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Production of Bioethanol Using *Saccharomyces Cerevisiae* by Utilizing the Banana Peel Wastes.

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ABSTRACT

Recently sugarcane, corn, wheat, sugar beets were used to produce ethanol and the production cost is more than twice the price of gasoline. The high feed stock possesses a major obstacle to a large scale implementation of bioethanol as a transportation fuel. An attempt was made on a bench scale with a different substrate source and to optimize the fermentation condition for an increased yield of bioethanol. A process involving the use of banana peel waste and barley/wheat malt as substrate and α-amylase respectively is presented in this report. After 72 hrs fermentation period, the end product bioethanol was recovered by simple distillation with castor oil the fermentation process resulted in the yield of 1.79% in an anaerobic wheat batch with 20 d old yellow banana. Finally, the optimized fermentation conditions were maintained in the maximum yield batch resulted in the yield of 1.85%.

Keyword: Anaerobic, banana peel, fermentation, simple distillation.

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INTRODUCTION

Ethanol can be made from mineral oil (petroleum ether) or from sugars or starches, cheapest of which are starches, and starchy crop with True Metabolizable Energy content (TME - 15.13 MJ Kg^{-1}) per acre is cassava (Manihot esculenta), which grows in tropical countries.

Bananas are the main fruit in international trade and the most popular one in the world for their contents which are used for the bioethanol production. In terms of volume they are the first exported fruit, while they rank second after citrus fruits in terms of value. Banana is a very delicate commodity on economic, social, environmental and political grounds. According to the Food and Agricultural Organization (FAO) of the United Nations statistics estimation, world total exports of banana accounted for 16.8 million tonnes in 2006. Around 98% of world production is grown in developing countries. Hence we consider the banana peel waste to exploit its valuable components such as starch, carbohydrates, total sugars for the production of bio-fuel.

Amylolytic enzymes have the ability to convert the total available starch in the different varieties of banana peels into reducing sugars. Some grains acts as the source of those enzymes like α & ß-amylase. We have utilized the barley and wheat malt as a two different sources of starch degrading enzymes, which provide the edible reducing sugar for the microorganisms, which has to be converted into bioethanol and carbon dioxide. Actually the barley and wheat are low in certain key enzymes (eg., α-amylase) and malting increases these levels. During mashing the malt enzymes are mixed with the starch to produce maltose and other fermentable sugars. Malt also provides various nutrients for the microbial growth including amino acids, vitamins and minerals.

The microorganism used in this study is *Saccharomyces cerevisiae* (yeast). Yeast is simple unicellular, facultative organisms belonging to the kingdom Fungi. *Saccharomyces cerevisiae* is a species of budding yeast. "*Saccharomyces*" derives from Latinized greek and means "sugar mold" or "sugar fungus", *Saccharo* – being the combining form "sugar", *myces* being "fungus" and *cerevisiae* comes from latin, means "beer". As a single celled organism *Saccharomyces cerevisiae* is small with a short generation time (doubling time- 1.2 to 2hours at 30ºC). Like all organisms yeast need energy to do the work of living (synthesis, transport, reproduction, etc.,). Yeast obtains this energy through the process called cellular respiration, which involves oxidation of organic molecules. Some of the energy produced by this oxidation is stored in the chemical bonds of adenosine tri phosphate (ATP) - the energy currency of the cell. There are two types of cellular respiration- aerobic and anaerobic.

Aerobic cell respiration: If oxygen is present, yeast continues respiration by oxidizing the 2 pyruvates produced by glycolysis to CO_2 and Ethanol.

Anaerobic cell respiration: When oxygen is unavailable, yeast carry out fermentation, a type of anaerobic respiration. Steps in anaerobic respiration are as follows:

Step 1 – pyruvate (from glycolysis) ⟶ acetaldehyde + CO_2
Step 2 – acetaldehyde ⟶ Ethanol

Because of these advantageous features, *S. cerevisiae* was chosen and studies focused on the ability of yeast to ferment the sugars in different varieties of samples and ethanol yield under different cellular respirations i.e., aerobic or anaerobic with fermentation conditions.

Applications of ethanol are wide spread and consider ethanol to be the answer to global warming. Bioethanol from agricultural and biodegradable wastes provides a viable solution to multiple environmental problems by simultaneously creating sink for waste and renewable energy production as well. Using ethanol blended fuel for automobiles can significantly reduce petroleum use and greenhouse gas emissions *(Wang et al., 1999).* The high feedstock cost poses a major obstacle to large scale implementation of bioethanol as a transportation fuel *(Reith et al., 2001).* But in this study, the feasibility of utilizing the cheaper substrate source, banana peel wastes for the production of bioethanol, checked and compare the results with the bioethanol production from cassava waste.

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MATERIALS AND METHODS

Sample collection

Different varieties of banana peel wastes were collected randomly in and around chengalpet market. Three different varieties were used in this study, namely

Musa paradisiaca-Yellow banana (20 days old)
Musa paradisiaca-Yellow banana (Fresh)
Musa acuminata-Karpoora valli
Musa balbisiana-Plantain

Samples were sorted by excluding its solid tips and then washed with distilled water. The sorted banana peels were milled into a mash with sterile distilled water.

