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Alcohol Production Potential of Locally Isolated Yeast Strain from Toddy Sap by Using Cassava Waste

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ABSTRACT

Cassava residue, starch-processing waste (tippi) from cassava starch plant, was used as a raw material in ethanol production. The experiment was performed into two steps (1) acid and enzymatic hydrolysis, the step in conversion of cellulosic materials and starch to fermentable sugar, and (2) ethanol fermentation, the step in conversion of fermentable sugar to ethanol by *Saccharomyces cerevisiae* NCIM3640, which was isolated from toddy sap. Cassava waste hydrolysed by using different concentrations of H_2SO_4. Highest yield (295.89 g reducing sugars/kg cassava waste) was obtained with 0.75% (v/v) H_2SO_4. Cassava residues were hydrolyzed by using independent and mixed-enzyme of cellulase and pectinase pH 4.5 at 28^{o}C for 1 hour then by α-amylase at 100^{o}C for 2 hours and finally glucoamylase at 60^{o}C for 4 hours. It was found that cassava residue with initial concentration of 11% non-water-soluble carbohydrate (w/v) could yield 456g reducing sugars/kg tippi. The increased amount of produced reducing sugars (497 g/ kg tippi) resulted from α-amylase. The optimum ethanol yield obtained was 0.28 (g/g) and 0.42 (g/g) for acid and enzymatic hydrolysates respectively. *S.cerevisiae* 3640 gave better results when compared with industrial strain of *S. pombe* and commercial strain of *S.cerevisiae* NCIM3570. Thus, utilization of cassava residue for ethanol production in this study could provide the most effective use of natural resources and eco-friendly energy.

Key words: Cassava waste, *Saccharomyces cerevisiae* 3640, acid and enzymatic hydrolysis, batch fermentation, ethanol

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INTRODUCTION

In recent years, fermentative production of ethanol from renewable resources has received attention due to increasing petroleum shortage. Cassava waste are readily available agricultural waste in India, yet they seem to be underutilized as potential growth medium for local yeast strains, despite their rich carbohydrate content and other basic nutrients that can support yeast growth. Potatoes and manioc (cassava – *Manihot esculenta* Crantz) can also be considered as plants suitable for starch-to-ethanol production. Cassava is considered a particularly attractive raw material for bioethanol production, as it is inexpensive and is not affected by food and feed shortage concerns [1,2]. The cassava wastes left over from these production processes are abundant and still contain a high amount of starch content. Most cassava wastes can be used as animal feed due to its high content of protein and other nutrients which are necessary for animal growth. In addition, cassava wastes can be used to produce ethanol. Use of cassava waste as raw material in ethanol production not only reduces waste material created from the cassava starch industry, but also lowers the cost of ethanol production [3,4]. Cassava is not only well-recognized as a food security supply being used as staple food in many regions, but it is also known as an energy security resource being used to produce liquid biofuel, i.e. ethanol. When cassava is used to produce ethanol, the starch in roots must be initially converted to sugars either by acid or, more preferably, by enzymes prior to sugar fermentation by yeasts [5]. By a two-stage enzyme hydrolysis, i.e. liquefaction by alfa-amylase and saccharification by glucoamylase, glucose is ultimately produced from cassava feedstock and then further fermented to ethanol by yeast. Approximately 60,000 tonnes of cassava starch factory residue (CFSR), a cellulo-starch byproduct, are discharged by the cassava (*Manihot esculenta* Crantz) starch factories of India [6,7]. In this study, hydrolysis of cassava waste was studied using both acidic and enzymatic methods followed by fermentation, in a lab scale, using the efficiency of ethanol production from *Saccharomyces cerevisiae* (NCIM 3640 & NCIM 3540) and *S.pombe* strains was evaluated.

