AN ENZYMATIC PROCESS OF BIOETHANOL PRODUCTION USING AGRICULTURAL WASTES BY *Saccharomyces cerevisiae* (MTCC 173) AND *Zymomonas mobilis* (MTCC 2427)

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ABSTRACT:

Ethanol has widespread use as a solvent of substances intended for human contact or consumption, including perfumes, flavours, colourings and medicines. The economics of ethanol production by fermentation is significantly influenced by the cost of raw materials, which accounts for more than half of production cost. In recent years efforts have been directed towards the utilization of cheap renewable agricultural resources such as banana peel, waste paper, sugarcane waste as alternative substrate for ethanol production. In this study, ethanol was produced from agricultural wastes by using two enzymes namely Amylase from *Aspergillus niger* and Cellulase from *Trichoderma viridae* to hydrolyse the starch and cellulose present in the raw materials. The hydrolysed and filtered extracts were fermented using *Saccharomyces cerevisiae* and *Zymomonas mobilis*. The fermented product was purified by primary distillation process at 80°C and the fractions were collected. The presence of ethanol was then determined by Alcoholmeter method. Results indicated that the *Zymomonas mobilis* organism yielded maximum ethanol where as minimum ethanol yield was recorded with *Saccharomyces cerevisiae* organism.

Keywords: Bioethanol, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, Enzymes, Fermentation.

INTRODUCTION:

Bioethanol is a domestically produced liquid fuel from renewable resources known as biomass. Ethanol is a gaining momentum as a viable fuel source due to recent fluctuations in the market of conventional fossil fuels. In addition to its common pharmaceutical and beverage uses, ethanol is being used as a fuel additive, gasoline and even as an alternative fuel source. It has a long history as a fuel for heat and light and also for internal combustion engines. Bioethanol can be produced from fresh fruits, vegetables and from many waste materials includes banana peel waste, sugarcane waste [1] [2] and waste paper [3] [4]. It can also be produced from a range of cellulose [5]. Ethanol is used in the manufacture of detergents, adhesives, lubricants, pharmaceutical dyes, pesticide resins and antifreeze agents.

In India ethanol is produced mainly by the fermentation of substrates containing a mixture of invert sugars and bound sucrose by using various strains of *Saccharomyces cerevisiae*. Apart from the use of *Saccharomyces cerevisiae*, bacteria named *Zymomonas mobilis* is also used to produce ethanol. In 1994, NREL developed, genetically engineered organism known as *Zymomonas mobilis* which enhances the fermentation of biomass sugars, leading to greater yields of ethanol and lower costs. The advantage that *Zymomonas mobilis* holds over traditional yeast processes lead to more economical and environmentally friendly methods of producing ethanol. Its ability to efficiently produce ethanol is of particular interest. *Saccharomyces cerevisiae* and *Zymomonas mobilis* are used together for improved ethanol production.

Banana is one of major constitute the principal food resources in the world and occupy the fourth world rank of the most significant foodstuffs after rice, corn and milk [6]. Banana peel an agrowaste was used as a substrate for ethanol production. Banana peels are readily available agricultural wastes yet they seem to be under utilized as potential growth medium for local yeast strain, despite their rich carbohydrate content and other basic nutrients that can support yeast growth [7] [8]. Paper waste which contains sugar materials is also used for ethanol production. The three categories of paper that can be used as stocks for making recycled paper mill broke, preconsumer waste and postconsumer waste. Mill broke is paper trimmings and other paper scrap from the manufacture of paper and is recycled internally in a paper mill but was discarded before it was ready for consumer use, such as old corrugated containers, old magazines and news papers, office papers, old telephone

directories and residential mixed paper. Cane sugar is a naturally occurring sweetener agent. Sugar cane is so far the most efficient raw material for Bioethanol production.

Selected strains of *Aspergillus niger* and *Trichoderma viridae* are used for the production of fermentable sugars from cellulosic wastes [9], and also they are used for the production of amylase and cellulase enzyme respectively. The amylase, which hydrolyze the starch are classified in various ways, depending on how they act on the starch molecules, Amylase include α amylase and β amylase. Cellulase catalyzing the hydrolysis of cellulose to cellulodextrins and glucose has been recommended for producing more fermentable sugar in brewer's mashes.