Yeast strain

Saccharomyces cerevisiae strain Y11857, used in this study were bought from MEDOX Biotech India Pvt. Ltd. and subcultured by using yeast malt extract broth (YEB). Yeast malt extract agar slants were also prepared for long time storage of the microbes. Since this strain (Y11857) was specific for aerobic respiration, it was used for aerobic fermentations.

For anaerobic fermentation, baker's yeast the commercial product was used. The granules were disaggregated in 10 mL of distilled water in a conical flask and 0.1 mL of the aliquot was inoculated in 100 mL of yeast malt extract broth. The flask was kept in rotary shaker at 80 rpm for about 24 hrs and store at 4^{o}C. The cells were sub-cultured in 50 ml of YEB by inoculating it with 0.1 mL of the mother culture. Finally, the freshly harvested cells were used for fermentation.

The malting process

Malt is a very convenient package containing 60-65% of the weight of malt is un-degraded starch. Barley and wheat are low in certain key enzymes (eg., α-amylase) and malting increases these levels. During mashing the malt enzymes were mixed with starch to produce maltose and other fermentable sugars. Malt also provides various nutrients for yeast growth including amino acids, vitamins and minerals.

Limited germination of the grains under aseptic conditions was done by malting process. It has three phase's viz., steeping, germination and kilning. Steeping encourages germination in which the starch gets converted to sugars, kilning stops the germination to ensure that very little of the starch was hydrolysed. Conversion of starch to maltose occurs in the mashing process.

Good malt ensures all the grains are germinated at the same rate, production of enzymes at good level, degradation of cell walls of starchy endosperm and the disruption of protein matrix.

Steeping

Steeping is the first stage of the malting process and has been taken 2-3 days in total. Barley and wheat seeds were surface sterilized by soaking in 1% sodium hypochlorite in distilled water for 20 mins and washed with distilled water. Steeping is the crucial phase of malting. By the end of steeping all the grains had been fully hydrated and just showed the signs of germination - the formation of a tiny rootlet or "chit", by producing heat and carbon dioxide.

Germination

Germination continues after the steeping for about 3days in a refrigerator. After germination, starch gets hydrolysed to simple sugars. The grain was turned regularly to maintain a loosely packed grain bed.

Kilning

During this phase, the germination was arrested by blowing large volumes of hot air through the grains in a hot air oven at 30°C for 24 hrs.

Then the dried grains were mashed with the help of mixer grinder and sieved in a 1 MM pore sized sieving tray.

Preparation of Substrate mash

Different varieties of banana peels were collected and stored separately. From the storage, 20 numbers of peels from each variety was taken, weighed, sliced into small pieces with knife and mashed in the mixer grinder by adding 100 mL of sterile distilled water.

The mashes were placed into respective 500 mL beakers and were sterilized in an autoclave at 212 lb / sq for 15 min and were stored in a refrigerator for fermentations. After the raw materials were processed and stored, the bench- scale fermenter set-up was prepared for different varieties of substrate mashes.

Bench-scale fermenter set-up

The materials used for the setting up of the bench scale fermenter are

1L conical flasks- fermentation vessel,
Rubber corks with double holes (6 mm)- to close the mouth of the flasks,
Silicone tubes with outer diameter 6 mm- to collect the samples and also to pass air,
Syringes - for sampling,
Syringe adaptors - for connecting the silicone tube and the syringe,
Fish pumps - for generating air,
M-seal and parafilm - for sealing the flasks.

By using these materials, the following steps for preparing the bench-scale fermenters. Sterile conical flask was filled with 125 g of mashes (substrate), 60 g of malt (barley / wheat), 50 mL of inoculum (5% of the volume of fermenter vessel) and nutrients such as 10 mL of ammonium hydroxide and 6 g of ammonium phosphate in 10 ml of distilled water. And finally, the slurry was made up to 1L with distilled water.

Two silicone tubes were inserted into the flask through the cork holes. In case of tube-1, one end of the tube was immersed into the slurry and the other end was fitted with the syringe via the adaptor for the purpose of samples collection.

In case of tube-2, one end of the tube was inserted into the flask above the slurry, whereas, the other end was inserted into the bottle, which was kept inverted inside the plastic jar containing water for anaerobic fermentations, but was connected to the fish pump in case of aerobic fermentation for the passage of air.

Finally the gaps were sealed in the corks and in adaptor connections with M-seal and parafilm respectively to prevent contaminations. This complete set-up was used as a bench-scale fermenter throughout the study.

Fermentation process

Fig-1 shows the flowchart of the fermentation process. This general work flow was followed for all the fermentation batch studies.

