Bioethanol Production from Agriculture Wastes by Acid Hydrolysis and Fermentation Process

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Abstract— Agricultural wastes such as carrot peel, onion peel, potato peel and sugar beet peel are products subjected to saccharification process by Penicillium sp. for the hydrolysis, this process was followed by the fermentation using yeast Saccharomyces cerevisiae for the production of alcohol which was fermented at 14, 21, 28 days to produce alcohol. The aim of the study was to determine alcohol content of fermented agricultural wastes based on different fermentation time. The fermented product was purified by primary distillation process at 78^{0}C and the fraction was collected. The ethanol was determined by dichromate method. High yield of ethanol was obtained from sugar beet peel 14.52% on 28th day and further confirmed by Gas chromatography and the yield of ethanol obtained on 28th day was 17.3%.

Keywords: Agricultural Wastes, Penicillium Sp., Saccharomyces Cerevisiae, Bioethanol

I. Introduction

One of the most characteristics of the 20th century is the production of biofuel for the automobiles used for personal and public transportation[1]. There are thousands of these vehicles in circulation today around the world; most of the engines are running on gasoline. In United States, the world's biggest ethanol producer utilizes corn as the substrate for ethanol[2]. Brazil, the second biggest ethanol producer, utilizes sugarcane as the substrate for ethanol[3]. Ethanol has attracted worldwide attention because of its potential use as an alternative automotive fuel. Carrot peel, onion peel, potato peel and sugar beet peel belong to this group of valuable biomass wastes which increased the yield of ethanol production by microbial fermentation, by appropriate fermentation of substrate by suitable process technology. Sugar cane is the major crop in Brazil while many other crop plants such as cassava, sugar-beet, wheat, rice, corn, barley, potato, and sorghum are also being utilized for bioethanol production in different parts of the world [4,5].

Bioethanol production was studied by Zhang et al, 2009[6] using Aspergillus Niger and Saccharomyces cerevisiae in simultaneous saccharification and fermentation. Similarly, studies were conducted by Zakpa and Kumar[7, 8] from lignocellulosic biomass. Ahmad et al.,[9] reported that sago and sweet sorghum substrates produced high amount of ethanol at the end of fermentation which was at 72h. The bioethanol production was due to the fact that saccharomyces cerevisiae growth was considered to be a non-associated growth since the ethanol was excreted as extracellular by the yeast. In India, bioethanol is mostly produced from sugarcane molasses which is a waste by-product obtained after the removal of sucrose from the sugarcane juice for sugar production [10]. Hence the present study was aimed at production of bioethanol using various agricultural wastes as substrates.

II. Material and Methods

A. Substrate Preparation, Pretreatment

A large amount of carrot peel, onion peel, potato peel and sugar beet peel were obtained from the different vegetables' traders in and around Bangalore, India and thoroughly washed with tap water and cut into smaller pieces. Thereafter substrates were sun dried for 3-5 days depending on moisture content and then milled sieved to size particle of 0.5-1mm. Pretreatment of the sample was then carried out by 1% NaOH for a period of 2 hours[11].

B. Microorganisms and Culture

The soil samples were obtained from the tomato fields in Bangalore. These soil samples were screened for the potent microorganisms to hydrolyze the substrate. The predominant fungal culture was isolated and identified by preparing a wet mount using lactose phenol cotton blue and confirmed by National Fungal Culture Collection of India (NFCCI), Pune. The fungus was cultured and maintained on Potato Dextrose agar medium at 30^{0}C. After optimum growth the culture was stored at 4^{0}C in refrigerator for further use[12].

C. Fermentation medium

The fermentation media used was 0.2% yeast extract, 0.2% $(NH_4)\ NO_3$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% KH_2PO_4, and 5 g of each substrate[13].