The purpose of the study is to produce Bioethanol using cheap substrates. And the study is targeted to produce high activity amylase and cellulase enzyme by using *Aspergillus niger* and *Trichoderma viridae.* This study compares the production of Bioethanol from cheap substrates such as banana peel, sugar cane waste and waste paper by using the strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis* grown in YEPD and RM medium.

MATERIALS AND METHODS:

Substrates: Agro industrial wastes such as Banana peel, Sugar cane waste and Paper waste were used for ethanol production.

Collection of substrates: Banana peels were collected from houses, and were dried and crushed to powder form. The second substrate, crushed Sugarcane waste was collected and made into small pieces by using mortar and pestle. Then, the Waste paper was collected and made into small pieces of approximately 2mm thick by using scissors. These three substrates were used for ethanol production.

Pre-treatment of substrates: The pretreatment of banana peel was carried out by using 2 ml/gm of 67%H_2SO_4 as a catalyst and steamed for 60 minutes. The collected lignocellulosic sugar cane waste were sieved and pretreated with 1N NaOH for 1hr at 100^0C [10]. The pretreated substrates were washed thoroughly with distilled water, dried at room temperature and stored in desiccators. Likewise, waste paper substrates were pretreated with 3%NaOH at 15lbs for 1hr. Then they were washed and neutralized with 0.1N HCl at p^H 5. Then they were dried at 45^0C in an oven after neutralization, and were subjected to enzyme treatment.

Source of Organisms: *Saccharomyces cerevisiae* (MTCC 173), *Trichoderma viridae* (MTCC 800) and *Zymomonas mobilis* (MTCC 2427) were obtained from IMTECH, Chandigarh. *Aspergillus niger* was isolated from spoiled bread sample and identified. The stock cultures of *Saccharomyces cerevisiae*, *Trichoderma viridae and Zymomonas mobilis were* maintained on Yeast Extract Peptone Dextrose Agar (YEPD), Malt Extract Agar (MA) and Rich medium (RM) respectively. Isolation and identification of the organisms were done on the basis of morphological, cultural and biochemical characteristics.

Enzyme production and Assay: *Aspergillus niger* and *Trichoderma viridae* were inoculated in Potato dextrose broth and incubated at room temperature for 7 days. After incubation, fermented media was filtered by Whatman No-1 filter paper and centrifuged and the supernatant was collected for enzyme assay. Amylase and cellulase enzymes were determined by Starch agar plate method and Dinitro Salicylic acid (DNS) method [11].

Enzymatic hydrolysis: 20g of Banana peel waste, Sugar cane waste and Waste paper were crushed and dissolved in 200ml of distilled water. Then the content was boiled and filtered. Then extract was sterilized after sterilization 5% of enzymes were added to the extracts and incubated at 37°C for 3 hours for hydrolysis process.

Determination of Total sugar and Residual sugars: The total sugar content of the samples were determined by Phenol sulphuric acid method [12] and the residual sugar content of samples were determined by Nelson-Somogyi method with glucose as standard [13]. The Reducing sugar (sugar utilized by the organism during fermentation) percentage was calculated by subtracting the residual sugar% from total sugar%.

Concentration of test = OD of test / OD of standard × Concentration of standard

Fermentation and Distillation: The hydrolyzed and filtered extracts were fermented using *Saccharomyces cerevisiae* and *Zymomonas mobilis* for seven days of incubation at room temperature in rotary shaker. After fermentation the content was filtered and the primary distillation was carried out in vacuum flask at 80^0C and fractions were collected. Estimation of ethanol content in the samples was determined by Alcoholometry method.