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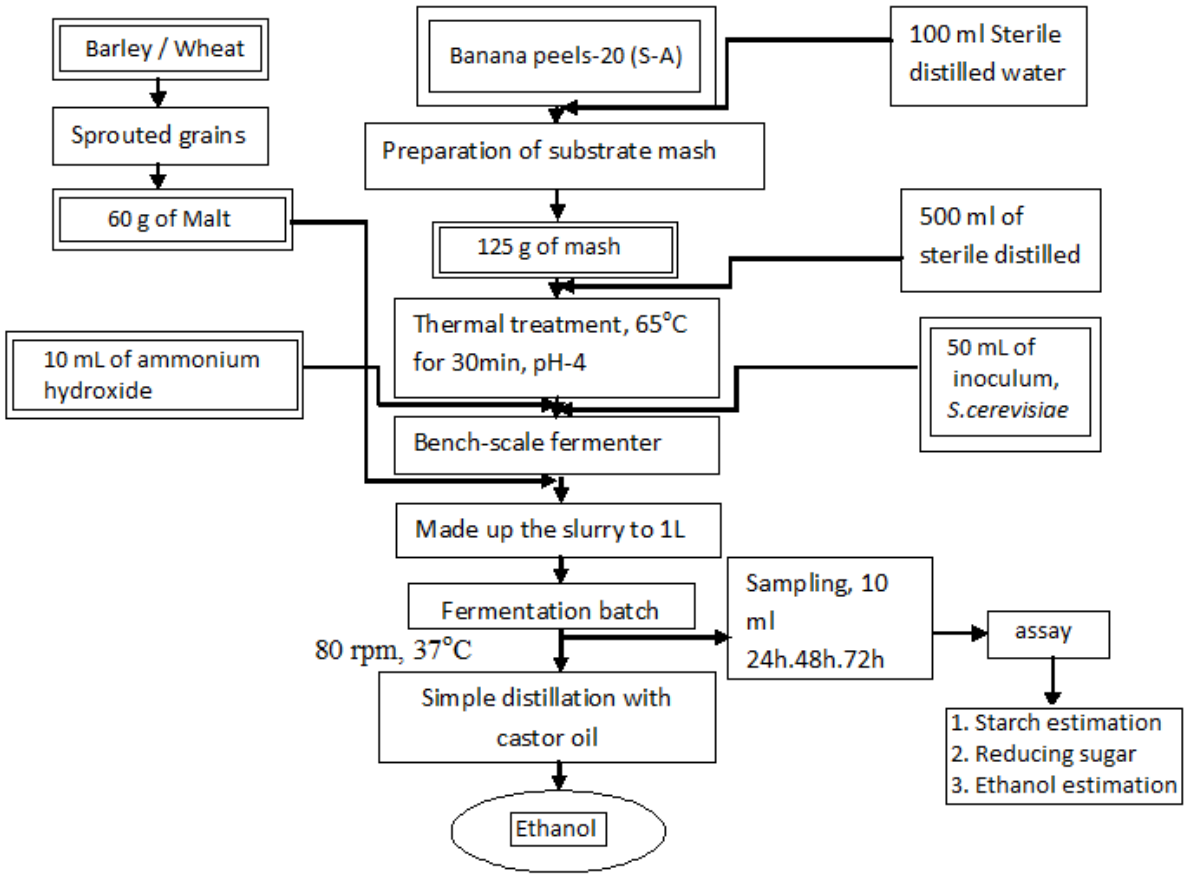

Fig-1: Flowchart of the fermentation process.

Fermentation conditions

Thermal treatment was carried out before the fermentation process to avoid the contamination. The contaminant, which could reduce the production quality, was found to be propenal (acrolein). The propenal formation in mashes was due to the growth and metabolism of sporulating bacteria. The following steps were followed during the treatment.

- The 1L flask containing the slurry (125 g peel mash + 500 ml sterile water) were kept at more than 65°C for a minimum of 30 min to reduce the microbial content as far as possible.

Before the temperature reaches 60°C, pH of the slurry was adjusted to 4.0 to avoid germination of spores during further processing; this was achieved by using concentrated sulfuric acid.

This kind of specific acidification of mashes was found to stop propenal (acrolein) formation.

Due to this thermal treatment method, bioethanol yield might be increased. Before fermentation, the pH and dissolved oxygen (DO) probes were used to note the pH and DO levels and was noted as 5.2 and 4.0 ppm respectively using pH and dissolved oxygen probes. After filling the flasks with the required sources for fermentation, they were kept in a rotary shaker at 80 rpm for 72 hrs incubation. These conditions were followed for all the fermentation batches.

Sampling

The samples were checked for starch, glucose and ethanol concentrations using anthrone, DNS and acid dichromate methods respectively. At regular intervals of time (24 hrs, 48 hrs, 72 hrs), 10 mL of the slurry was taken out with the help of syringe and centrifuged at 3500 rpm for 10 min and resulting supernatant was used for the following assay.

ISSN: 0975-8585

Starch measurement

To 1mL of the supernatant, 4 mL of anthrone reagent was added and noted the colour change from green to dark green. The content was allowed to stand for 10 mins at room temperature and the absorbance was read at 620 nm and recorded.