MATERIALS AND METHODS

Microrganisms and preculture conditions

Saccharomyces cerevisiae (NCIM 3640) strain isolated from toddy sap and *S. cerevisiae* (NCIM 3570) strain obtained from NCIM, Pune. *Schizosaccharomyces pombe* strain obtained from Alfa Laval India Pvt. Ltd, Pune, India. Both strains of *S.cerevisiae* were maintained on YEPD agar slants (dextrose, yeast extracts, peptone, and agar, pH 5.0). *S. pombe* strain was maintained on Sabouraud dextrose agar medium and in liquid medium of same composition (without agar) by periodic transfers. For preparation of inoculum development, the seed culture of *S. cerevisiae* strains were grown with shaking at 30^{o}C in YEPD medium (the medium composition for cell growth is as follows (g/L): dextrose 20, yeast extracts 10, peptone 20, and agar, pH 5.0) and *S.pombe* strain was grown in Sabouraud dextrose medium with shaking at 30^{o}C up to its exponential phase. Then the cells were harvested by centrifugation. The pre-culture medium for inoculation into the cassava-based medium was composed of liquefied cassava with a total sugar concentration of 100 g/L and 0.1% of urea and diammonium phosphate. The growth

culture and pre-culture were performed in a 500-mL Erlenmeyer flask containing 200 mL of the medium and were cultivated at 30°C for 24 h.

Preparation of liquefied cassava (Tippi) and fermentation medium

Cassava waste (tippi) was collected from Sri Jyothi Sago Factory, Rasipuram, Namakkal District, TamilNadu, India. Tippi was sun dried and weighed. It was reduced by 5% and determined the moisture content of tippi. It was pulverized using hammer mill, sieved and finally separated as fine and coarse powder. Two technologies used to convert cellulose and hemicellulose to fuel ethanol are acid and enzymatic hydrolyses.

(a) Acid hydrolysis of cassava with different concentrations of sulphuric acid

Cassava waste (1kg) was hydrolyzed with 5000 ml (1:5 w/v) of various concentrations of H_2SO_4 at 60-120^oC. The hydrolysates were separated to obtain any suspended or unhydrolysated materials. Each sample was neutralized by 2 M NaOH solution for analytical processing [8].

(b) Enzymatic hydrolysis of cassava

Cassava waste was hydrolyzed with various enzymes (i.e. α- amylase, glucoamylase, cellulose, pectinase) which were obtained from Himedia laboratories. Several hydrolysis conditions, including cellulase 15 units per gram cassava at pH 4.8, 40^oC for 20 min, pectinase 4.7 units per gram cassava at pH 3.5, 20^oC for 30 min, mixture of cellulase and pectinase at pH 4.5, 28^oC for 1 h, α- amylase 24.02 units per gram cassava at pH5.5, 100^oC for 2 h, and glucoamylase 0.66 units per gram cassava at pH 4.5, 60^oC for 24 h, were tested.

After the liquefying steps were completed, the resulting liquefied cassava mash was centrifuged at 10,000×g and the supernatant, which contained approximately 207 g/L total sugar, was diluted with distilled water to the desired total sugar concentration. Samples of liquefied cassava containing 100 g/L total sugar was used as the fermentation medium for the ethanol production. Batch fermentation was carried out in 250 ml Erlenmeyer flask containing 100 ml of the fermentation medium was yeast inoculums grown overnight (5% v/v), then incubated at room temperature for 48 hr. At the end of the fermentation period, the alcohol was separated from the extract using simple distillation procedure at 78.3 - 80°C [8].

Analytical methods

The determinations were cell viability using the methylene blue method [9]. Total reducing sugar in acid hydrolysate (1.2M HCL for 10 min at 60^oC), using the 3,5-dinitrosalicylic acid method [10]. The sugar concentration was calculated using standard curves and expressed as gram sugar per litre. For the biomass assays, cells were washed by vacuum filtration and dried 70^oC until constant weight and expressed as grams per litre in the final medium.