D. Saccharomyces Cerevisiae

The yeast Saccharomyces cerevisiae was isolated from soil samples collected from vineyards rich in waste materials which include fallen and discarded grapes. The soil samples were suspended in sterile distilled water and allowed to settle, then the supernatant was diluted by serial-10-fold dilutions and the samples were inoculated on to sterile Yeast- extract, Peptone and Dextrose (YEPD) plates contained dextrose (6%), peptone (0.5%) and yeast extract (0.5%) [14]. Yeast cells pre-grown in inoculum medium for 48 h under shaking condition (150 rpm). The grown yeast isolates were identified as Saccharomyces cerevisiae by studying some of the morphological, biochemical and physiological characteristics[15] and confirmed by National Fungal Culture Collection of India (NFCCI) Pune. The organism was maintained on potato dextrose agar slants at 4ºC.

III. Analytical Methods

A. Estimation of Reducing Sugars

Somoyogi (1952)[16] estimated reducing sugars of the samples with glucose as standard. 10g of the agricultural waste sample was taken and the sugars were extracted with hot 80% ethanol twice (5ml each time). Supernatant was collected and evaporated keeping it on a water bath at 80°C. 10ml of water was added to dissolve the sugars. Aliquots of 0.2, 0.4, 0.6,

0.8, and 1ml of the working standard solution was pipetted out into a series of the test tubes. Volume of standard and sample tubes were made up to 2ml with distilled water. 2ml of distilled water was pipetted out in a separated tube to set a blank. 1ml of alkaline copper tartrate reagent was added in each tube. All the tubes were placed in a boiling water bath for 10 min. Then the tubes were cooled and 1ml of arsenomolybdic acid reagent was added into each tube. The optical densities of the standard and test sample were measured at 660nm and a graph was plotted. Test sugar sample was performed like the standard and the amount of reducing sugar present was calculated in the sample.

B. Determination of Protein

The protein content of carrot peel, onion peel, potato peel and sugar beet peel were determined by Bradford's method (1976)[17] with bovine serum albumin as standard.

C. SDS-PAGE of crude extract of carrot peel, onion peel, potato peel and sugar beet peel

Crude extract of carrot peel, onion peel, potato peel and sugar beet peel obtained from inoculating Penicillium was centrifuged (10,000 g) for 10 min. SDS PAGE was carried out to determine the homogeneity and molecular weight of cellulase enzyme as it was found to be predominant in the conversion of complex polysaccharides into simple sugars. SDS-PAGE was carried out with the crude enzymes produced from all the four substrates inoculated with Penicillium sp. Polyacrylamide gel electrophoresis in the presence of SDS separates the polypeptide chains according to their molecular weight. To determine the molecular weight of agricultural waste substrates used SDS-PAGE was performed on a 5% stacking and a 12% separating gel resolving gel according to the method [18].

D. Fermentation

The chemically pre-treated substrates were used for all the experiments. In order to optimize bioethanol production, the substrates were taken in different variations. Saccharification and fermentation studies were performed in 250 ml Erlenmeyer flasks in which 5 grams of substrate was taken in each flask and fermentation was carried out (Fig. 1). For fermentative production of bioethanol (stationary method) Saccharomyces cerevisiae was employed. After 14, 21, 28 days of saccharification mycelia of Penicillium was removed under aseptic conditions and 10% of Saccharomyces cerevisiae culture was added to all the flasks[14]. The substrates which were saccharified with different time and fermentation of 14, 21 and 28 days were taken and filtered through muslin cloth. The extracts recovered were distilled at 78ºC for the collection of ethanol.

E. Ethanol Determination

Ethanol was estimated by Potassium dichromate method [19]. The process was carried out for a period of 14, 21, 28 days at 30^0C and these samples were estimated for bioethanol and further confirmed by Gas Chromatography

IV. GAS CHROMATOGRAPHY (GC-MS)

A. Quantitative Ethanol Determination

A Gas Chromatography (Shimadzu 2010) was used in the quantitative analysis of ethanol in all the fermentation media. Gas Chromatography settings and characteristic features were selected to enable ethanol separation from the injected supernatant. The supernatant solution (0.5 mL) was dispensed into an l- mL caped sample vial, and then 5 mL of 1% internal standard solution (equivalent to 50 mg) was added. After mixing, 0.1 µL of the sample solution was injected directly into a GC with syringe. This sample solution was suitable for injecting directly into a GC for determination of ethanol content [20].