Determination of ethanol concentration

10 mL of the acid dichromate solution was transferred to 250 mL conical flask with matching rubber stopper and 1ml of the supernatant was transferred into the sample holder. The sample holder was suspended over the dichromate solution and kept in place with the rubber stopper. The sample flask was stored overnight at 28˚C in an incubator and after overnight incubation the flask was bring down to room temperature.

Loosen the stopper carefully and removed and discarded the sample holder. The walls of the flask were rinsed with 100 mL of distilled water and 1mL of potassium iodide solution and swirled to mix the solution.

3 blank titrations were prepared by adding 10 mL of acid dichromate solution to a conical flask, adding 100 mL of water and 1 ml of potassium iodide solution and swirling to mix. A burette was filled with sodium thiosulfate solution and titrate each flask with sodium thiosulfate. When the iodine colour fades from brown to yellow, 1 mL of starch solution was added and titrated until the blue colour disappears. The blank flasks were titrated first, and repeated until concordant results were obtained.

Calculations for the determination of ethanol concentration

The volume of sodium thiosulfate solution, used for the sample and blank titration, was used to determine the alcohol concentration. The no. of moles of sodium thiosulfate in this volume was calculated. Using the equations, the relationship between the moles of sodium thiosulfate and the moles of ethanol were determined.

- as 1 mol of $S_2O_3^{2-}$ is equivalent to 6 mol of $Cr_2O_7^{2-}$
-and 2 mol of $Cr_2O_7^{2-}$ is equivalent to 3 mol of C_2H_5OH
-1 mol of $S_2O_3^{2-}$ is equivalent to 0.25 mol of C_2H_5OH

By using this ratio, the moles of alcohol in different no. of samples were calculated. Finally, the answer was converted from moles per litre to percentage. These assays were performed during the 72 hrs fermentation period.

Product recovery by simple distillation

Distillation is a common wet-chemical technique for separating organic compounds based on differences in their boiling points.

The boiling point of the compounds was too similar in the fermented slurry, so the vapor produced at any given temperature will be a mixture of two compounds. i.e., azeotropic mixture of ethanol and water.

After fermentation period, to recover the end product, the simple distillation with castor oil based on the differential miscibility of water in castor oil (*Renaldo V. Jenkins of Langley Research Center)* was performed.

The fermented slurry was filtered to remove the solid particles, and the filtrate was mixed with 50 mL of castor oil and heated in a round bottomed flask with heating mantle. The more volatile compounds (ethanol, 78.2^{o}C) will vapourize first, leaving the higher boiling compounds (water, 100^{o}C) behind in the mixture.

The vapour produced was condensed with the help of tap water, and the bioethanol was recovered.

ISSN: 0975-8585

RESULTS AND DISCUSSION

Results

In the fermentation process, production of bioethanol was done using different varieties of banana peel waste as substrate. In the fermentation process of production of bioethanol from cassava, dried cassava flour was used as a substrate, with α-amylase and *S. cerevisiae*. In the fermentation process, banana peel mash was used as a substrate with wheat/ barley malt as enzyme source and *S. cerevisiae (Table-1).*

Table-1: Comparison of ethanol fermentation process with cassava wastes and the ethanol fermentation process with different varieties of banana peel wastes.

Conditions	Fermentation with cassava wastes	Fermentation with banana peel wastes
Substrate source	Cassava flour	Banana peel mash
Enzyme source	α-amylase	Barley/wheat malt-60 g
Microorganism	5% *S. cerevisiae*	5% *S. cerevisiae*
Distilled water	1L	1L
Fermentation period	3 days	3 days
Ethanol yield	1.05%	1.85%

Starch and glucose absorbance

The starch and glucose absorbance were read at 620 nm and 540 nm for different varieties of samples during the fermentation period to observe the conversion rate of starch to glucose and then from glucose to bioethanol. The recorded values were plotted against the varieties as shown in the (fig-2 to fig-9).

