
The alcoholic fermentative efficiency of medicinal fungus *Ustilago maydis* to unravel mechanisms of fruit waste deconstruction with the aid of Separate Hydrolysis and Fermentation (SHF) method

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ABSTRACT

The insistence for the fossil fuels is expanding day by day; this is to be met to fight for the economic development of the country. The environmental imprint and commercial performance of the existing suite of biofuel production approaches make them unsatisfactory to dislocate fossil fuels and diminish their influence on the account of greenhouse gas. To combat the decrease in economic growth and increase in the demand of people, researches opted for biofuels. First generation biofuels are acknowledged to be made from agro products, the second generation biofuels are made from lingo cellulosic biomass and the third and fourth generation of biofuel production encompasses “algal conversion into biofuels”. So, to combat this issue of reliance on other countries and food versus fuel issue one has to move forward for the production of sustainable energy using reuse and recycle strategy. In this research paper we had focused on environmental remediation process with the aid of fruit pomace residues.

Keyword: Fossil, Biofuel, Cellulosic, Pomace, Remediation

Introduction

Energy catastrophe and climatic changes are crucial subjects around the globe. The hunt for alternate energies has provided number of ways to produce electrical energy, such as wind farms, hydropower machinery, or solar cells. Earlier it was foretold that in near future, the crude oil will route out within 40 to 70 years, and natural gas will get finish within 50 years [1]. Subsequently, fossil fuel, which is principally used in automobile sector, should be swapped with non- or less-emitted carbon dioxide fuels. Worldwide average temperature is prophesied to upsurge from 1.4 to 5.8°C by the year 2100 plus carry on to intensify long after that [2].

Utilization of renewable biomass possessions to harvest liquid biofuels such as bio-ethanol offers striking explanations in the reduction of GHG emissions, further declining dependence on overseas oils, addressing energy security concerns, establishing rural and agronomic economies, and

growing sustainability of the world transport system [3]. Biofuel denotes to numerous liquid, gaseous and solid fuels resulting from organic i.e. biological materials known as biomass [3, 4]. These comprises of product such as bio-ethanol, bio-methanol, oils derived from vegetable, bio-diesel, bio-synthetic gas (bio-syngas), bio-char, bio-oil, bio-gas, bio-hydrogen and Fischer-Tropsch liquids [3]. First generation biofuels are fuels manufactured from agrarian crops such as oil seed, sugar crops, and grains [4, 5]. Second generation biofuels are retrieved from non-food biomass i.e. lignocellulose materials such as energy crops (vegetative grasses), forest residues, husks, stalks and straws [4, 6]. Third cohort biofuel are fuels shaped from photosynthetic algae also acknowledged as oilgae [5].

In the course of the process of fermentation, partial oxidation of biological complexes occur and for this reason less energy is acquired when equated with aerobic oxidation of the compounds. In contradiction, term industrial fermentation typically states to either aerobic or anaerobic processes, whereas fermentation in biological context defines a stringent anaerobic process, which occurs if pyruvic acid do not move in the Krebs cycle and if electrons from glucose metabolism pathway do not enter electron transport system (ETC). This in turn will enhance the production of ethanol as well as help in the process of environment remediation in the end. The main objective of the present study is to explore the novel organism *Ustilago maydis* for the production of ethanol as it has not been explored earlier for the production of alcohols and to remediate the environment from waste materials as well as to fight with the growing issue of global warming introducing the carbon neutral fuels. Thus, the study will also revolve around the issue of food vs. fuel concern, GHGs emissions, reliance on fossil fuels, etc.

Materials and Methods:

MATERIALS

Study was done at SOBT, Rajiv Gandhi Proudyogiki Vishwavidyalaya, Bhopal, Madhya Pradesh. **Fungus:** *Ustilago maydis* (MTCC 1474) was procured from Microbial Type Culture Collection (MTCC), Microbial Type Culture Collection, and Gene Bank, Institute of Microbial Technology, Chandigarh and was grown in malt extract agar (MEA).

Fruit pomace: The desired fruit wastes Apple (*Malus domestica*) and Guava pomace (*Psidium guajava*) were procured from the local fruit juice markets.

METHODS

1. Fungal Strain and Growth Media Preparation

Strain selection was done using bioinformatics approach, to retrieve a proper direction towards the experimentation process.

2. FUNGIpath Tool

FUNGIpath is a new tool dedicated to metabolism pathway exploration in fungi. In this tool, data for 50 fungi species was given along with their metabolic pathways and resemblances with the other species specifically *Saccharomyces cerevisiae* thus, the desired species came out to be fungus *Ustilago maydis*.

3. KEGG Database

After the selection of the desired species, its descriptive pathway and the activated enzymes were confirmed using Kyoto Encyclopedia of Genes and Genome (KEGG) database.