V. RESULTS AND DISCUSSION

A. Isolation of the Fungal Culture

Isolated fungal culture was identified depending on its morphological, culture, biochemical and physiological characteristics and confirmed as Penicillium sp. The results were confirmed by National Fungal Culture Collection of India (NFCCI), Pune.

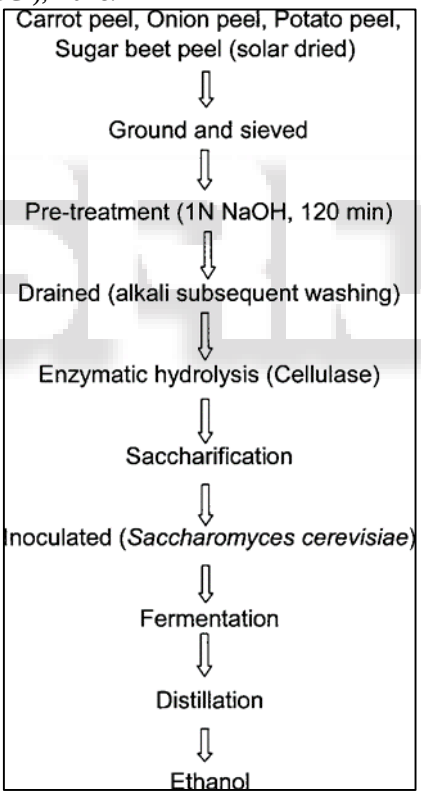

Fig. 1: Flow diagram for production of ethanol using cellulase enzyme

Penicillium are omnipresent dominant and problematic in pathogen environment. They cause major loss in export market due to fruit decay. Conidia are small, lightweight and static allowing for attachment into any surface and surrounding environment [21, 22].

B. Estimation of Reducing Sugars

The sugar released, varied from substrate to substrate, highest amount of sugars was released by onion peel through saccharification when compared to other substrates. (Fig. 2) Maximum amount of sugar released from onion peel (486.25

µg/mL) followed by carrot peel (428.31 µg/mL), potato peel (377.98 µg/mL) and sugar beet peel (315.81 µg/mL). Tewari et al.[23]; Gomathi et al.[24] reported that reducing sugar concentrations can be further increased by pre-treatment of the cellulosic wastes. Raghavendra et al.[25] studied that the wheat straw, had recorded reducing sugar (127.7 mg/g), non-reducing (49.77 mg/g) and total sugar of 177.47 mg/g. Among fungal cultures, Trichoderma reesei released maximum reducing sugar of 22.30 mg/g in paddy straw, 25.56 mg/g in wheat straw, 28.26 mg in sugarcane bagasse and total sugars of 72.22 mg/g, 76.89mg/g and 80.23 mg/g respectively. Phanerochaete chrysophobia recorded maximum reducing sugars of 14.55 mg /g in paddy straw 18.11 mg/g in wheat straw, 20.96 mg/g in sugarcane bagasse and total sugar of 60.45 mg/g, 69.03 mg and 66.61 mg/g. Hence our results are in agreement with the above data given by the researchers.