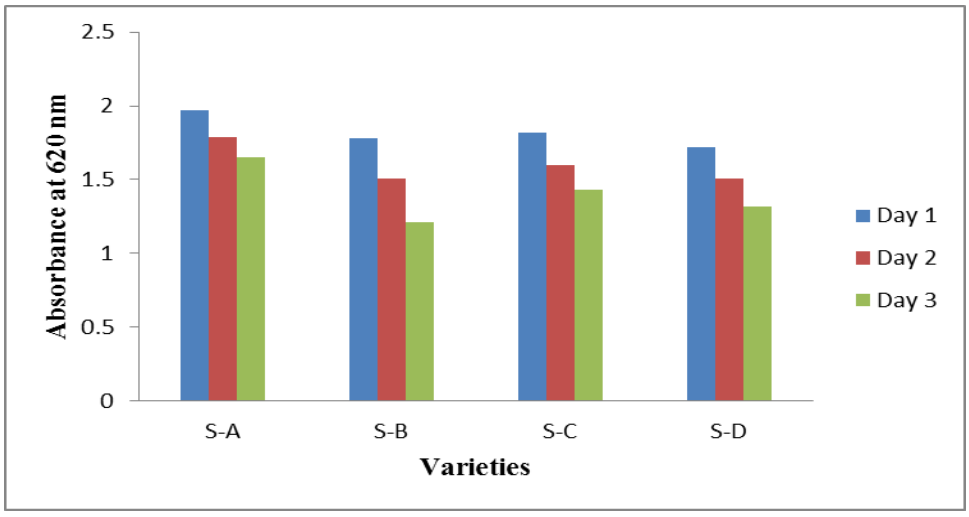

Fig-2: Starch (absorbance) - anaerobic barley batch

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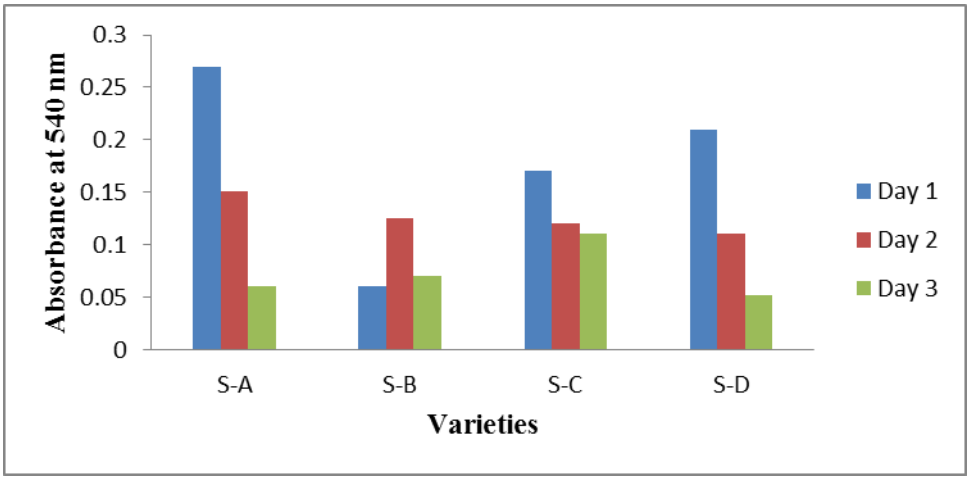