4. Revival of culture and culturing on agar plates (triplicates) [7]

Fungus *Ustilago maydis* MTCC 1474 was obtained as lyophilized culture from IMTECH, Chandigarh that was further revived on malt extract and was cultured over malt extract agar for its utilization.

5. Collection of the fruit wastes [8]

Fruit wastes were obtained from the local fruit markets else fruit juice corners situated in the city. These wastes were then washed in 5% Potassium permanganate (KMNO₄) & thus were rinsed well with distilled water.

6. Detection of the substrate for the fermentation process [9]

- Benedict's test** (2014b www.elearning.najah.edu): Reducing sugars are oxidized by the copper ions in the Benedict's solution to form a carboxylic acid and a reddish precipitate of cuprous oxide within three minutes in alkaline medium.

- Anthrone test for the estimation of total carbohydrates** (2014b www.elearning.najah.edu): Anthrone test was carried out in which furfural reacts with anthrone reagent to give a blue-green complex. The test is based on the dehydration of monosaccharide to furfural derivatives and thus is a colorimetric test.

7. Statistical analysis

Statistical analysis was done with the help of MINITAB software (v. 14). This software gives a tremendous array of results that could aid in the prediction of effective data points obtained after the experimental design. It is based on coefficient of regression values that should lie between 0-1 at 95% confidence interval.

8. Fermentation, Recovery and Analysis of the product [10]

After hours of fermentation process, a small amount the sample was taken out to carry out the ethanol detection. The sample taken out was either centrifuged or filtered through the filter paper. The supernatant collected was analyzed using analytical as well high performance methodologies (Gas Chromatography).

Analytical Tests-

Lugols Test: Iodoform Reaction (Identification Test)

(Laboratory Manual Chapter 10- Energetics, Fermentation, and Respiration)

Dichromate Test for the determination of ethanol [11]

Concentration of ethanol in a sample was determined by back titration with acidified potassium dichromate. When the sample with an excess of potassium dichromate is taken, all ethanol is oxidized to acetic acid. When alcohol vapor makes contact with the yellow dichromate-coated crystals, the color changes from yellow to green.

Ethanol was also detected with the help of Gas Chromatography [12]

Results and Discussion:

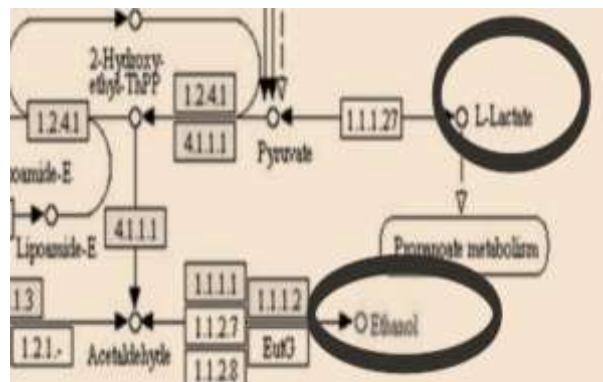
1. Strain Selection

Metabolomics of fungus: *Ustilago maydis*

Strain selection was done using bioinformatics tools and databases. FUNGIpath was used, which is an online tool that is used to study the metabolic potential of fungal species in brief

**Figure 1 (A and C): Metabolic Pathway for fungus *Ustilago maydis* taken from KEGG database
(B): Microscopic view of *Ustilago maydis***

A)



B)



C)

Taxonomy	Selected genomes	1.1.1.1	1.1.1.27	1.2.1.12	2.7.1.11	2.7.1.40	2.7.2.5	2.7.7.9	4.1.1.1	4.1.2.15	4.2.1.11	5.3.1.1	5.3.1.9	5.4.2.1	5.4.2.2
Acom.	Doth.	-	-	1.	Oxidoreductases	-	-	-	-	-	-	-	-	-	-
	Eerot.	-	-	1.1.	Acting on the CH-OH Group of Donors	-	-	-	-	-	-	-	-	-	-
	Leot.	-	-	1.1.1.	With NAD(P)H or NADPH as acceptor	-	-	-	-	-	-	-	-	-	-
	Sord.	-	-	1.1.1.27	L-lactate dehydrogenase	-	-	-	-	-	-	-	-	-	-
	Sacch.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Tapir.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Baud.	Agar.	Homeb.	-	-	-	-	-	-	-	-	-	-	-	-	-
		Trem.	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pucc.	Micro.	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ustil.	I	-	0%	-	-	-	-	-	-	-	-	-	-	-
	Blastocladiomycota	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chytridiomycota	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Mucoromycota	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Mitosporidia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Outgroup	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total percent in selected genomes:	0 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %

Metabolic pathway of the fungus *Ustilago maydis* again confirms that the fungus is devoid of the lactate dehydrogenase activity, i.e. it does not comprises of the enzymes that are essential for the production of lactic acid.

