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Optimization of Bioethanol Production from Fruit Wastes using Isolated Microbial Strains

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ABSTRACT

Production of Ethanol fermented from renewable sources for fuel or fuel additives are known as bioethanol. Bioethanol as a fossil fuel additive to decrease environmental pollution and reduce the stress of the decline in crude oil availability is becoming increasingly popular. Its production mainly utilizes three types of raw materials- sugar juice, starchy crops, and lignocellulosic materials. This research work investigate ethanol production from fruit juices of four different fruits such as *Vitis vinifera (grapes), Sugarcane (Saccharum officinarum), Citrus cimetta (Mosambi)* and *Citrullus canatus (Watermelon)* using *Saccharomyces cerevisiae* for fermentation and optimizing several factors that influence the process for bioethanol production such as temperature, pH and sugar concentration.

Keywords: Bioethanol production, Agricultural wastes, Process optimization, Fermentation process

1.0 INTRODUCTION

Bioethanol is being widely investigated as a renewable fuel source because in many respects it is superior to gasoline fuel [1]. Ethanol provides energy that is renewable and less carbon intensive than oil. It is a biofuel produced from biomass via biochemical procedures. An efficient ethanol production requires four components: fermentable carbohydrates, an efficient yeast strain, a few nutrients and simple culture conditions.

Approximately 80% of world supply of alcohol is produced by fermentation of sugar and starch containing crops or byproducts from industries based on such crops. Among the widely used substrates for ethanol production are the molasses of sugarcane and sugar beet. Several studies have shown that sugarcane-based ethanol reduces greenhouse gases by 86 to 90% [2, 3]. Sugar-based bioethanol production- such as sugarcane and sugar beet- is a simple process and requires one step less than starch-bioethanol, this is because they are ready for conversion with limited pre-treatments as compared with starchy or cellulosic materials. Generally, the process is based on extraction of sugars (by means of milling or diffusion), which may be then taken straight to fermentation. The

wine is distilled after fermentation, such as in starch-based production.

The most well-known and commercially significant yeasts that been primarily used for bioethanol production are the related species and strains of *Saccharomyces cerevisiae* [4]. These organisms have long been utilized to ferment the sugars of rice, wheat, barley, and corn to produce alcoholic beverages and in the backing industry [5]. One yeast cell can ferment approximately its own weight of glucose per hour. Sugars from sugar cane, sugar beets, molasses, and fruits can be converted to ethanol directly [6].

In order to produce ethanol in large quantities and reasonable costs, the optimization of various physico-chemical parameters is important. The important parameters that could affect ethyl alcohol fermentation may be mentioned: availability and ferment ability of the substrate, possible isolation of new potent strain and improvement of the available strain towards higher productivity, improvement in fermentation technology and reduction in by-product formed during the fermentation process.

2.0 MATERIALS AND METHODOLOGY:

The chemicals that are used in this research are potassium dichromate, hydrochloric acid, *Saccharomyces cerevisiae* strains, different fruits such as mosambi, sugarcane, watermelon and grapes. The equipments used are hot plates, incubators, distillation unit and hydrometer. Fermenting yeast *Saccharomyces cerevisiae* (MTCC 171) procured from MTCC, Institute of microbial technology, Chandigarh was used in the present study. The microbial growth media YEPD is used under aerobic condition at temperature 30 ^{0}C.

2.1 Preparation of the substrate for the fermentation process

All four fruits (sugarcane, watermelon grape, and mousambi) were collected from different places. These fruits were rinsed in water and juice of each one of them was prepared and collected. The fermentation system was set up by taking ten conical flasks and in each set up different substrate (fruit juice) of 200 mL was used. Hydrochloric acid was used to adjust the initial pH prior to inoculation and process was carried out at room temperature.

2.2 Inoculum and inoculation

The yeast inoculum was prepared in YEPD broth. The fermentation system was inoculated with 2 mL culture broth /200 mL of substrate. The fermentation system was left undisturbed for about a week. The fermentation was carried out at varying temperature, pH, reducing sugar concentration, agitation and immobilized condition. During incubation, specific gravity of the sample was noted frequently by using a hydrometer. When the specific gravity reaches a steady value, it indicates the end of the fermentation process. The incubation period varies for each fruit juice sample.

2.3 Extraction of Ethanol by Steam Distillation

Fermented broths were removed at 48 hours of interval and contents were analyzed for total sugar and ethanol. Simple distillation unit at a temperature of 78 – 96°C separated the mixture of ethanol and hot water. In this method 80% of pure ethanol is obtained, which rectified by using rectifier units to obtain 99.2% pure ethanol [7].

2.4 Alcohol Estimation

Samples were first distilled, and the resultant ethanol concentration was measured using a dichromate reagent [8].