Fig-3: Glucose (absorbance) - anaerobic barley batch

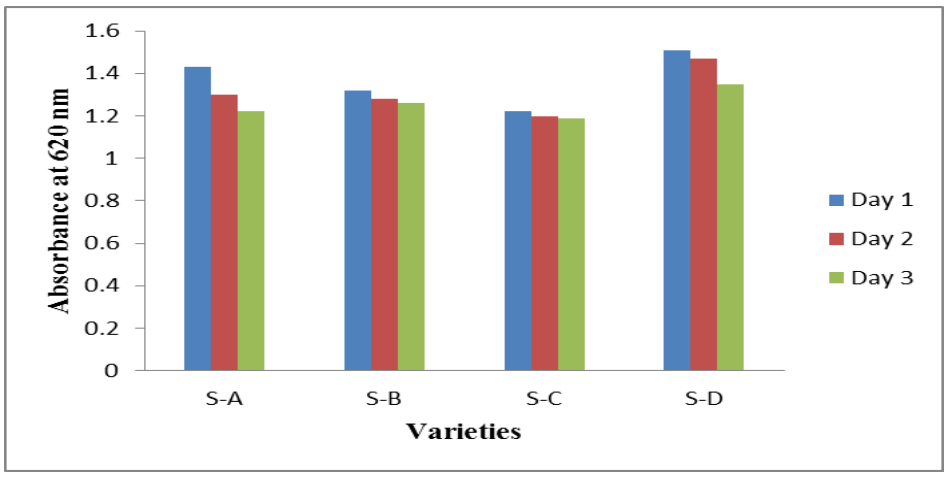

Fig-4: Starch (absorbance) - anaerobic wheat batch

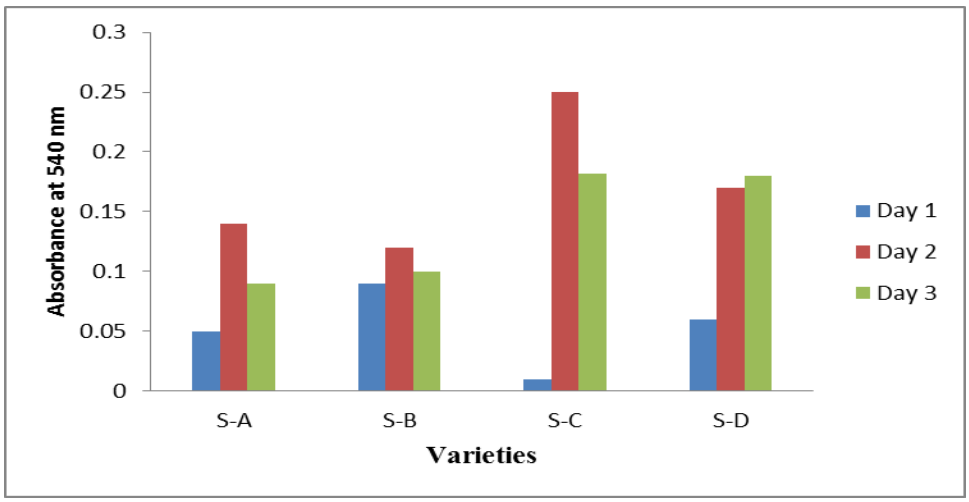

Fig-5: Glucose (absorbance) - anaerobic wheat batch

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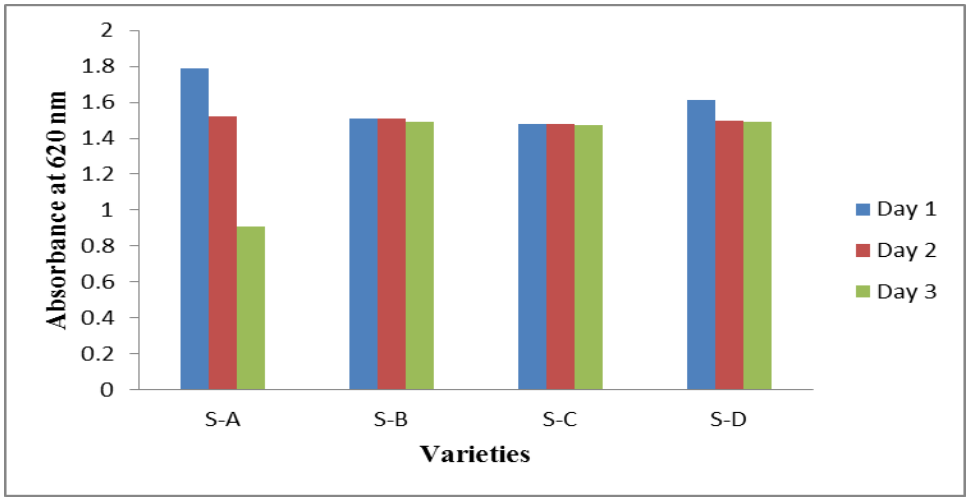