RESULT AND DISCUSSION:

Figure 1: Total sugar percentage of untreated substrates

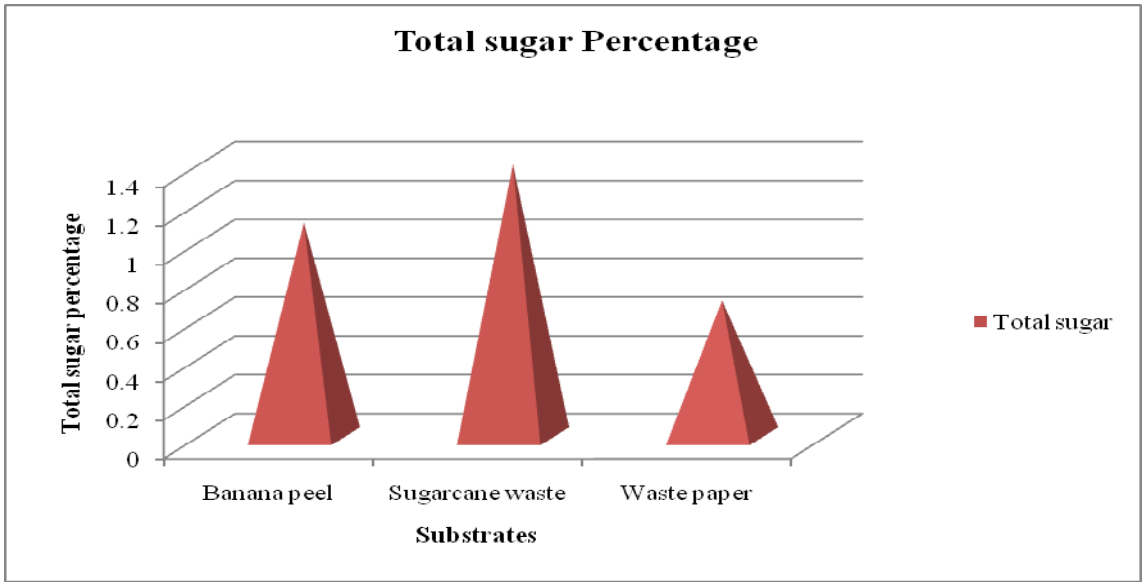


Figure 2: Cellulase enzyme assay by DNS Assay method