Table 1: Percentage of Ethanol Obtained

S. No	Samples	Specific Gravity	Percentage Ethanol
1	Grape	0.820	11.25
2	Mousambi	0.864	6.23
3	Watermelon	0.835	10.10
4	Sugarcane	0.818	12.15

Table 2: Ethanol Concentration obtained from different fruit samples

Samples	Ethanol Conc (Mg/Ml)	Ethanol Conc (µg/Ml)
Sugarcane	0.52	520
Grape	0.47	470
Watermelon	0.39	390
Mousambi	0.32	320

2.5 Optimization of fermentation process

Fermentation process carried out by yeast is known to vary with respect to various factors such as-substrate concentration, temperature, pH, N-source and inoculum size. It is therefore imperative to optimize the fermentation conditions for yeast cells so that the production efficiency increases. Various factors were investigated affecting ethanol production from fruits.

2.6 Effect of Temperature

Temperature plays a major role in the production of ethanol, since the rate of alcoholic fermentation increases with the increase in temperature. The fermentation process is always accompanied with evolution of heat that raises the temperature of the fermenter. As a result it becomes necessary to cool the large fermenters in the distilleries. To optimize the fermentation temperature, fermentation was carried out at 15, 20, 25, 30 and 35ºC. Fruits diluted to 20% sugars and supplemented with nitrogen and phosphorus were used as production media and fermentation was carried out at different temperatures. The periodic samples were analyzed for reducing sugars and ethanol content.

2.7 Effect of pH

pH of solution 5.0, 6.0, 7.0 and 8.0 were tested for fermentation using fruit sample with 20% sugar concentration and temperature of 29 ± 1ºC. Low pH inhibits the yeast multiplication.

2.8 Effect of Sugar concentration

Initial sugar concentration is an important influencing parameter as it has the direct effect on fermentation rate and microbial cells. The actual relationship between initial sugar content and the fermentation rate is rather more complex. Generally, fermentation rate will be increased with the increase in sugar concentration up to a certain level. But excessively high sugar concentration will exceed the uptake capacity of the microbial cells leading to a steady rate of fermentation. In batch fermentation, increased ethanol productivity and yield can be obtained at higher initial sugar concentration, but it takes longer fermentation time and subsequently increases the recovery cost [9]. To study the effect of sugar concentration on ethanol production by *S.cerevisiae*, the production media was prepared to sugar concentration of 5, 10, 15, 20, 25, and 30 percent with distilled water and filtered through ordinary filter paper to remove suspended particles. Fermentation was carried out in 250 ml conical flasks. A twenty four hour old inoculum of yeast was added at the rate of 6 percent to the medium. Samples were withdrawn after every 12-hour interval and estimated for residual sugars [10] as well as ethanol content in the media [11]. GC method for estimating the percentage of ethanol was employed. The initial sugar concentration that was efficiently utilized by the yeast for ethanol production was selected and maintained in fermentation media for further use.

2.9 Analytical methods

2.9.1 Spectrophotometric determination of ethanol [11]

One millilitre of the fermented wash was taken in 500ml Pyrex distillation flask containing 30 ml of distilled water. The distillate was collected in 50 ml flask containing 25 ml of potassium dichromate solution (33.768 g of $K_2Cr_2O_7$ dissolved in 400 ml of distilled water with 325 ml of sulphuric acid and volume raised to 1 litre). About 20 ml of distillate was collected in each sample and the flasks were kept in a water bath maintained at 62.5ºC for 20 minutes. The flasks were cooled to room temperature and the volume rose to 50 ml. Five ml of this was diluted with 5ml of distilled water for measuring the optical density at 600nm using a spectrophotometer. A standard curve was prepared under similar set of conditions by using standard solution of ethanol containing 2 to 12% (v/v) ethanol in distilled water. Ethanol content of each sample was estimated and graph was made.

2.9.2 Estimation of reducing sugars

The DNS method of was used to estimate reducing sugars. One ml of appropriately diluted solution (500-1000 μg ml^{-1}) sample was taken in a test tube to which 3 ml of DNS reagent was added. The tubes were boiled in a boiling water bath for 15 minutes.

One ml of Rochelle salt was added to these test tubes and tubes were cooled to room temperature and used for measuring optical density at 575 nm. A standard curve of glucose was prepared by using 100-1000 g concentration prepared in distilled water [10].

2.9.3 Gas chromatography

Ethanol in the fermentation broth was estimated by gas chromatography method. A computer related Nucon series gas chromatograph equipped with flame ionization detector (FID) was employed for the separation and quantification of ethanol. A stainless steel column (5m × 2mm) was fitted into the instrument to provide on column injection. The column packing was Porapak Q. The detector and injector temperature was maintained at 200°C. The gas chromatograph was connected to an integrator and computer system to determine area of ethanol and internal standard peak.