C. Protein Estimation

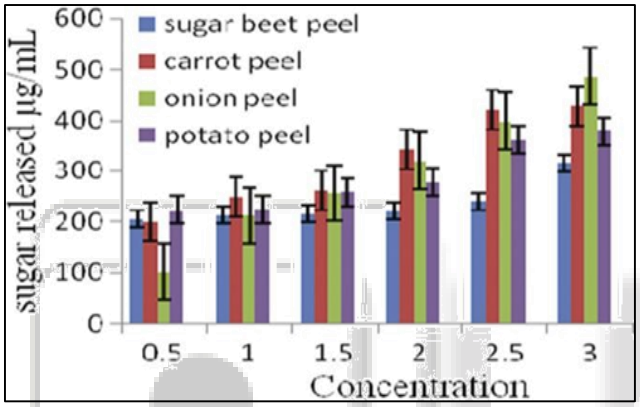

Fig. 2: Release of reducing sugars from various substrates tested

The utilization of the protein from agricultural wastes can be of immense importance for bioethanol production. Protein content of carrot peel, onion, potato peel, and sugar beet peel, varies from 10.56 µg/ml, 12.19 µg/ml, 14.52 µg/ml and 17.3µg/ml respectively without addition of microorganism. Then the protein content with the addition of Penicillium sp. was observed in carrot peel, onion, potato peel, and sugar beet peel, varied from 1858.35µg/ml, 2457.14µg/ml, 2427.6 µg/ml and 2161.38 µg/ml. Maximum protein content without the addition of microorganism was observed in potato peel which contains high starch when compared to other substrates, whereas maximum protein content with Penicillium sp was observed in onion peel 2457.14(µg/ml) which enhanced the biomass production for bioethanol production .Yabaya and Ado[26] reported the mycelial protein production by Aspergillus Niger using banana peel. Steinkraus[27] used edible substrates for the production of microbial biomass protein. Lignocellulose biomass with Aspergillus Niger was 0.195±1.10 (mg/g) with mycelial protein of about 52.5±0.26 (mg/g). The Cladosporium sp. lignocellulosic biomass was about 0.224 (mg/g) with mycelial protein of about 60.6±1.12 (mg/g) reported by Poludasu et al. (2013)[28].

D. SDS Page

The protein present in various agricultural wastes substrates showed several bands ranging from 30 to 150 kDa in (Fig. 3). The crude enzyme extract of onion peel, sugar beet peel, carrot peel and potato peel confirmed their homogeneity and enzyme was resolved on 5% stacking and 12% running gel. Protein was evident by three bands of onion peel corresponding to 50 kDa, 60kDa and 80kDa on SDS-PAGE; sugar beet peel presents 5 bands corresponding to 47kDa, 50 kDa, 60 kDa and 100kDa; carrot peel presents 3 bands corresponding to 43 kDa, 50 kDa and 100 kDa; potato peel presents 4 bands corresponding 40 kDa, 50 kDa and 60 kDa.

Arifin et al.[29] identified 29 kDa alkaline cellulase and 30-65 kDa cellulase was reported in Bacillus sp and Bacillus pumilus, whereas Giorgini [30] reported 60- 70 kDa cellulase has been obtained in 10% Native PA GE. Chen et al.[31] have reported CMCase of 94 kDa in Sino rhizobium fredii while Arffin et al.[29] found 83 and 50 kDa CMCase in Aspergillus Niger wild type strain Z10. Our findings support theoretical expectation and practical experiences that Penicillium sp produce cellulolytic and ligninolytic enzymes at the initial stage of composting process and reach their peak within a few days, making the substrate available for biodegradation

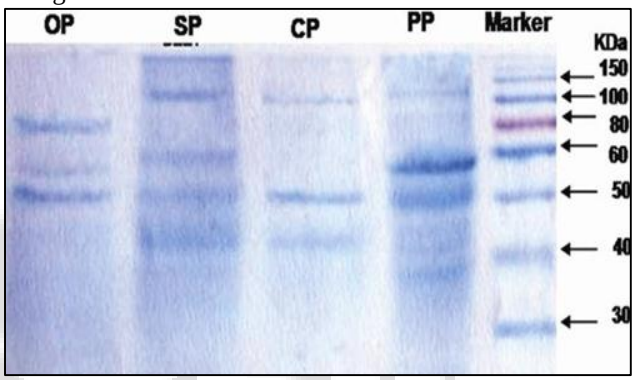

Fig. 3: Determination of molecular weight of substrates by SDS PAGE

E. Ethanol obtained by dichromate method

Various raw materials and different methods for bioethanol production have been observed and lignocellulosic materials were focused for bioethanol production. The maximum level of bioethanol varied from day to day fermentation. During the fermentation period, the ethanol yield of substrates was found to increase gradually from the 14th, 21st and 28th day (Table 1). Maximum alcohol content 3.75 % (v/v) and 5.23% were observed in potato peel, at 14 days and 21 days respectively. Bioethanol obtained from sugar beet peel was found to be 14.52 % (v/v) and confirmed by Gas Chromatography which yielded 17.3 % the Retention Time [min] was 1.54, Area [mV.s] was 6173.16, Height [mV] 263.2 and Area [%] 92.8.(Fig. 4). The alcohol content obtained was 15.34 % and 14.4 % by Gas Chromatography by