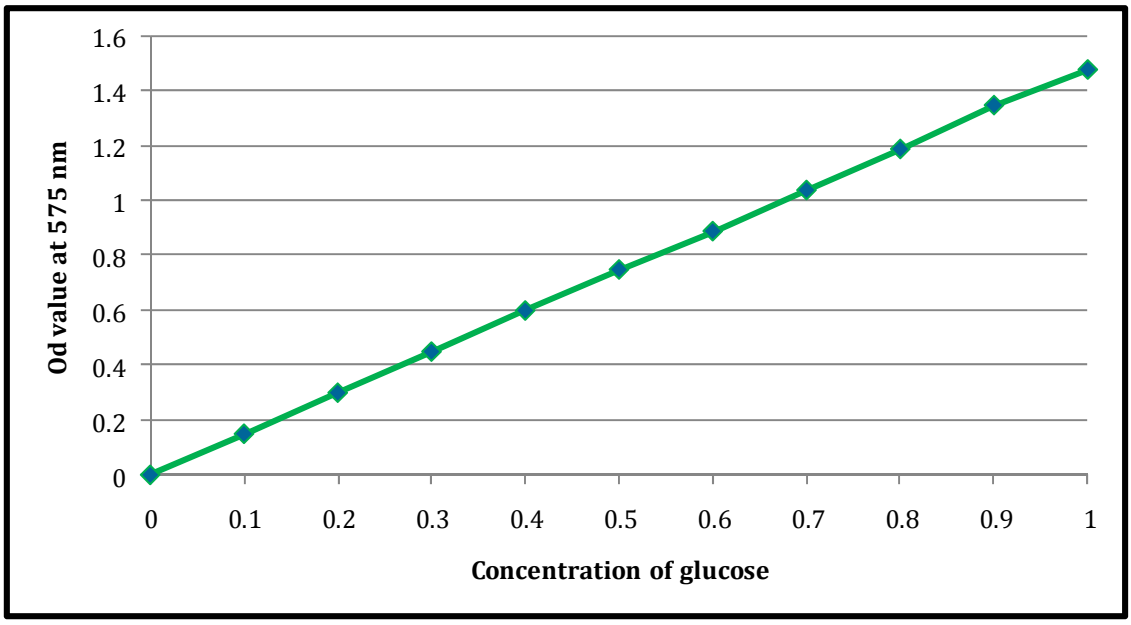


Figure 3: Residual sugar percentage in fermented substrates

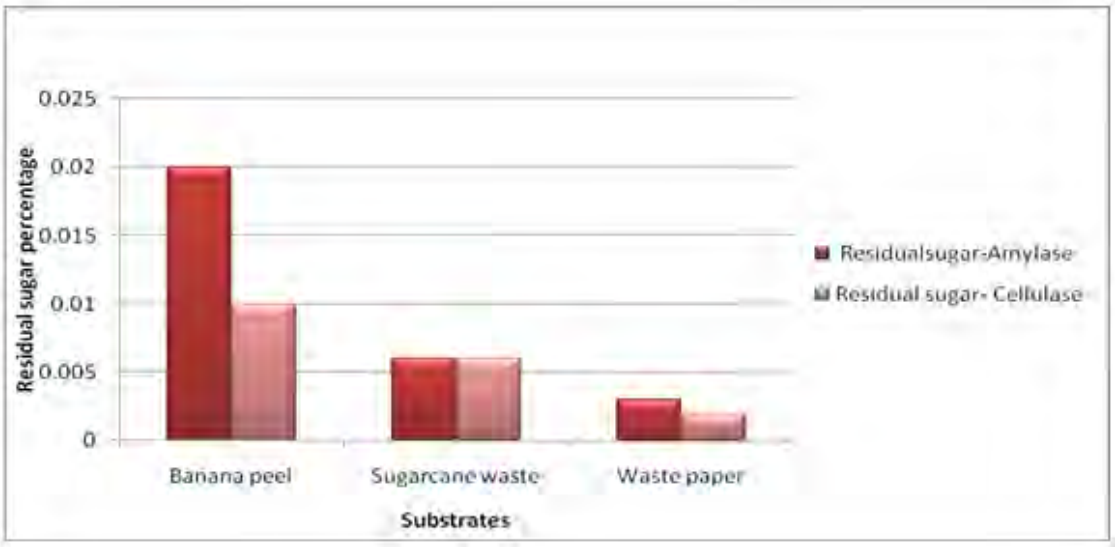
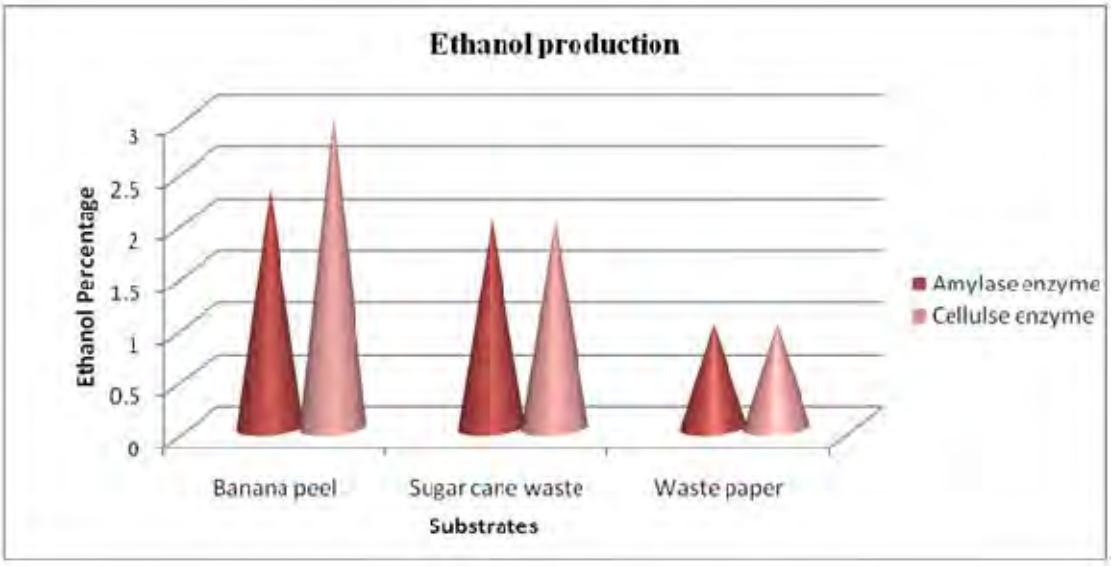