2. Growth profile of *Ustilago maydis*

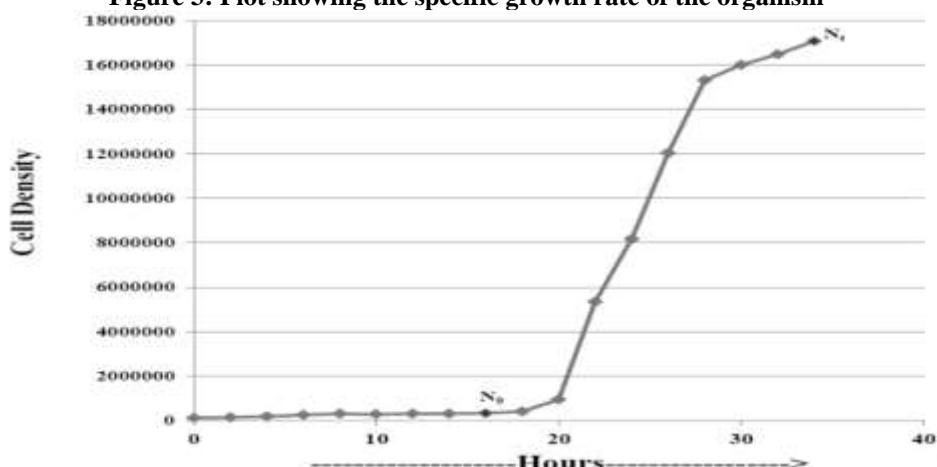
When fungal cells of *Ustilago maydis* were inoculated into the nutrient medium and incubated

under optimal physical growth conditions, a typical batch growth curve resulted.

3. Specific Growth Rate (SGR)

The *exponential phase* is a period of logarithmic cell doublings and constant, maximum specific growth rate (μ_{max} , in dimensions of reciprocal time, 1/h), the precise value of which was dependent.

Figure 3: Plot showing the specific growth rate of the organism



4. Identification Tests for Carbohydrates

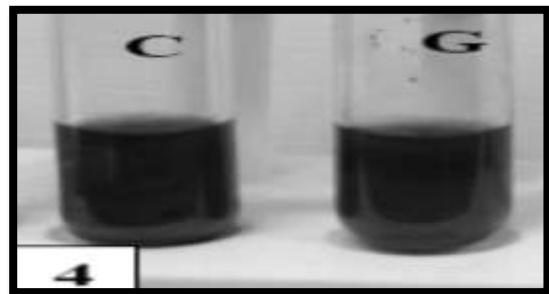
Benedict's Test

Figure 4: Benedict's Test for the identification of sugars (Apple [1] and Guava [2])

Depending on the concentration of sugar in the solution yellow to green Color is developed.



Figure 5: Fehling's test for the identification of sugars (Apple [3] and Guava [4])
Formation of the colored precipitate indicated the presence of reducing sugars in the test solution.



5. Confirmatory test for the analysis of carbohydrates

Table 1: Observation table to plot the standard curve of anthrone for glucose analysis

Test tube	Conc. of glucose (mg)	Glucose as standard (ml)	Water (ml)	Anthrone (ml)	O.D. (600 nm)
Blank	0	0	1	4	0.000
1	0.2	0.2	0.8	4	0.163
2	0.4	0.4	0.6	4	0.306
3 (Known)	0.5	0.5	0.5	4	0.421
4	0.6	0.6	0.4	4	0.512
5	0.8	0.8	0.2	4	0.683
6	1.0	1.0	-	4	0.852
S1 (MALT)	-	0.5	0.5	4	0.029
S2 (APPLE)	-	0.5	0.5	4	0.012
S3 (GUAVA)	-	0.5	0.5	4	0.019

6. Calculations for the total Reducing Sugars

- Anthrone Test (Malt)**

0.1 g malt contains 0.0094 g glucose, therefore calculating the amount of glucose in 100 g of malt extract was 9.4 g.