	% bioethanol by dichromate method			% of bioethanol by Gas Chromatography
Substrates	14 days	21 days	28 days	28 days
Sugar beet peel	2.66	5.21	14.52	17.3
Carrot peel	0.48	1.77	2.78	2.2

Onion peel	3.44	4.20	8.50	14.4
Potato peel	3.75	5.23	11.06	15.34

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

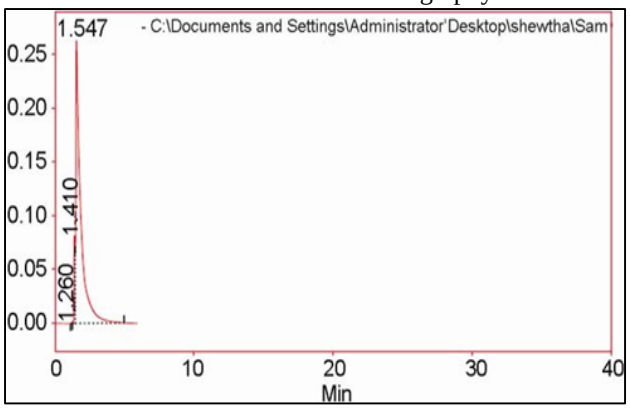

Fig. 4: Gas Chromatogram of Penicillium sp with sugar beet peel at 28 days.

using potato peel and onion peel as substrates. The maximum concentration of ethanol was achieved at 28 days of fermentation and started to level off. From the results obtained on bioethanol production potential of various lignocellulosic wastes varied, and can be concluded that sugar beet peel was a very promising raw material for bioethanol production. Sugar beet peel has high reducing sugar and total sugar content compared to other lignocellulosic materials that have been examined for bioethanol production by pretreatment of the substrates. The fermentation activity of Saccharomyces cerevisiae significantly depends on the composition of sugar in the substrate. In the present study an attempt was made to use the fungal culture Penicillium sp as source of cellulase enzyme in saccharification step which hydrolyzes complex cellulosic substrates by the release of extracellular cellulase enzyme and release simple sugars. The sugars obtained after hydrolysis of agricultural wastes by Penicillium sp to ferment activity of Saccharomyces cerevisiae was significantly dependent on the composition of the sugar. These results have demonstrated that agricultural wastes are potential source for the production of bioethanol by enzymatic activity of microorganisms. These wastes should be converted to useful products like biofuel. Srichuwong et al. [32] reported the maximum ethanol yield of 16.61% (v/v) in the bench scale SSF process under the optimal conditions in 61.5 hours. Nimbkar et al.[33] studied the effect of different inoculum size viz., 2,4,6,8 and 10% on the ethanol production from unspecialized juice of sweet sorghum and obtained maximum alcohol concentration of 12.45 and 12.23% (v/v) at 6% and 2% respectively. Chandel et al.[34] reported the cost of cellulases and fermentation sugars after enzymatic saccharification are the important factors for ethanol process. Itelima et al.[35] obtained maximum ethanol in corn cobs of 1.87% (v/v), on the first day and gradually increased to 10.08% (v/v). The qualitative and quantitative confirmation of the ethanol obtained by Potassium dichromate method and Gas Chromatographic Analysis showed complete correlation. There are currently several regionally important bioethanol feedstocks, all in need of innovations that reduce pre-treatment costs and improve processing efficiency.

VI. Conclusion

The use of alternate sources (underutilized agricultural waste carrot peel, onion peel, potato peel and sugar beet peel) for the production of ethanol has been found to be economical and effective. This process of utilizing the solid waste those are very rich in cellulose, hemicellulose and lignin, gives rise to zero waste generation techniques. The maximum alcohol obtained from sugar beet peel was 17.3% at 28th day. The fermentation extract confirms the presence of ethanol.

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