Figure 4: Comparison of ethanol production using banana peel, sugar cane waste and waste paper



DISCUSSION:

Total sugar percentage: In this experiment, the total sugar percentage was carried out by using untreated substrates and the maximum percentage was found to be high in both sugar cane wastes (1.4%), banana peel (1.1%) where as in waste paper (0.07%), the total sugar percentage was low when compared to the other two substrates.

Table 1: Total sugar percentage in substrates

Substrates	Total sugar (mg/100ml)	(%)
Banana peel	**1075.5**	**1.1**
Sugar cane waste	**1392.5**	**1.4**
Waste paper	**0.715**	**0.7**

Cellulase enzyme assay: The results [Table 2 and Figure 2] show that the constant increase of glucose released from the substrates.

Table 2: Cellulase Enzyme Assay by Dinitro Salicylic Acid Method

Particulars (ml)	B	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	E
Glucose standard	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	-
Distilled water	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0	-
Enzyme	-	-	-	-	-	-	-	-	-	-	-	1
0.05M citrate buffer	1	1	1	1	1	1	1	1	1	1	1	1
Incubated at 60°C for 10 minutes												
DNS solution	1	1	1	1	1	1	1	1	1	1	1	1
Boiled at 100°C for 5 minutes												
Rochelle salt solution	1	1	1	1	1	1	1	1	1	1	1	1
OD at 575 nm	0	0.15	0.3	0.45	0.6	0.75	0.89	1.04	1.19	1.35	1.48	0.51

Residual sugar percentage: In this experiment, residual sugar percentage was carried out by using the fermented substrates and it was found that the percentage of residual sugar was more in banana peel waste when compared to others.

Table 3: Residual sugar percentage in substrates

Substrates		Residual sugar (mg/100ml)	(%)
Banana peel	Amylase	21.7	0.02
	Cellulase	14.9	0.01
Sugarcane waste	Amylase	6.2	0.006
	Cellulase	6.12	0.006
Waste paper	Amylase	3.1	0.003
	Cellulase	2.9	0.001

Ethanol production: The result [Table 4 and Figure 4] shows that the maximum ethanol yielded by *Saccharomyces cerevisiae* in the substrates such as Banana peel (2.3%) and Sugarcane waste (2%) that were treated with amylase, where as in Waste paper (1%) the percentage of ethanol was found to be reduced when compared to other substrates. The ethanol yielded by *Zymomonas mobilis* in substrates such as Banana peel (3%) and Sugar cane waste (2%) that were treated with cellulase, where as in waste paper (1%).

Table 4: Ethanol production from Banana peel, Sugarcane waste and Waste paper by *Saccharomyces cerevisiae*

Substrates	Fermenting organism	Enzyme used	Ethanol (%)
Banana peel			**2.3**
Sugar cane waste	***Saccharomyces cerevisiae***	**Amylase**	**2**
Waste paper			**1**

Table 5: Ethanol production from Banana peel, Sugarcane waste, Waste paper by *Zymomonas mobilis*

Substrates	Fermenting organism	Enzyme used	Ethanol (%)
Banana peel			**3**
Sugarcane waste	***Zymomonas mobilis***	**Cellulase**	**2**
Waste paper			**1**

CONCLUSION:

The maximum ethanol yield was recorded by the fermented organism, *Zymomonas mobilis* using the enzyme cellulase. The fermented organism, *Saccharomyces cerevisiae* was produced the minimum yield of ethanol by using amylase enzyme. The substrates such as Banana peel and Sugarcane waste had more capability to produce ethanol as compared to Waste paper.

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