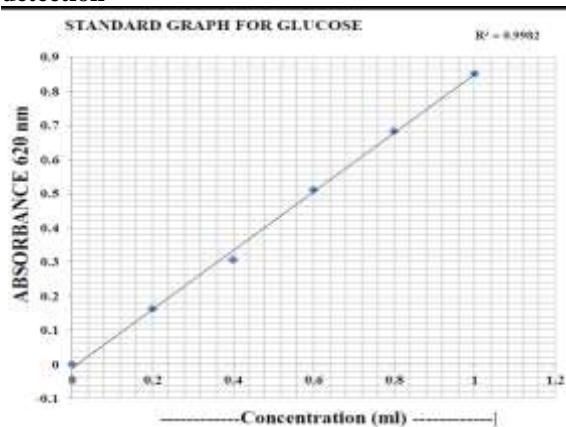
- Anthrone Test (Apple)**

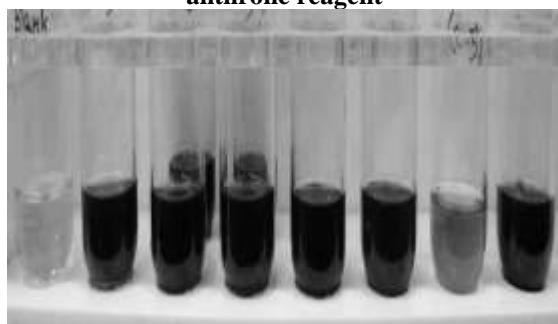
0.1 g apple pomace contains 0.004 g glucose, therefore calculating the amount of glucose in 5 g, 10 g and 15 g of apple pomace is 0.2 g, 0.4 g and 0.6 g respectively.

- Anthrone Test (Guava)**

0.1 g apple pomace contains 0.0063 g glucose, therefore calculating the amount of glucose in 5 g, 10 g and 15 g of guava pomace is 0.315g, 0.63 g and 0.94 g respectively.

Figure 6 (A): Standard graph for anthrone detection



(B): Color change reaction of sugar with anthrone reagent

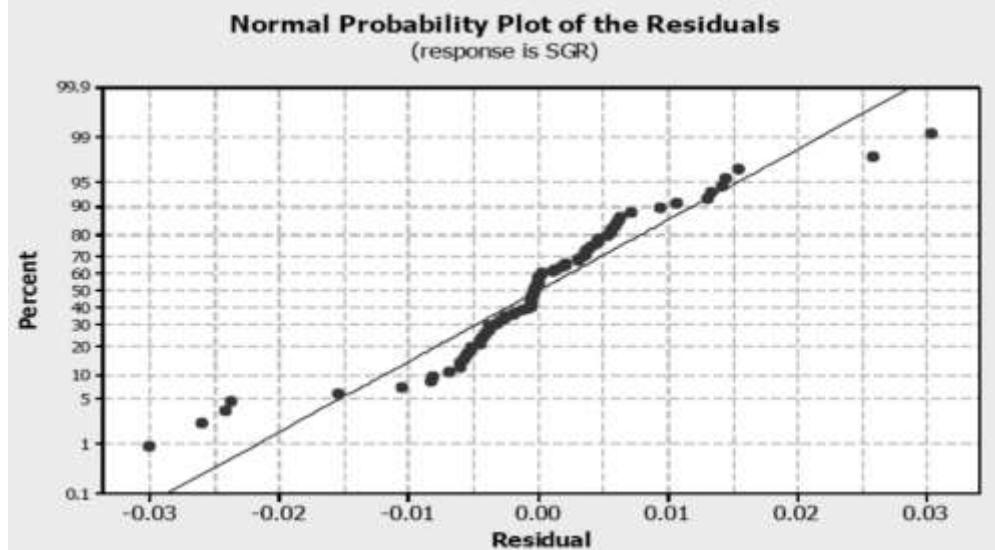
On looking on to the graphs obtained (Anthrone test for malt extract, apple pomace and guava pomace), it can be concluded that for 100 g of malt contained 9.4 g of reducing sugar which came in between of the range given in literature (7-10 g / 100 g).

7. Statistical Analysis Design of Experiments (DOE)

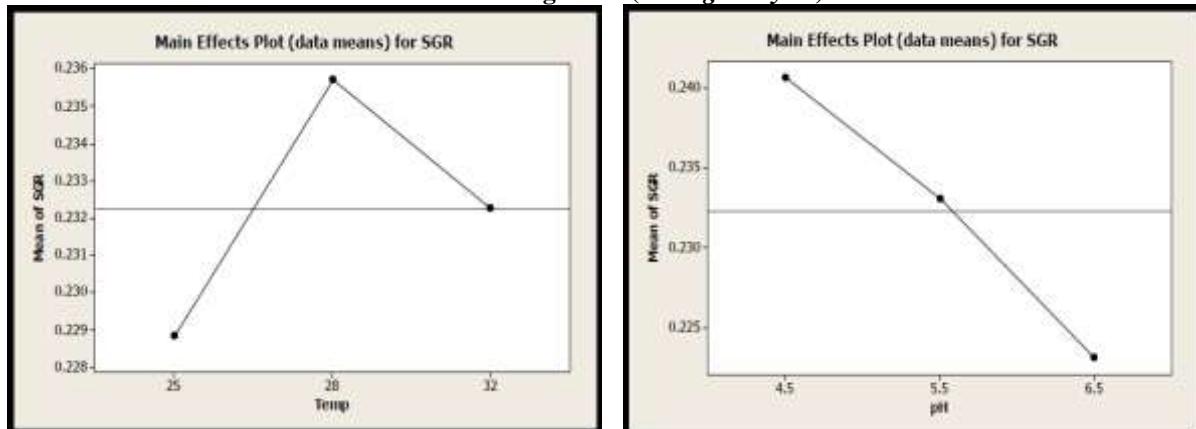
Designing of the experiments was done using the statistical software Minitab 14 via general full factorial design. In total 27 experimental set-ups (triplicates $27 \times 3 = 81$) were designed using the MINITAB software for each substrate (apple pomace and guava pomace).

