



Bioethanol Production with the Inoculation of Yeast Using Vegetable and Fruit Wastes as Substrate

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Abstract: In the current climate change situation, finding alternative energy sources is critical to reduce the usage of fossil fuels. Bioethanol, as a biofuel has got an exceptionally role in the world. Second-generation bioethanol can be made from undesired biomass such as fruit and vegetable wastes, and it is a promising alternative to fossil fuels. Bioethanol production using five fruit and vegetable wastes, banana (*Musa paradisiaca*), papaya (*Carical papaya*), sapota (*Manilkara zapota*), pomegranate (*Punica granatum*) and potato (*Solanum tuberosum*) were studied in submerged fermentation using their autohydrolysis liquor by fermenting with yeast isolated from grapes. The greatest amount of bioethanol produced differed from day to day throughout fermentation. The ethanol yield of substrates increased gradually from 24 to 72 hours during the fermentation period. Maximum alcohol content 18.5%(v/v) and 17.4%(v/v) were observed in potato peel and banana peel at 72hrs respectively. Bioethanol obtained from sapota peel, pomegranate peel and papaya peel were found to be 16.5% (v/v), 15.2% (v/v) and 13.2% (v/v) respectively.

Key words: Biomass, Bioethanol, Fossil Fuel, Second-generation Production, Autohydrolysis Liquor, Yeast.

I. Introduction

Global energy consumption has gradually expanded in tandem with the growth of the human population and the rise of industrial affluence. Import of transport fuel is affected by limited reserves of fossil fuel. Within the next few years, annual global oil production will begin to decline. Renewable energy sources could be an alternative option in this circumstance. Renewable energy sources include wind, water, sun, biomass, and geothermal heat, whereas fuel production and the chemical sector may rely on biomass as an alternative source in the near future. Renewable biomass fuels, such as bioethanol, biodiesel, and biohydrogen, generated from sugarcane, corn, switchgrass, algae, and other sources, can be used to replace all petroleum-based fuels¹. Worldwide ethanol demand is steadily expanding as a result of fast population expansion and industrialization. Due to their primary value as food and feed, conventional crops such as corn and sugarcane are unable to supply the global demand for bioethanol production. Second-generation bioethanol production necessitates the development of cost-effective and sustainable processes that use renewable lignocellulosic biomass as a starting material. Therefore, agricultural wastes are attractive feedstocks for bioethanol production. These wastes are cost effective, renewable and abundant. Underutilized organic wastes such as fruit and vegetables have biological and chemical potential in producing bioethanol. Some biological potentials include the possibility of indigenous microorganisms like *Candida* spp². Fruit and vegetable wastes, on the other hand, contain chemical potential due to large levels of complex saccharide in the form of lignocellulose. Lignocellulose from a rich source might be hydrolyzed into D-glucose and D-xylose, which could then be converted to bioethanol by microorganism³. In many Indian towns, the demand for fruits and vegetables is driving up the volume of organic wastes. In a traditional market, waste from fruits and vegetables is the most common type. It was found that banana, papaya, sapota, pomegranate, and potato wastes were dominated in most of the markets. The characteristics of organic wastes at traditional markets were affected by waste dominance, with water content of 84.46 percent, dry matter 15.54 percent, volatile content of 91.80 percent, ash content of 8.2 percent, C-organic content of 68.62 percent, total nitrogen level of 2.22 percent, and C/N ratio of 30.912⁴.

Lignocellulosic materials are renewable, low cost and are abundantly available, which includes crop residues, grasses, sawdust, wood chips, etc. In the last two decades, extensive research has been done on ethanol generation from lignocellulosics⁵. As a result, bioethanol production could be a viable option for effectively utilising agricultural waste. Lignocellulose consists of 30-50% of cellulose, 20-40% of hemicellulose and 10-15% lignin⁶. Cellulose is the main structure of lignocellulosic based biomass which is a glucose homologous polymer connected by β -1,4 glycosidic bond⁷. Cellulase enzymes such as exoglucanase, endoglucanase and β -glucosidase could be produced by microorganisms to hydrolyze cellulose into glucose⁸. After all the sugar sources are hydrolyzed into glucose and other simple sugars, the bioconversion continues until bioethanol is produced.

