Biomedical & Pharmacology Journal Vol. **4**(1), 135-140 (2011)

Ethanol Production from Cassava by Co-Cultures of *Aspergillus oryzae* and *Rhizopus oryzae*

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(Received: March 10, 2011; Accepted: April 22, 2011)

ABSTRACT

Ethanol has been known for a long time, being perhaps the oldest product obtained through traditional biotechnology. Ethanol production from cassava starch by co-cultures of selected such as *Aspergillus oryzae* and *Rhizopus oryzae* under submerged fermentation. The present study was aimed to evaluate the role of some fermentation parameters like ethanol yield, residual sugar, cell density, pH and temperature on ethanol production from cassava waste by *Aspergillus oryzae* and *Rhizopus oryzae*. At 1%, 1% and 10% substrate concentration, 10% gave a better ethanol production. *Aspergillus oryzae* inoculated medium gave better ethanol production compare to *Rhizopus oryzae* inoculated medium. The maximum ethanol yield was noted in the combination of *Aspergillus oryzae* and *Rhizopus oryzae* 6.2±0.17, residual sugar 2.7±0.17, celldensity 2.9±0.57. The highest ethanol yield was achieved at pH 6(8.7±0.17) and temperature (7.4±0.17) for *Aspergillus oryzae.*

Key words: Ethanol, submerged fermentation, *Aspergillus oryzae, Rhizopus oryzae,* cassava.

INTRODUCTION

Total demand for ethanol is expected to reach amount $3X10^{11}$ in 2010[1] . However due to increasing petroleum shortage, fermentation production of ethanol from renewable resources has received considerable attention[2] . Now-a-days, fermentation technology produces nearly 80% ethanol as clean fuel. Ethanol production by fermentation process has been studied extensively for several decades. Over the last few years, new approach with great potential for ethanol production from starchy materials and lignocellulosic biomass have been used, that is simultaneous saccharification fermentation (SSF) process[3] and Separate Hydrolysis and fermentation (SHF) process[4].

Cassava (Manihot esculenta crantz), a species of sub-bush, is perennial plant which grows a circular roots that are greatly different from quantity ant size because of different variety. Cassava root contains high starch content while it holds less protein, lipids and ash. The fresh root of some good variety of cassava includes 25%-32% starch content on which cassava is shown as a good raw material for starch. Cassava pulp, a fibrous residual material, is a by product of starch manufacturing; It generates as solid waste and result in creation of around 10-25% of the original cassava root weight[5]. It is used as a carbohydrate source in balanced feed, replacing wheat flour and synthetic binders and forming depending on the manufacture. Ethanol is generally produced by the fermentation of sugar, cellulose, or converted starch and has a long history. The first type of plant will produce a strong alcohol from cassava, but this will have an odor as the distilling process is very crude. It is potential source of ethanol production based on the high yields and low cost[6] reported the ethanol production by two steps from cassava pulp.

Submerged fermentation define does not have the problem of contamination, pH adjustment etc., which is a great problem in solid state fermentation[7] . Supplementation with medium components during growth of the organism is easy[8]. Suggested that the advantages of Submerged fermentation. They are high water content and more dilute nature makes temperature control easier. Product purification may be easier. Fermentation is life without air[9].

Aspergillus oryzae is a group of molds, which is found everywhere world wide, especially in the autumn and winter in the northern hemisphere. Cassava was successfully converted to ethanol by separate enzymatic hydrolysis with the aid of fermentation by *Aspergillus oryzae*.

The ability of *Rhizopus oryzae* strains of ethanol production 80% of initial moisture content consisted of 16g cassava. The amylolytic fungus, *Rhizopus oryzae* produces extracellular amylase and liquefy starch to oligosaccharides and glucose. Respectively ethanol under oxygen limiting condition[10] . The present study was aimed at ethanol production by the co-cultures of *Aspergillus oryzae* and *Rhizopus oryzae* under Submerged Fermentation and optimized fermentation parameters also.

MATERIAL AND METHODS

Source of Organisms

The soil samples were collected from a paddy field at Mannargudi, Thiruvarur (DT) Tamil Nadu, India and stored in a sterile polythene bags. Then the soil sample was immediately brought to the laboratory for the isolation of fungi[11] . *Aspergillus oryzae* and *Rhizopus oryzae* strains were obtained from serial dilution technique. The organisms were maintained on potato dextrose agar slants at 4°C.

Sample Preparation and Pretreatment

Cassava samples were collected from milling centers in Mannargudi, Thiruvarur (DT), Tamil Nadu. The samples were converted into fine powder by milling and sieving. Pretreatment of the sample was then carried out by refluxing the powder with 0.2M NaoH for 2 hours and then neutralized with HCL. The pretreated sample was dried in an oven at 65° C.