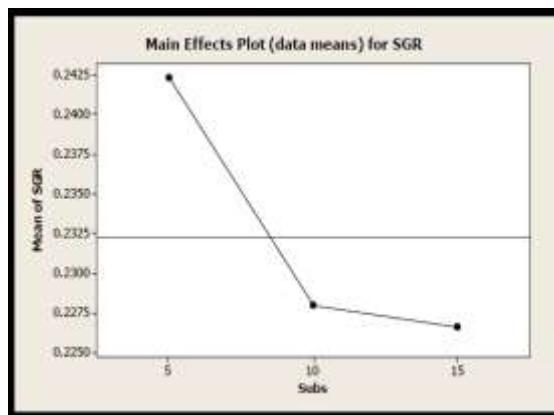
Figure 7: Parameters selected in DOE

Parameters	Symbol	Level	Coded Values	Range
Temperature C	Temp	3	123	25-28-32
pH	pH	3	123	4.5, 5.5 6.5
Substrate Conc	RS	3	123	5-10-15

8. Main Effects Plot for Specific Growth Rate**Figure 8 (A): Normal probability plot of specific growth rate (*Psidium guajava* as substrate)**

Further, parameters that were focused for the process of fermentation (*Psidium guajava+malt media set up*) are as follows:

Figure 8 (B): Graphs indicating the effect of temp, pH, and substrate concentration on specific growth rate of the organism (*Ustilago maydis*)



Plots indicated that at pH 4.5 there was a significant increase in the growth of the organism indicating that the organism is efficient enough to survive in the acidic environment i.e. at low pH (pH 2) which was the validation of the data given in the cited

literature [14]. Furthermore, at 28 °C of temperature there has been a tremendous increase in the growth of the organism. In the plot for main effect of substrate, it has been observed that at 05 g of guava pomace, organism is flourishing indicating the organism is utilizing some essential constituents of guava pomace besides glucose

9. Major Experimental Setups

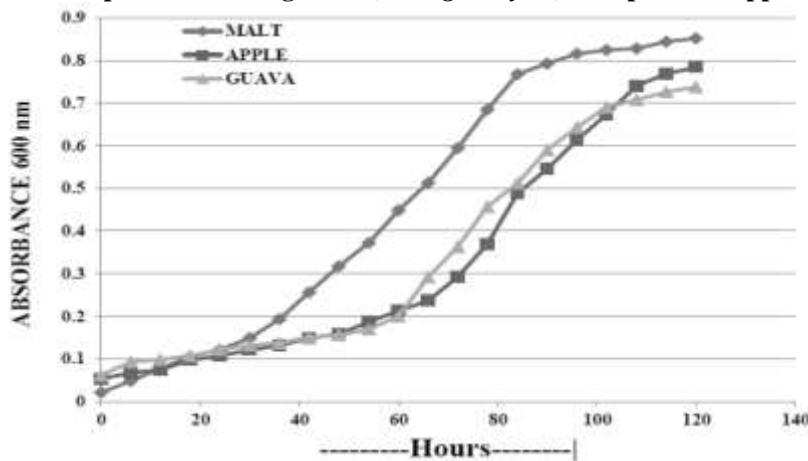
Out of the 81 setups of fermentation for each guava pomace and apple pomace, the best possible results were taken into account for the scale up process (01 ltr). Hence, at regular intervals glucose consumption was measured along with the ethanol concentration for each combination of fungus + malt extract; fungus + guava pomace and fungus + apple pomace. The selected set ups are described below:

Table 2: Effective data points obtained from the experimental model for the fermentation process after general full factorial method

Media	pH	Temperature (°C)	Reducing Sugar Conc. (g)	Specific Growth Rate (per hour)
MALT	5.5	28	10	0.2189
GUAVA	4.5	28	5	0.2882
APPLE	4.5	28	10	0.2700

10. Growth profile of the fungus in different media compositions

Figure 9: Growth profile of the organism (*Ustilago maydis*) with pomace supplementation



Graph for apple pomace depicted that there was still an increase in the growth of an organism but at a steady pace, reason being the organism was new to its environmental conditions (apple pomace). This was the slow adaptation phenomenon of the organism. Similar was the case with guava pomace

slow adaptation was there but as soon as the organism adjusted itself to the nutrient media enriched with the guava extracts, remarkable increase in growth was there, indicating its improvement in growth.