Fig-6: Starch (absorbance) - aerobic barley batch

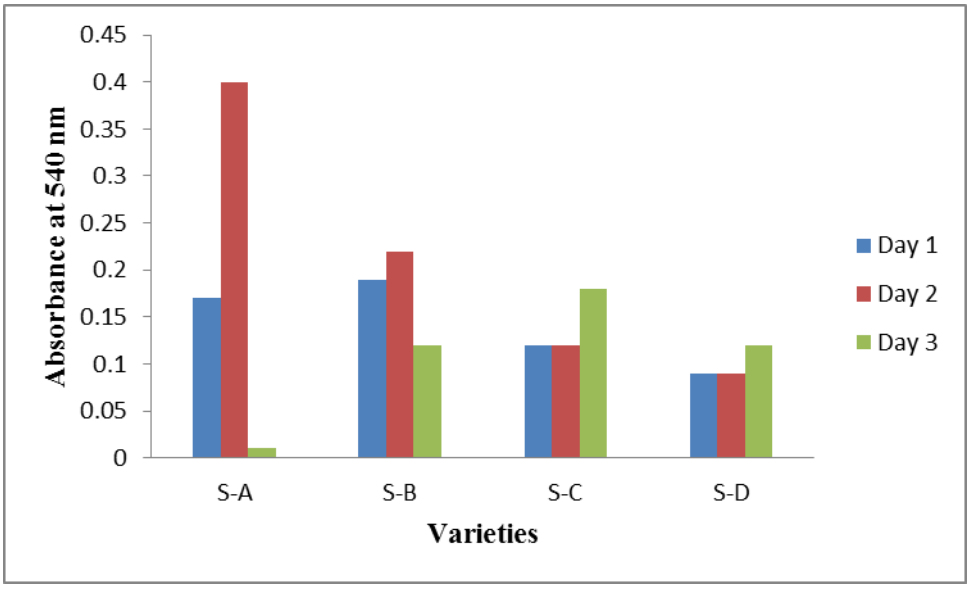

Fig-7: Glucose (absorbance) - aerobic barley batch

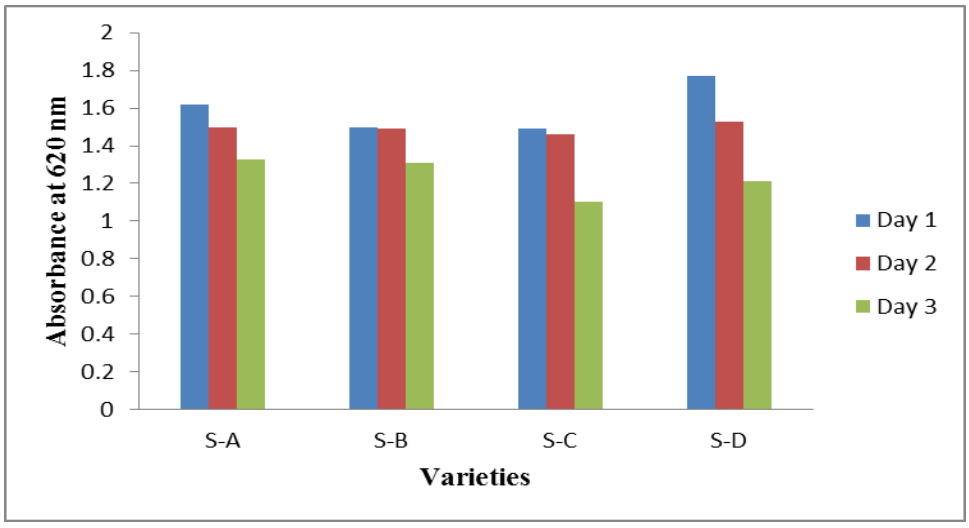

Fig-8: Starch (absorbance) - aerobic wheat batch

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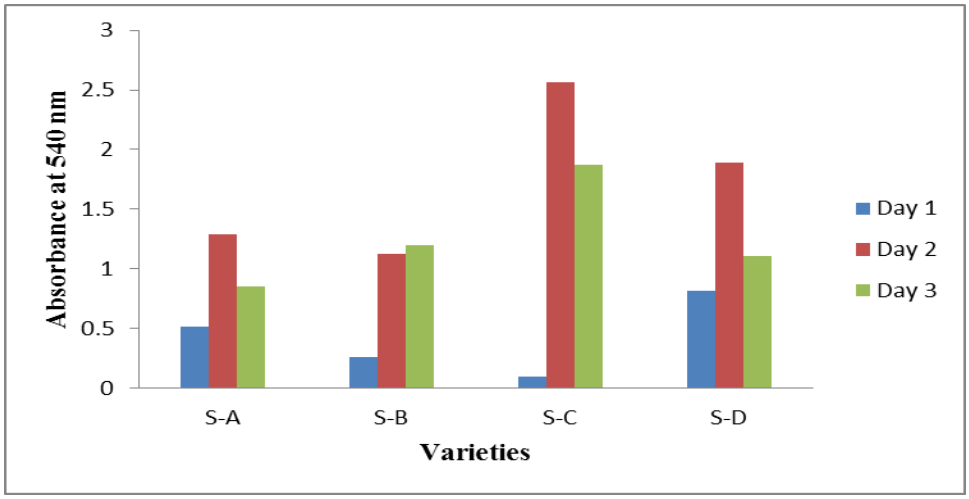

Fig-9: Glucose (absorbance) - aerobic wheat batch

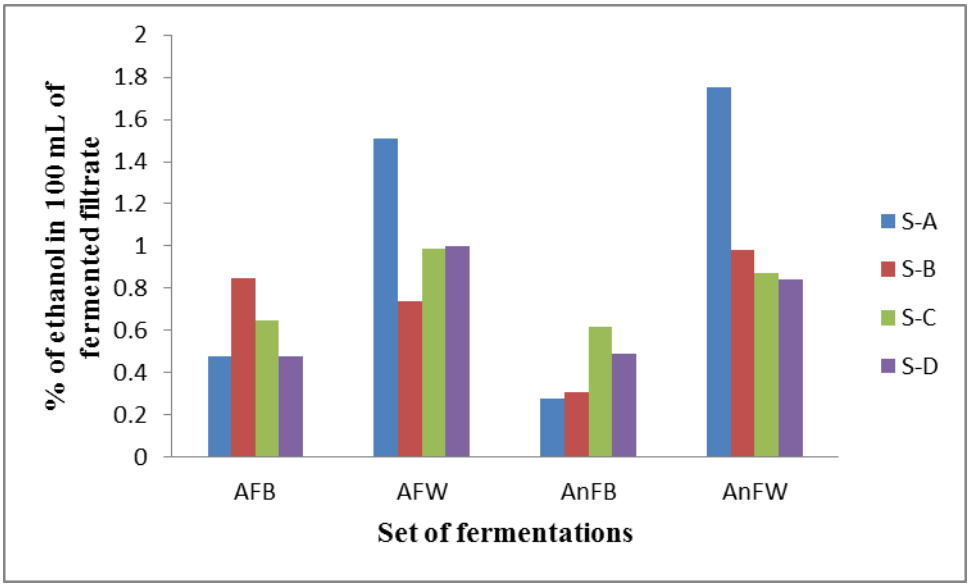

Fig-10: Graphical representation of Ethanol concentration in fermentation batches
AFB - Aerobic fermentation with Barley AFW - Aerobic fermentation with Wheat
AnFB - Anaerobic fermentation with Barley AnFW - Anaerobic fermentation with Wheat

Table - 2: Bioethanol concentration values determined for 16 fermentation batches

VARIETIES	Aerobic(wheat) %			Aerobic(Barley) %			Anaerobic (Wheat) %			Anaerobic(Barley) %		
	1	2	3	1	2	3	1	2	3	1	2	3
Old *Musa paradisiaca*	0.12	0.85	***1.56***	0.37	0.28	0.49	0.14	0.92	***1.79***	0.24	0.33	0.36
Fresh *Musa paradisiaca*	0.40	0.75	0.88	0.16	0.25	***0.96***	0.32	0.72	1.05	0.03	0.26	0.27
Musa acuminata	0.01	0.95	1.05	0.34	0.84	0.76	0.02	0.84	0.97	0.09	0.17	***0.77***
Musa balbisiana	0.29	0.90	1.09	0.19	0.97	0.53	0.19	0.72	0.94	0.42	0.51	

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Bioethanol concentration values

The values determined for 16 fermentation batches by acid dichromate method were given in the table-2. Bioethanol concentration estimated using acid dichromate method for 16 fermentation batches were plotted as graph (Fig-10).