Culture condition for ethanol production

A synthetic medium containing yeast extract base glucose broth and a complex medium containing the pretreated cassava were used for ethanol production under Submerged Fermentation. The synthetic medium was prepared by dissolving 6.7 g of yeast extract and 10 g of glucose in a liter of distilled water. The complex medium was prepared with the cassava at a concentration of 10 g / liter. Supplemented with 3g $FeNH_4\ (SO_4)_2$, 5g $(NH_4)_2\ HPO_4$, 6g urea and 10g peptone. The pH of the medium was adjusted to 5.0, sterilized in an autoclave and filtered. Another set of media containing 100g/L of glucose and cassava were similarly prepared. The media were dispensed into 500 ml Erlenmeyer flasks each containing 250ml of the medium. The flask containing synthetic medium were inoculated with *Aspergillus oryzae* and the another synthetic medium were inoculated with *Rhizopus oryzae* while these containing complex medium were inoculated with both *Rhizopus oryzae* and *Aspergillus oryzae.* The flasks were incubated at 25°C for 5 days[12].

Effect of pH on ethanol production

10g of cassava was prepared and dispensed into different flasks. The pH of the broth was set as 4, 5 and 6 in a flask and sterilized. A loopful of *Aspergillus oryzae* was inoculated into 3 flask and *Rhizopus oryzae* was inoculated into 3 flask. All the 6 flasks and incubated at 28° C for 5 days. After incubation to determine the percentage of ethanol production.

Effect of temperature on ethanol production

10g of cassava was prepared and dispensed into different flasks. The temperature of the broth was set as 25° C, 30° C and 35° C in a flask and sterilized. A loopful of *Aspergillus oryzae* was inoculated into 3 flasks and *Rhizopus oryzae* was inoculated into 3 flasks and incubated at 28° C for 5 days. After incubation to determine the percentage of ethanol production.

Analytical methods

At 24 hour intervals, samples were taken aseptically from the fermentation media determine growth, residual sugar and ethanol concentration.

The growth was determined by measuring the cell density (optical density) at 650nm, The residual sugar was determined using dinitrosalicylic acid (DNS) method described by[13] and ethanol was determined after standard distillation using the method described by[14].

Statistical analysis

The results obtained in the present investigation were subjected to statistical analysis like mean (X) and standard deviation [15]

RESULTS AND DISCUSSION

In this present study producing organisms, *Aspergillus oryzae* and *Rhizopus oryzae* were isolated from paddy field soil sample. Ethanol production was estimated by *Aspergillus oryzae* and *Rhizopus oryzae* using cassava waste. High ethanol production was observed in 10% cassava containing medium than the 10% gucose containing medium.

The results of the ethanol yield from the synthetic medium containing glucose and complex medium containing cassava are shown in (Table1). At 1%, 1% and 10% glucose concentration, the synthetic medium gave a maximum ethanol yield of 4.4±0.05, 3.9±0.12 and 5.6±0.28 respectively. While at the same concentrations of cassava, the complex medium yielded 5.0±0.05, 4.7±0.17 and 6.2±0.17 ethanol. It was observed that at all concentrations of substrates. The ethanol yield increased steadily reaching the peak after 72 hrs of fermentation and then declined. The Cassava at 1% and 10% concentration gave a better ethanol yield compared to glucose. This shows that the cassava as one of the cheaper substrates for ethanol production[16].

The results in Table 2 showed the pattern of residual sugar during fermentation period. At 1%, 1% and 10% glucose concentration, the synthetic medium gave a maximum residual sugar was 1.4±0.17, 0.9±0.17 and 1.7±0.17 respectively. While at the same concentrations of cassava, the complex medium yielded 2.4±0.17, 2.1 ±0.17 and 2.7±0.17. The residual sugar in the fermentation media was

Table 1: Ethanol Yield from Synthetic and Complex Media

Fermentation	SM (Glucose)			CM (Glucose)		
Time	**1% *A.oryzae***	**1% *R.oryzae***	**10% *A.oryzae* & *R.oryzae***	**1% *A.oryzae***	**1% *R.oryzae***	**10% *A.oryzae* & *R.oryzae***
24	2.3± 005	1.9± 0.05	3.5± 0.11	3.5± 0.11	3.3± 0.11	5.3± 0.22
48	3.7± 0.05	3.3±0.17	4.8±0.05	4.4± 0.11	4.3 ±0.09	5.9± 0.11
72	4.4±0.05	3.9± 0.12	5.6±0.28	5.0± 0.05	4.7± 0.17	6.2 ± 0.17
96	3.8±0.09	3.5± 0.05	4.5± 0.05	4.7± 0.11	4.2± 0.17	5.7± 0.17
120	3.2±0.12	2.9±0.11	4.0± 0.05	4.1± 0.17	3.7± 0.05	5.2±0.19

Values are expressed as Mean ± Standard deviation

SM → Synthetic medium

CM → Complex medium

Table 2: Residual sugar obtained during fermentation of the synthetic and complex media