Figure 10: Glucose consumption during the process of fermentation

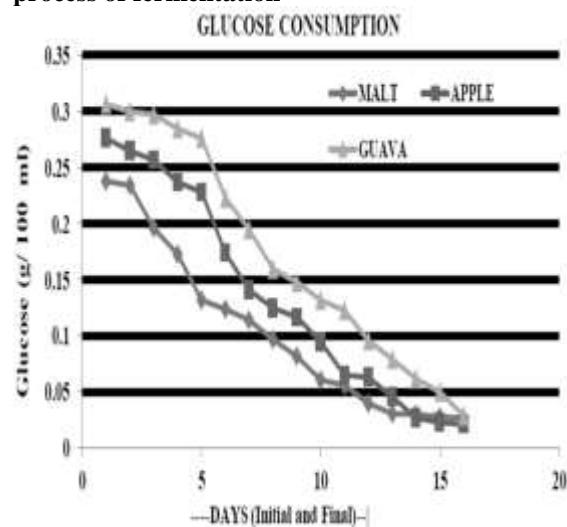
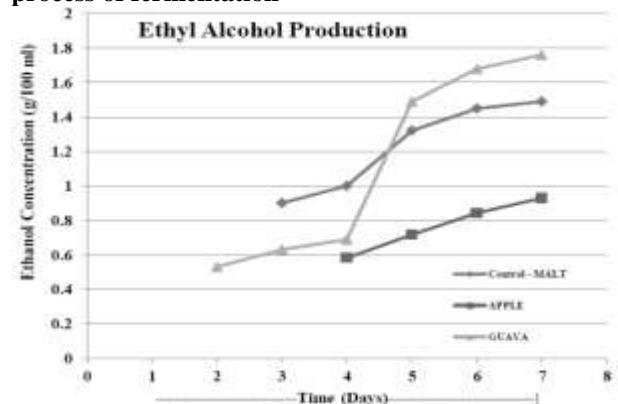


Figure 11: Ethyl alcohol production during the process of fermentation



11. Fermentation, Recovery, and Analysis of the product

Lugols Test

Lugols test exemplifies the reaction between alcohol and iodine present in the lugols reagent. This test was conducted as an initial test for the detection of alcohols

Dichromate Test for the Detection of Ethyl Alcohol

Table 3: Observations after detecting the samples via chromic acid assay

Test tube	Standard ethanol conc. (%v/v)	Water (ml)	Chromic acid(ml)	O.D. (590 nm)
1	0 (Blank)	1	1	0.000
2	1 (0.2 ml)	0.8	1	0.201
3	2 (0.4 ml)	0.6	1	0.345
4	2.5 (0.5 ml)	0.5	1	0.347
5	3 (0.6 ml)	0.4	1	0.455
6	4 (0.8 ml) KNOWN	0.2	1	0.577
7	5 (1ml)	-	1	0.734
8	S0 MALT	0.2	1	0.273
9	S1 GUAVA	0.2	1	0.323
10	S2 APPLE	0.2	1	0.171

The results obtained directed that with 05 g of substrate concentration, efficient amount of ethanol (1.76 g/100 ml) can be produced. Additionally, guava pomace will act as a promising substrate for the production of biofuel.

Gas Chromatography

Analysis of GC peaks was done with the help of retention time consideration.

Table 4: Sample details considering the retention time in minutes

S.No.	SAMPLE DETAIL	RETENTION TIME (minutes)
1.	Standard Ethanol	0.650
2.	Ethyl acetate	0.617
3.	n-Butanol	0.983
4.	Control (Malt)	0.683
5.	Sample (Guava)	0.683
6.	Sample (Apple)	0.733