Spontaneous alcoholic fermentation of grape must is a complex process owing to metabolic activities of different groups of indigenous microorganisms including filamentous fungi (i.e., *Botrytis* spp.), yeasts, and bacteria (lactic and acetic acid bacteria) originating from grapes, soil⁹. These complex microbial consortia's physiological properties lead to the synthesis of metabolites and the alteration of grape molecules, altering the sensory properties (colour, aroma, flavour, structure, and body) of the final product¹⁰. The outcome of spontaneous alcoholic fermentation is difficult to anticipate due to the sequential action of different yeast species/strains naturally present on the berries grapes or in the winery. As a result, results are often unreproducible¹¹.

The indigenous yeast obtained were then used to ferment sugar in fruit and vegetables waste autohydrolysis liquor as substrate and produce bioethanol. The aim of the study was to determine the utilization of fruit and vegetable waste's autohydrolysis liquor towards bioethanol production with the use of indigenous yeasts as starter.

II. Materials and Methods

Indigenous yeasts isolation from grapes:

In the collection bag, grapes were manually squeezed and then incubated at ambient temperature (23-28°C). Partially fermented musts were serially diluted and spread-plated on malt extract agar after 5 days. After a 5-day incubation period at 28°C, colonies with distinct morphology and/or colour were chosen. Cultures were purified by repetitive streaking on malt extract agar. Yeast cultures were preserved on malt extract agar slants, stored at 4°C and sub-cultured every 3 months. Before each test, strains were cultured twice in YPD (yeast extract 10 g/l, peptone 20 g/l, dextrose 20 g/l).

A new strategy for bioethanol production in submerged fermentation (SmF) using fruit and vegetable waste's autohydrolysis liquor:

Each of the selected fruit and vegetable wastes (banana, papaya, sapota, pomegranate and potato peels) and water are mixed in a closed and pressurized vessel in order to obtain a liquid/solid ratio of 50:1 w/w. The system is autoclaved at 121°C for 30 min. The liquid phase liquor (soluble hemicelluloses rich fraction) is separated from the solids by filtration and used directly as liquid substrate^{12 & 13} for bioethanol production (figure A). After cooling, the pH was adjusted to 5.5 for fermentation by yeast. 2ml of potent yeast inoculum was inoculated to the fermentation media containing 25 ml of fruit and vegetable waste hydrolysate in conical flasks. No other carbon or nitrogen sources were added to the media in this study. Submerged state fermentation was carried out in separate conical flasks for 72 hrs. After the stipulated time of fermentation, estimation of various parameters were carried out for each sample.

Estimation of Ethanol Concentration:

Total ethanol conc. in the medium was estimated by chromic acid method i. e. measuring absorbance at 584 nm using a spectrophotometer¹⁴. Acidified potassium dichromate is a good oxidizing agent which oxidizes ethanol to ethanoic acid accompanied by color changes from orange to green. Based on the above principle, a standard curve is prepared with known concentration of ethanol and the volume of each standard is made up to 1 ml by distilled water and 4 ml of $K_2Cr_2O_7$ added to each test tube and mixed thoroughly with a vortex mixer. Incubated at 55°C for 10 min and read absorbance was at 600 nm. A standard curve of absorbance against concentration was prepared. To 1 ml of the test is subjected to the ethanol concentration determination described above. The concentration is determined from standard curve.

Estimation of Reducing sugars:

Reducing sugars in autohydrolysis liquor was calculated before and after fermentation by DNS method¹⁵. Sample was mixed with 3ml of DNS and boiled for exactly 5 min, the optical density was checked at 540 nm to measure the color intensity. The reducing sugar as glucose was expressed in µg per ml. the standard curve of glucose is used for reducing sugar fermentation.

Titrateable acidity:

When each sample was collected, the titrateable acidity of the fermentation broth was measured. Titrateable acidity was evaluated using 0.1N NaOH and phenolphthalein as an indicator on 5ml samples of fermented broth. The amount of acid produced was calculated as a percentage of lactic acid using the following formula:

$$\%lacticacid = \frac{mlof\ 0.1N\ NaOH * normality\ of\ NaOH * Mol.Wt.\ of\ acid}{mlof\ sample * 10}$$

Soluble protein assay:

Protein contents of the culture supernatant was assayed by folin ciocalteau method of ¹⁶, after preliminary precipitation with three volumes of 10% TCA. Bovine serum albumin was used as standard.