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Estimation of alcohol

Gas chromatography (SHIMADZU, GC-2014, Kyoto, Japan) with a flame ionization detector was used for ethanol analysis of the samples. The samples were filtered through 0.2µm microfilters for ethanol concentration before injection. (i) Column: Rtx^R-5 Crossbond^{R}5% diphenyl/95% dimethyl poly siloxane (ii) Column temperature -120^{o}C (iii)Injector temperature -150^{o}C (iv) Detector temperature - 200^{o}C (v) Flow rate of carrier gas- 5µl/min. Ethanol concentration was expressed as g/l. Ethanol productivity, defined as grams of ethanol per liter per hour, was calculated based on initial concentration. Ethanol yield was calculated based on initial sugar concentration and reported as percentage of theoretical yield.

RESULTS AND DISCUSSION

Both enzymatic and acidic hydrolysis methods can be used to convert cassava waste into fermentable sugar, however the enzymatic method is preferred due to its safety and environmental aspects. Cassava waste hydrolysates are obtained from saccharification using commercial amylase and amyloglucosidase enzymes under the optimum conditions was fermented by *S.cerevisiae* NCIM 3640, *S. pombe* and *S.cerevisiae* NCIM 3570 (used as a reference strain). The residual reducing sugars represented in table 1&2 after treating the substrate with different concentration of acid and various enzymatic hydrolysis. Application of high concentrations of H_2SO_4 resulted in more solubilisation of cassava but no significant increase was observed in reducing sugars. Also, browning or charring of hydrolysate occurred with increasing acid concentrations. Both the dilute and concentrated acid processes have several drawbacks. Dilute acid hydrolysis tends to yield some undesirable by-products in addition to the sugars [11]. They are furfural and 5-dihydroxymethyl furfural, which are known to inhibit fermentation [12]. These compounds are reported to be produced in very small concentration but they may be toxic to fermentation. Concentrated acid hydrolysis forms fewer by-products but for economic reasons the acid must be recycled. The separation and reconcentration of the H_2SO_4 adds more complexity to the process [13].

Table 1. Acid hydrolysis of cassava with different concentration of sulphuric acid

Sulphuric acid concentration (%)	Reducing sugars (g/kg)
0.25	130.24
0.50	198.25
0.75	295.89
1.10	264.20
1.5	256.26
2.0	248.35
0 (Control)	92.65

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Table 2. Enzymatic hydrolysis of cassava treated with different enzymes

Enzyme used	Reducing sugars (g/kg)
Amylase	497.00
Cellulose	350.54
Glucoamylase	405.14
Amylase + glucoamylase	416.24
Amylase + glucoamylase + cellulose	456.54
Control	95.65

In addition to the above fact, H_2SO_4 is highly corrosive and difficult to handle. Neutralization of hydrolysate is required before the fermentation, which leads to sludge formation at the bottom. This is then removed by the solid–liquid separation. The concentrated and dilute sulphuric acid processes are performed at high temperatures like 150–180°C, which can degrade the sugars, reducing the carbon source and ultimately lowering the ethanol yield [14]. The two acid technologies are old techniques; whereas, the enzymatic process is relatively new. Since thippi contains mostly starch (70%) and some protein, the rest being a poorly defined mixture originated from root and pectin peelings [15]. Three different enzymes amylase, pectinase and cellulase were used for optimization process. Amylase was then selected for further studies.

A number of authors have proposed the use of *Z. mobilis* in place of traditional yeast for ethanol production from starch substrates. *Z. mobilis* ferments hexoses at very high rate under anaerobic conditions and ethanol yields are very close to theoretical [16]. The abundance of low-cost feedstock and the cost-effective technology are of great importance for reinforcing industrialization of bioethanol for fuel use as sustainably-sourced and eco-friendly energy. Indigenously yeast strain was isolated from toddy sap to produce ethanol was investigated. *C. tropicalis* is known to produce ethanol from starch at a low rate as it produces glucoamylase [17]. Since this organism produces starch-decomposing enzyme at a low rate, this property can be used to develop a fermentation process by further optimizing the process, where *C. tropicalis* can be grown directly on thippi slurry (without hydrolyzing it). Amylase alone was not sufficient for complete starch fermentation by *S. cerevisiae* or other microorganisms like *Z. mobilis*, which lacks glucoamylase activity, while *C. tropicalis* can completely convert starch into ethanol if the substrate is already treated with amylase [18].The hydrolysate is a complex mixture of sugars. Therefore, mixture of *Z. mobilis* and *C. tropicalis* was assessed as compared to a single strain. This was an attempt to exploit the advantage of each strain to improve the utilization of reducing sugars and the ethanol production. Optimization of few parameters like increased agitation and increased concentration of $(NH_4)_2SO_4$ resulted in reduction of fermentation time. Similar results were shown with *Z. mobilis* in cassava starch [19].