Figure 12: Chromatogram for standard ethanol

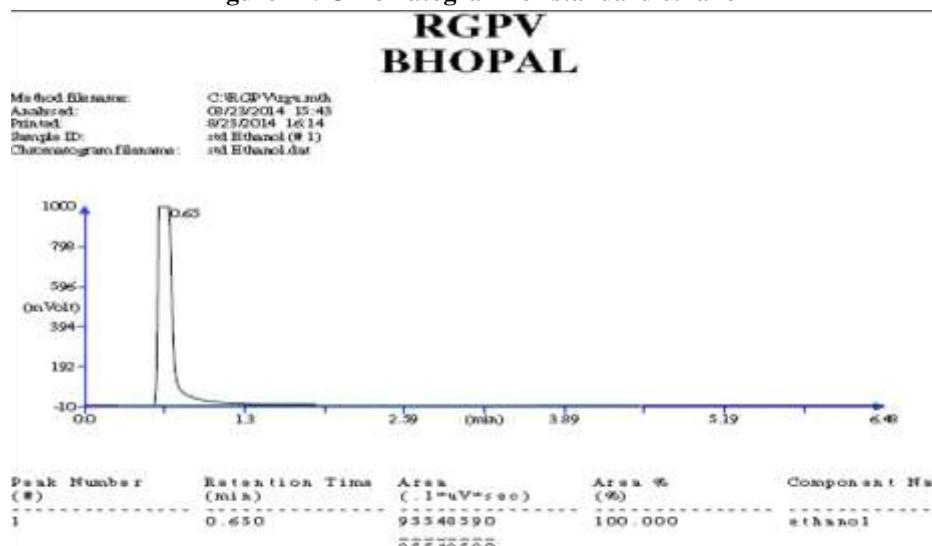


Figure 13: Chromatogram for malt substrate in fermentation media (control)



Figure 14: Chromatogram for guava substrate in fermentation media

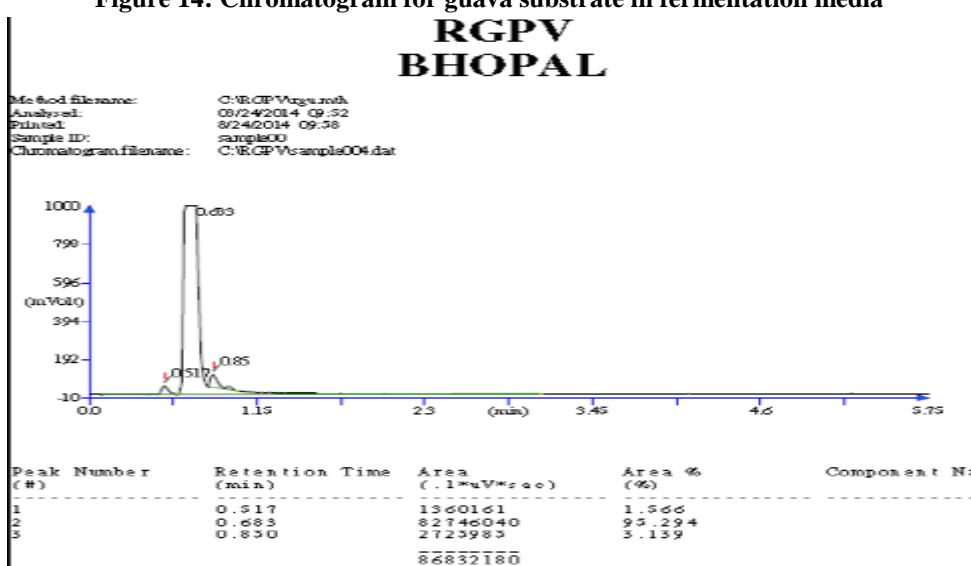


Figure 15: Chromatogram for apple substrate in fermentation media



Conclusions

Mounting energy costs, growing demand for energy, uncertainty in oil trading countries, and anxieties for the environment, arouse curiosity in fuels such as ethanol. As gasoline charges continue to upsurge and more gravity is put on the administration to capitalize in or reassure production of unconventional fuels, agrarians, companies, co-ops, and investors have shown more attentiveness in the probability of producing ethanol. Literature suggests the feasibility of generating ethanol concentrated on food based materials in an assortment of geographical locations. Various farmers efficaciously harvest heaps of crops every year in an edict to sell. Conversely, most of these ranchers are facing problem of lots of leftover and do not even know how to efficiently use the surplus amount of crops produced.

Therefore, it became one of the important issues to reconnoiter and find supplementary applications of these crops. However, because of ethical issues such as food vs. fuel, this has been deserted in research. This study therefore affords insights of how else waste could be used instead of being disposed as waste without harming the environment.

The evaluation demonstrates that ethanol production from selected alternative feedstock's: apple pomace and guava pomace along with malt extract to compare as such to avoid the disposal of the leftovers as garbage. Only with a profound understandings of growth conditions and a thorough classification of the organism it was possible to improve the fermentation by unraveling specific problems with the stability, shortening the fermentation time and find high titres of the end product.