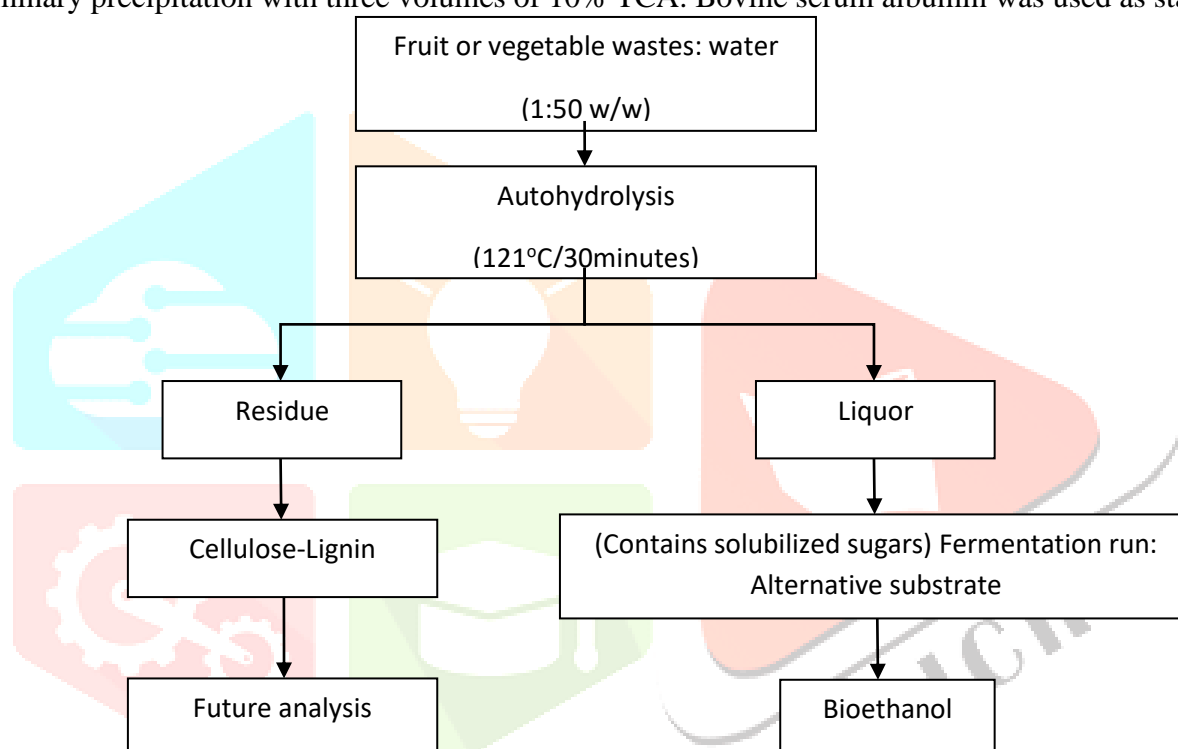


Fig. A: Scheme of extraction of soluble sugars from fruits and vegetable wastes.

III. Results and Discussion:**Indigenous yeasts isolation from grapes:**

Indigenous yeast colonies were isolated from partially fermented must of grapes (Fig:1 to 4). The selected colonies were subcultured on malt extract agar medium and stored at 4°C. For the production of bioethanol 24hrs culture of yeast grown in YPD broth was used as inoculum.

A new strategy for bioethanol production in submerged fermentation (SmF) using fruit and vegetable waste's autohydrolysis liquor:

Five agro wastes which include fruit and vegetable wastes (banana peel, papaya peel, pomegranate peel, sapota peel and potato peel) were used as substrates for bioethanol production. These wastes were autohydrolysed to collect liquid liquor rich in reducing sugars for bioethanol production in submerged fermentation.

Estimation of Ethanol Concentration:

The greatest amount of bioethanol produced differed from day to day throughout fermentation (Table:1 and figure 5). The ethanol yield of substrates increased gradually from 24 to 72 hours during the fermentation period. Maximum alcohol content 18.5%(v/v) and 17.4%(v/v) were observed in potato peel and banana peel at 72hrs respectively. Bioethanol obtained from sapota peel, pomegranate peel and papaya peel were found to be 16.5% (v/v), 15.2% (v/v) and 13.2% (v/v) respectively.

