

Ethanol is widely explored fuel and will be in future also. The researchers are trying to produce higher yield of ethanol at a cheaper rate and in less time. Alterations in the Stress tolerance potentiality of strain by different parameter like temp, pH, substrate quantity, metal ion quantity and agitation etc. Genetic engineering also can be done on different level. Profiling of the organism is a leftover task that can be for the upcoming future needs Different substrate can be chosen so that better results can be obtained higher than *Carica papaya*.

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