The composition of apple and guava pomace, their variability and enzymatic digestibility were considered in order to appraise their potential as raw

materials for manufacturing desired products. Alcohol-soluble complexes were principally made up of glucose and fructose (two carbon sources readily fermentable by microorganisms). Apple pomace as well as guava pomace had shown high susceptibility to thermal hydrolysis at temperature 60°C. Owing to the compositional differences among the samples, the final glucose content of thermal hydrolyzates obtained under the same operational conditions may differ in up to some percent. The conversion of pomaces into reducing sugars with the aid thermal hydrolysis offers the potential for radical technical encroachment through solicitation of modern biotechnology techniques to comprehend truly low costs for the production of bioethanol.

In the present study, experimental design using statistical software was considered since the effect of pH, temperature, reducing sugar concentration and their interactions was the main motive to work on to the metabolism of organism. During the work conducted, SGR of the organism was considered with the supplementation of dried pomaces in the media. Apple pomace when compared with the earlier studies, it was concluded that the batch of apples considered were having lesser sugar concentration (glucose, 4 mg/ 100 g) as compared to the literature cited (glucose, 6.5 mg/ 100 g).

Moving ahead with the glucose concentration of guava pomace it came out to be 6.3 mg/100 g. Further, *Ustilago maydis* was new to the field of bioprocess technology as it was never used earlier for the production of bioethanol, therefore, its

adaptation to the environment and its consumption of the glucose was major concern.

Considering the application of general linear model on to the experimental design, it was seen that at specific growth rate for 18 hours, very fluctuating cell densities were observed, this might be due to the reason that organism is being provided with different conditions and diverse interactions. This approach tremendously proved its efficiency as with interactions being prominent, effective data points for the substrate was analyzed.

Considering, Walsh et al., when media was supplemented with guava pomace, it was observed that specific growth rate of the organism came out to be 0.2882/ hour which was in very close vicinity to the value given in the literature that is 0.29-0.30/ hour, later when the study was conducted on apple pomace it was observed that specific growth rate of the organism (0.2700) came out to be less than obtained earlier with the guava pomace. These results were taken in comparison to the control media for which the specific growth rate came out to be 0.2189. Further these results were confirmed their authenticity when the regression (R^2) values were calculated with the F-distribution analysis. Coefficient of determination for guava pomace came out to be 0.8554 (85.54 %) and for apple it came out to be 0.8291 (82.91%). The results gave the present study a peculiar direction that the regression line approximates the real data points. And somewhere around the best interaction is considered by the ANOVA analysis which is why the probability values are less than 0.05, indicating the results are significant at 95 % confidence interval statistically. Moreover, the effective data points from the experimental model obtained after the statistical analysis for guava pomace were 28°C, pH 4.5 and 5 g sugar concentration. Similarly for apple pomace, data points at which maximum specific growth rate observed were 28°C, 4.5 pH units and 10 g of reducing sugar concentration. These probable models predicted that the organism is active at acidic pH unlike other organisms and is very susceptible to the carbohydrate concentration since at 5 g of sugar concentration its specific growth rate increased and when 10 g of substrate utilization was there very less but specific growth rate decreased. These results were carried further for ethanol production, which is the desired product of the study.

Nevertheless, on fermentation at the probable conditions discussed earlier, ethanol production with the consumption of reducing sugars of guava pomace came out to be 1.76 g/100 ml, followed by apple pomace was 0.93 g/100 ml and further malt extract broth that was the control provided 1.49 g/100 ml ethanol. This gave the current study a track that with fewer quantity of substrate (5 g) effectual amount of ethyl alcohol (1.76 g/100 ml) can be produced and also guava pomace is an encouraging

and comparable substrate for the production of bioethanol.

Furthermore, 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 samples it came out to be near the range of ethanol i.e. for malt (control) it came out to be 0.683 minutes, for guava and apple in the fermentation media it came out to be 0.683 minutes and 0.733 minutes respectively. Concluding remarks state that if stringent bans are made on the manufacture of food ethanol, ethanol out of waste and lignocellulosic ethanol will blossom well and due to this, the costs of enzymes for hydrolysis may fall. In addition, a breakthrough in genetic engineering will direct an organism, which will directly convert waste into ethanol.

Future Upcoming

- Sequencing of the promising plants that can be used for the biomass.
- Molecular & chemical description of the structural design of the cell walls of plants and metabolic process and regulatory process indulged in the cell wall synthesis and its breakdown for the retrieval of biofuel.
- Molecular, Physical and chemical description of the microbes and the communities of microbes from the environment for the degradation of lignocellulose present in the biomass for the production of second generation biofuels.
- It has been predicted that the use of genetically engineered raw materials with higher carbohydrate content combined with the improvement of conversion technology could reduce the cost of ethanol over the next 10 years.

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