The time course of mixed culture fermentation was compared at 1 and 10 litre level. Reducing sugars utilization and ethanol production studies showed that the mixed culture consumed maximum reducing sugars in 8–18 h. In the 10 litre fermentor, maximum sugar was utilized for cell growth resulting in less ethanol yield. While *Z. mobilis* consumed maximum reducing sugars in 12–24 h, yeast *C. tropicalis* showed maximum reducing sugar consumption in

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22–32 h. The impact of mixed cultures on biomass to ethanol processes has been explained in a previous study [20].

The main aim of this research work was to develop a feasible technology for ethanol production from the abundantly found starchy waste material, thippi. The results of this study show that acid hydrolysis has several drawbacks and hence enzymatic treatment was optimized for thippi hydrolysis. Enzymatic hydrolysis using amylase yielded very high amounts of reducing sugars, and proved thippi to be a potential substrate for ethanol production. A higher ethanol yield was obtained with *S.cerevisiae* NCIM 3640 during thippi hydrolysate fermentation than the commercial strain and industrial strain (Table 3 & 4). By increasing the inoculum size and agitation, the fermentation time could be reduced effectively to 24-48 h. *C. tropicalis* has a property to produce starch-decomposing enzyme and complete fermentation of thippi can be obtained without hydrolyzing the substrate. Therefore, direct fermentation process can be developed, which will be helpful in cost reduction of the ethanol production. Analysis of fermentation data indicates that *C. tropicalis* yielded 214.2 g ethanol/kg thippi (61.2 g ethanol/l). There are very few reports showing such a high value of ethanol production by starch conversion with *C. tropicalis*. Ruiz *et al* reported that alcohol production using glucose syrups from enzymatically-hydrolyzed starch was carried out with a wild strain of *Candida sp* isolated from sugar cane juice, obtaining volumetric ethanol productivities around 1.8-3.2 g $L^{-1}h^{-1}$ [21]. The results obtained from this research would be beneficial for the application of ethanol fermentation by *S. cerevisiae* NCIM 3640 from cassava waste.

Table 3. Ethanol production from acid hydrolysate of cassava supplemented with 1% urea and diammonium phosphate using different strains at 30°C, 48hr.

Strain used	Cassava acid hydrolysate	P (g/l)	Q_p (g/l/h)	Y_{ps} (g/g)	E_y (%)	Biomass (gl^{-1})
NCIM 3640	100	28	0.77	0.28	54.7	1.67
S. pombe	100	27	0.75	0.27	52.8	1.58
NCIM 3570	100	24	0.66	0.24	46.9	1.52

P: ethanol concentration; $Q_{p:}$ volumetric ethanol productivity; $Y_{ps:}$ ethanol yield, Ey (%) :fermentation efficiency.

Table 4. Ethanol production from enzymatic hydrolysate of cassava supplemented with 1% urea and diammonium phosphate by using different strains at 30°C, 48hr.

Strain used	Cassava enzymatic Hydrolysate (g/l)	P (g/l)	Q_p (g/l/h)	Y_{ps} (g/g)	E_y (%)	Biomass (g/l)
NCIM 3640	100	42	1.16	0.42	82.1	1.89
S. pombe	100	40	1.11	0.40	78.2	1.78
NCIM 3570	100	37	1.02	0.37	72.4	1.62

P:ethanol concentration; $Q_{p:}$ volumetric ethanol productivity; $Y_{ps:}$ ethanol yield, Ey (%) :fermentation efficiency.

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