3.0 RESULT AND DISCUSSION:

3.1 Growth studies and effect of sugar concentration

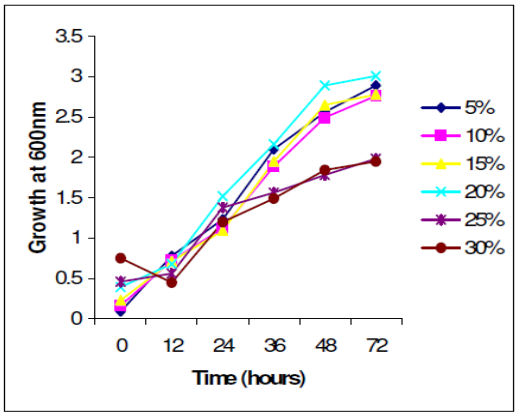
The growth of *S.cerevisiae* in gradually increasing concentrations of sugar showed an increase in optical density upto 20% sugar concentration in YEPD medium as shown in Table 1. However on increasing the sugar concentration beyond 20%, the growth was inhibited as shown by the optical density measured. Samples were taken every 12 hours for the study of growth kinetics. The growth was measured at 600nm.

Moaris *et al* (1996) also studied viability of *Saccharomyces* sp. in 50% glucose and reported a viability of 10-98.8% in different strains of yeast [12]. The detrimental effect of high sugar concentration on ethanol production was studied by Gough *et al* (1996) in *Kluyveromyces marxianus* and a sucrose concentration more than 23% in molasses was found to affect ethanol production [13]. Therefore, in the present study growth and fermentation were carried out with sugar concentrations upto 20%.

Table1 Effect of increasing sugar concentration on *S.cerevisiae*

Time (hrs)	Sugar concentration (%)					
	5%	10%	15%	20%	25%	30%
0	0.09	0.16	0.23	0.39	0.46	0.75
12	0.78	0.72	0.71	0.68	0.56	0.45
24	1.23	1.12	1.09	1.52	1.38	1.2
36	2.1	1.89	1.95	2.16	1.56	1.49
48	2.56	2.49	2.65	2.89	1.78	1.84
72	2.89	2.76	2.78	3.01	1.98	1.95

Graph 1. Effect of increasing sugar concentration on growth of *S.cerevisiae* at 6 pH and 30°C temperature



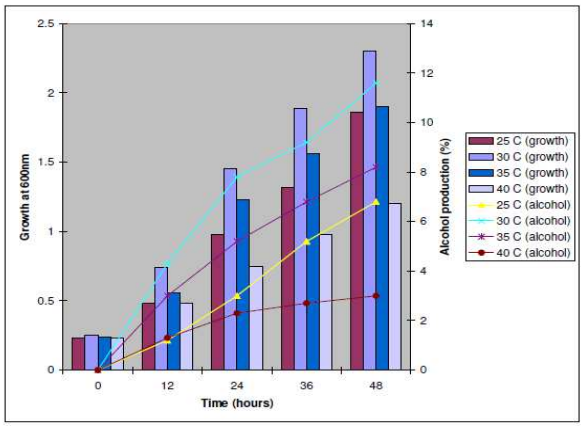
3.2 Effect of temperature on ethanol yield

Temperature is one of the major constraints that determine the ethanol production. To know the optimum temperature for ethanol fermentation, the solutions were kept at 25, 30, 35 and 40°C with 20% initial sugar concentration. Two parameters were simultaneously studied, the growth of *S.cerevisiae* and the ethanol yield. Samples were withdrawn every 12 hours and the fermentation was carried out for 48 hours. A low ethanol yield of 6.8% was observed at 25°C in 48 hours. As shown in Table 2 at 30°C ethanol yield was maximum and turned out to be 11%. However increasing the temperature beyond 30°C the growth as well as concentration of alcohol decreased. This
decrease was pronounced at 40°C so 30°Cwas selected as optimum temperature for ethanol production.

Temperature tolerance was also been found to depend upon sugar concentrations of the medium as in the case of, fermentation of molasses at 35°C was possible when sugar concentration was 20%(w/v) with no fermentation when sugar concentration was 22%(w/v) [14].