Estimation of reducing sugars:

The amount of sugar released differed depending on the substrate (Table: 2 and figure 6). Highest amount of reducing sugars were released by potato peel (700 μ /ml) after autohydrolysis, when compared to other substrates. Maximum amount of sugars released from banana peel (620 μ /ml), followed by sapota peel (540 μ /ml), pomegranate peel (480 μ /ml) and papaya peel (320 μ /ml).

Soluble protein estimation:

The utilization of the protein from agrowastes can be immense important for bioethanol production. Protein content varied from substrate to substrate (Table:3). Highest content of protein were found in potato peel (80 μ /ml) and banana peel (65 μ /ml) without the addition of microorganisms. Then the protein content with the addition of yeast culture was observed in potato peel and banana peel varied from 140 μ /ml to 120 μ /ml respectively. Maximum protein content without the addition of yeast was observed in potato peel which contains high starch when compared to other substrates, whereas maximum protein content with yeast was observed in potato peel which enhanced the biomass production for bioethanol production. Yabaya and Ado¹⁷ reported the fungal protein production by *Aspergillus niger* using banana peel. Steinkraus¹⁸ used edible substrates for the production of microbial biomass protein. Lignocellulosic biomass with *A.niger* was 0.195 (mg/g) with mycelia protein of about 52.5 (mg/g).

Titrateable acidity:

As lactic acid bacteria proliferated, acidity increased, presumably due to the fermentation of sugars. The acidity that developed favoured the growth of yeasts which subsequently multiplied rapidly at about 24hrs of fermentation. Association between lactic acid bacteria and yeasts is common in many food and beverage fermentations¹⁹. Titrateable acidity (expressed as percent lactic acid) increased throughout the fermentation (Table:4).

The bioethanol production capacity of various agricultural wastes was found to be variable, and it was decided that potato peel was a particularly promising raw material for bioethanol production. When compared to other agro wastes that have been investigated for bioethanol production, it has a high reducing sugar concentration.

The results have demonstrated that agro wastes are potential source for the production of bioethanol by enzymatic activity of microorganisms. These wastes should be converted to useful products like biofuels. The findings showed that agro wastes can be used to produce bioethanol through the enzymatic activity of microorganisms. These wastes should be converted to useful products, such as biofuels.



Fig:1 Partially fermented must



Fig:2 Yeast colonies on malt extract agar



Fig: 3 Subculturing of yeast

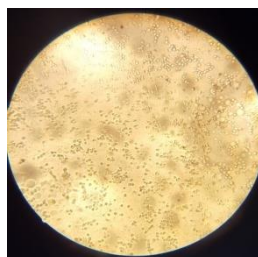


Fig: 4 Microscopic observation



Fig: 5 Bioethanol production



Fig: 6 Reducing sugars

Table 1: The quantity of bioethanol (%) by dichromate method

Substrate	% bioethanol by dichromate method		
	24hrs	48hrs	72hrs
Banana peel	4.8	8.2	17.4
Papaya peel	2.6	5.8	13.2
Pomegranate peel	3.2	6.7	15.2
Sapota peel	3.5	7.4	16.5
Potato peel	5.7	10.4	18.5

Table 2: Reducing sugars in autohydrolysis liquor before fermentation

Substrate	Reducing sugars concentration ($\mu\text{g/ml}$)
Banana peel	620
Papaya peel	320
Pomegranate peel	480
Sapota peel	540
Potato peel	700

Table 3: Protein concentration on 72hrs of incubation period before and after fermentation

Substrate	Protein concentration ($\mu\text{g/ml}$)	
	Before fermentation	After fermentation
Banana peel	65	120
Papaya peel	40	80
Pomegranate peel	45	84
Sapota peel	52	98
Potato peel	80	140

Table 4: Titratable acidity

Substrate	% lactic acid		
	24hrs	48hrs	72hrs
Banana peel	0.1	0.4	0.8
Papaya peel	0.1	0.2	0.4
Pomegranate peel	0.1	0.2	0.5
Sapota peel	0.1	0.3	0.7
Potato peel	0.4	0.6	0.9

IV. Conclusion:

Alternative sources (underutilised agro wastes such as fruit and vegetable wastes) have been shown to be cost-effective and efficient in the production of bioethanol. Zero waste generation techniques are made possible by utilising solid waste that is high in cellulose, hemicellulose, and lignin.

Acknowledgement:

Grateful acknowledgements to Telangana Social Welfare Residential Degree Colleges for Women, Warangal East, Telangana for the infrastructure support.

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