Fermentation	SM (Glucose)			CM (cassava)		
Time	**1% *A.oryzae***	**1% *R.oryzae***	**10% *A.oryzae* & *R.oryzae***	**1% *A.oryzae***	**1% *R.oryzae***	**10% *A.oryzae* & *R.oryzae***
24	0.2±0.05	0.1±0.05	0.4±0.05	1.1±0.05	1.0±0.05	1.5±0.05
48	0.5± 0.11	0.4±0.01	0.7±0.11	1.6±0.11	1.4±0.11	2.1±0.11
72	1.4±0.17	0.9±0.17	1.7±0.17	2.4± 0.17	2.1±0.17	2.7±0.17
96	0.9±0.05	0.7±0.05	1.4±0.05	2.0±0.09	1.9±0.09	2.4±0.05
120	0.6±0.09	0.4± 0.1	0.8±0.09	1.8±0.05	1.6±0.05	2.2±0.09

Values are expressed as Mean ± Standard deviation
SM → Synthetic medium
CM → Complex medium

Table 3: Growth (cell density) obtained during fermentation of the synthetic and complex media

Fermentation	SM (Glucose)			CM (cassava)		
Time	**1% *A.oryzae***	**1% *R.oryzae***	**10% *A.oryzae* & *R.oryzae***	**1% *A.oryzae***	**1% *R.oryzae***	**10% *A.oryzae* & *R.oryzae***
24	0.27± 0.01	0.24± 0.01	1±0.07	0.39±0.01	0.35± 0.01	1.6± 0.77
48	0.29± 0.11	0.27± 0.02	1.1±0.40	0.42±0.02	0.41± 0.02	1.9± 0.11
72	0.34± 0.13	0.31±0.12	1.4±0.17	0.49±0.02	0.46± 0.02	2.1± 0.33
96	0.38± 0.03	0.36± 0.03	1.6±0.23	0.56± 0.03	0.53± 0.03	2.3 ± 0.23
120	0.43±0.03	0.42± 0.03	2.0± 0.28	0.59± 0.02	0.57± 0.03	2.9 ± 0.57

Values are expressed as Mean ± Standard deviation
SM→ Synthetic medium
CM→ Complex medium

observed to decrease with increase in fermentation time. This could be attributed to the utilization of the sugar as carbon source for the growth and subsequent ethanol production. Cassava at 1% and 10% concentration gave a better residual sugar compared to glucose. The both *Aspergillus oryzae* and *Rhizopus oryzae* inoculated medium gave a better residual sugar compare than individually inoculated medium.

The growth (cell density) of the organisms was also observed to increase steadily during the fermentation period (Table 3). At 1%, 1% and 10% glucose concentration, the synthetic medium gave a maximum cell density was 0.43±0.03, 0.4±0.03 and 2.0±0.28. While at the same concentrations of cassava, the complex medium yielded 0.57±0.03, 0.57±0.03 and 2.9±0.57. This may be due to the fact that the organisms are utilizing the nutrients present in the media for growth and ethanol production. However, the media with 10 % substrate concentrations supported more growth of the organisms. Also the cassava seem to support higher growth of the organisms compared to glucose. The both *Aspergillus oryzae* and *Rhizopus oryzae* inoculated medium gave a better Growth compare than individually inoculated medium. Similarly reported by[17] in corn cobs. This may be due to the presence of other compounds in the cassava such as non starch carbohydrates, proteins, amino acids and other compounds which support more growth

Simultaneous Saccharification Fermentation (SSF) process using co-cultures between starch decomposing microbe and ethanol producing microbe is an alternative route for ethanol production from starch. Because this procedure leads to save for energy and low cost in fuel ethanol production from renewable feedstock. Ethanol production from an efficient microbes will be an important role in the new area.

Table 4: Optimization of parameters

S. No	Organisms	pH			Temperature		
		4	5	6	25°C	30°C	35°C
1.	*Aspergillus oryzae*	7.2±0.05	8.0±0.09	81±0.17	6.6±0.05	6.9±0.09	7.4±0.17
2.	*Rhizopus oryzae*	4.1±0.04.	8±0.09	5.4±0.17	5.9±0.05	6.4±0.09	7.1±0.17

Effect of pH concentration of Ethanol yield

The ethanol yield significantly influenced by different pH such as 4,5 and 6. The maximum ethanol yield was 8.7±0.17, 5.4±0.77 at pH 6 such as *Aspergillus oryzae* and *Rhizopus oryzae* respectively. The *Aspergillus oryzae* gave a better ethanol yield compared to *Rhizopus oryzae*.(Table 4). The similar results were reported by[18] .

Effect of temperature concentration of Ethanol yield

The ethanol yield significantly influenced by different temperature such as 25°C, 30°C and 35°C. The maximum ethanol yield was 7.4±0.17, 7.1±0.17 at 35°C. The *Aspergillus oryzae* gave a better ethanol yield compared to *Rhizopus oryzae*.(Table4). The optimal temperature of ethanol production was determined to be around 32° C. The related results were observed by[19] reported the maximum ethanol production was found to be 30° C.

CONCLUSION

The results of this study showed that cassava are good substrate for ethanol production compared to glucose. The substrates at 10% concentrations supported higher yield of ethanol and increases with fermentation time and peaked at 72 hours. Therefore findings of this work suggest that ethanol can be produced from agricultural wastes such as cassava. Cassava is the cheapest raw materials for ethanol production. Ethanol production from an efficient microbes will be an another important role in the area.

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