Table 2 Effect of temperature on ethanol production

Time (hrs)	Growth (In O.D.)				Alcohol (in %)			
	25°C	30°C	35°C	40°C	25°C	30°C	35°C	40°C
0	0.23	0.25	0.24	0.23	0	0	0	0
12	0.48	0.74	0.56	0.48	1.2	4.3	3	1.3
24	0.98	1.45	1.23	0.75	3	7.8	5.2	2.3
36	1.32	1.89	1.56	0.98	5.2	9.2	6.8	2.7
48	1.86	2.3	1.9	1.2	6.8	11.6	8.2	3



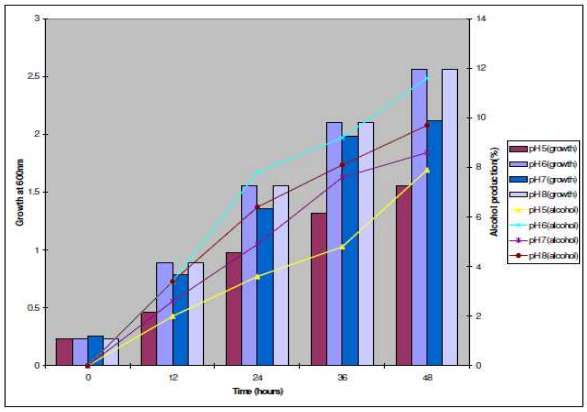
Graph 2 Effect of temperature on alcohol production at pH 6and 20%sugar concentration.

3.3 Effect of pH on ethanol yield

Initial sugar concentration of 20% and optimum temperature of 30°C was selected for further studies and subjected to pH treatments 5, 6, 7 and 8. The results are shown in table 3. At pH 5, fermentation took place but it gave low ethanol content. Best results were obtained at pH 6 where maximum ethanol production was noticed. Yadav *et al* (1997) found an increase in alcohol concentration, productivity as well as efficiency with an increase in pH from 4.0-5.0 and found that the optimum pH range for *S.cerevisiae* strain HAU-1 to be between pH 4.5-5.0 [15]. Based on fermentation efficiency the pH 6 was selected for further experimentation.

Table 3 Effect of pH on ethanol production

Time (hrs)	Growth				Alcohol			
	pH 5	pH 6	pH 7	pH 8	pH 5	pH 6	pH 7	pH 8
0	0.23	0.23	0.26	0.23	0	0	0	0
12	0.46	0.89	0.79	0.89	2	3.4	2.6	3.4
24	0.98	1.56	1.36	1.56	3.6	7.8	4.9	6.4
36	1.32	2.1	1.98	2.1	4.8	9.2	7.6	8.1
48	1.56	2.56	2.12	2.56	7.9	11.6	8.6	9.7



Graph 3 Effect of pH on ethanol production at 20% sugar concentration and 30°C temperature

3.4 Increase in ethanol yield using fermenter

After optimizing the various parameters like pH, temperature, sugar concentration etc. the experiment was scaled up from shake flask to fermenter. The optimum of previous experiments was taken i.e. the sugar concentration of 20%, pH 6 and temperature 30°C to further carry the experiment on fermenter. Fermenters are designed to provide best possible growth and biosynthesis conditions for industrially important microbial cultures. In fermenter, it is easier to control various parameters like temperature, pH that increases the ease of obtaining the desired product, ethanol in present study. After carrying out the fermentation, the samples were analysed using GC and compared with the standard run of absolute ethanol. From the broadness of peak it was inferred that there was continuous increase in ethanol production till 48 hrs.

4.0 CONCLUSION

In our present study we obtained ethanol from four ripened fruits *Vitis vinifera* (grapes), *Saccharum officinarum* (Sugarcane), *Citrus Cimetta* (Mosambi), *Citrullus Canatus* (Watermelon) collected from local markets around Bangalore .With the aid of yeast strains *Saccharomyces cerevisiae* (MTCC NO. 171) procured from MTCC, Institute of microbial technology, Chandigarh, ethanol was produced by the process of fermentation. After two week we could obtain 520 µg/ml and 470µg/ml of ethanol from 100 mL of fruit juices of Sugarcane, Grape and then followed by Watermelon and least being the Mousambi after distillation and maintaining a pH of 4 and temperature of 35°C. We could infer that more concentrated form of ethanol could be obtained by re-distillating the product ethanol obtained initially using a higher grade of distillation setup. A higher percentage (v/v) of ethanol could be obtained if the ethanol tolerance capability of yeast species is improvised by mutating the yeast species. This more concentrated form of ethanol could be used as a biofuel, which releases no toxic gases out in the environment. This process is environment friendly and the left over residues after fermentation can be disposed in the soil acting as a fertilizer for the soil. So even a common man may develop this process and produce it on commercial basis.

The fermentation of Sugarcane using *S.cerevisiae* (distillery strain) under optimized conditions i.e. pH 6, sugar concentration 20% and temperature 30ºC revealed an increase in ethanol production with good fermentation efficiency. However fermentation efficiency deceases after 48 hours of fermentation time. This might be due the either substrate limitations or due to product inhibition. S. cerevisiae reportedly showed the decrease in growth with increase in ethanol concentration in the medium.

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