Observation

- The maximum yield was observed in the anaerobic wheat batch S-A as 1.79%.
- Hence this anaerobic wheat batch S-A was subjected to optimization studies.

Optimization studies

The 5 flasks which have been used for optimizing the fermentation parameters in the anaerobic-wheat batch in the sample (S-A) shown in the table-3 i.e., 20d old yellow banana which have been resulted in maximum yield as 1.79%. The critical parameters of this process are given in Table-3.

Table-3: Variations in optimization conditions and incubation period in optimizing the maximum yield batch

Conditions / flasks	1	2	3	4	5
Substrate sample (S-A)	50 g mash	*50 g mash*	125 g mash	125 g mash	250 g mash
Enzyme source (wheat malt)	60g malt	60 g malt	*50g malt*	*40 g malt*	60 g malt
S.cerevisiae	50ml inoculum	50ml inoculum	50ml inoculum	50ml inoculum	*100ml inoculum*
Salts	NH_4OH- 10 ml, *phosphate 6 g*	NH_4OH- 10 ml	NH_4OH- 10 ml	NH_4OH- 10 ml	NH_4OH- 10 ml
Incubation time	3 days	3 days	*5 day*	3 days	3 days

Table - 4: Bioethanol concentration for the optimization batches of anaerobic wheat S-A batch

FLASK/DAYS	1	2	3	4	5
1	0.006	0.065	0.19	-	-
2	0.39	0.77	***1.85***	-	-
3	0.35	0.53	1.49	1.39	1.22
4	0.16	0.52	0.72	-	-
5	0.02	0.03	0.09	-	-

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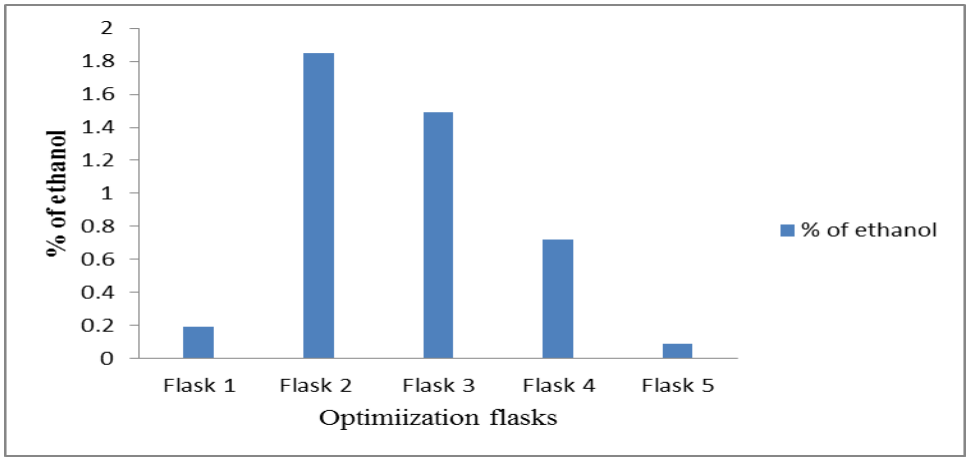

Fig-11: Graphical representation of bioethanol concentration in optimization flasks

Results of optimization batches

Bioethanol concentration for the optimization batches of anaerobic wheat S-A batch were given in Table-4. Bioethanol concentration estimated for optimization flasks by acid dichromate method were plotted as graph (Fig-11).

Observation:

From the above results, it was concluded that the maximum yield was observed in flask 2 as 1.85% i.e., 1.85 ml of bioethanol per 100 ml of fermented slurry. The fermentation condition followed for flask 2 is as follows.

substrate sample (S-A) - 50 g mash,
Enzyme source (wheat malt) - 60 g malt,
S.cerevisiae (baker's yeast) - 50 mL inoculum,
Ammonium hydroxide - 10 mL,
Sterile water - 1L,
Incubation period - 3 days.

20d old yellow banana peel or 125g mash in 1L slurry resulted in 18.5 mL of bioethanol using simple distillation via differential miscibility with castor oil.

CONCLUSION

The current investigation shows that older the banana (*M.paradisiaca*) peel waste, higher was the yield of bioethanol during fermentation.

The fermentation process with banana peel wastes led to reduction in time for substrate preparation. It is cost effective due to the elimination of α-amylase from the cassava fermentation process.

From this study, it can be deduced that banana peel wastes from banana processing may serve a good source of carbon for yeast fermentation to produce bioethanol.

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