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Studies on Bioethanol Production from Spoiled Starch Rich and Cellulose Rich Vegetables by Two Stage Batch Fermentation

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Exhaustion of non-renewable energy resources and environmental impacts (pollutants) are becoming serious problems for societies. India is the second major producer of fruits and vegetables in the world. Fruits and vegetables are more prone to spoilage than cereals due to their nature and composition. So we made an attempt to produce ethanol from spoiled starch rich vegetables like wild potato (*Solanum tuberosum*), sweet potato (*Ipomoea batatas*), which possess abundant starch. Saccharification (Baterial/Enzymatic) was done in the presence of amylase producing microorganisms (*Bacillus Subtilis*, *Saccharomyces cerevisia)*. In this process starch is converted into monosaccharide i.e glucose. This glucose was subjected to alcoholic fermentation in the presence of *Saccharomyces cerevisiae*. This process of fermentation was followed by distillation at 78°C for alcohol extraction. We conducted sacharification and fermentation with single and mixed cultures. We got a yield of ethanol is about 7.5 mg/ml with an enzyme mediated saccharification followed by immobilized yeast fermentation. We also performed fermentation with whole vegetable waste (including cellulose rich vegetables) with single and mixed microbial cultures. Finally, we got 9.98 mg/ml of concentrated ethanol with immobilized mixed culture.

Key words: Spoiled starch rich vegetables, Starch saccharification, Baker's yeast, Alcoholic fermentation, Distillation, Immobilization, Ethanol.

Renewable biomasses are available for conversion to liquid fuel, ethanol[1-4]. Ethyl alcohol can be made of three main, abundant renewable feedstock sources, saccharines, starch materials and cellulosic materials[5-7]. Low value agricultural residues which can easily be converted to fermentable sugars, agricultural by-products of starch industry such as potato pulp and from raw starch hydrolysis are attractive resources for economical production of' ethanol[7,8,10,11].

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Human activity, primarily through the combustion of fossil fuels, has released hundreds of millions of tons of so-called 'green house gases'(GHGs) into the atmosphere[9]. GHGs include gases such as carbon dioxide (CO_2) and Methane (CH_4). This situation makes it more important than ever to investigate ways of extending the available petroleum and produce cleaner burning fuel, preferably from a renewable source. The effective alternative for these constraints is the utilization of biomass as renewable source. One such promising fuel source is the production of ethanol from carbohydrate (biomass) [11-13]. Biochemical conversion option can be divided into digestion (Production of biogas, mixture of mainly methane

and carbon dioxide) and fermentation (production of ethanol). Ethanol is primarily produced from two major routes: the catalytic hydration of ethylene (synthetic ethanol) and the fermentation of agricultural feed-stocks which investigate beneficial microorganisms. The maximization of amylase productivity at reduced cost is possible either by improving the strain or by the optimization of fermentation conditions. Keeping the above parameters in view, the present study was designed to screen the microbes, which are capable of digesting raw starch from soil, selection of cultures, saccharification of starch by using microbial amylases and conversion of sugars into ethanol by employing yeast[13].

MATERIAL AND METHODS

Screening of microbes for amylase production

The bacterial cultures capable of producing raw starch digestive enzymes were isolated from the local soil by serial dilution method. The soil suspension of 10^{-4} dilution was added to raw starch solution and incubated for 48 hrs for the specific growth of amylase producing bacteria. This sample was serially diluted and inoculated into Starch agar plates by pour and spread plating methods. Starch agar medium was used to facilitate the growth of amylase producing bacteria. Biochemical tests were used to identify the microorganism as *Bacillus subtillis.*

Substrate Preparation

Spoiled starch rich vegetables *Solanum Tuberosum, Beta vulgaris, Ipomoea batatas, Colacacia sp. & Amorphophallus sp.* were collected. These were left out for two more days for further spoilage at room temperature. These spoiled starch rich vegetables were cut into pieces and 500 grams was weighed, in combination of all. This substrate was subjected to different processing methods i.e boiling, mashing, autoclaving .Then bacterial mediated and enzyme mediated sacharification was done. The process of converting glucose to ethanol by employing yeast is referred to as alcohol fermentation.

Estimation of glucose concentration by DNS method

For the estimation of glucose (mg) Dinitrosalicylic acid method (DNS method) of Dubious *et al.* (1961) was employed during this study.

Estimation of ethanol by potassium dichromate method

Alcohol standard was prepared by dissolving absolute ethanol in water to get 10 mg/ml concentration.$K_2Cr_2O_7$ solution was prepared by dissolving 10 gm of $K_2Cr_2O_7$ in distilled water in a 100 ml standard flask and make up the volume to mark.

Standard graph for ethanol from following procedrue

Standard solution of 10 mg/ml prepared then different concentrations of Ethanol of 1-10 mg/tube (0.1-1 ml) and make up the volume to 5ml with distilled water. Then 1 ml of Potassium dichromate reagent was added. All the test tubes were kept in ice water and 4 ml of conc. Sulfuric acid added to each tube gently through the walls. Then the optical density (OD) was measured at 660 nm. Standard graph plotted with those values. Similarly OD values for unknown samples were measured. Then concentrations of unknown samples, corresponding to the obtained OD values were taken.

RESULTS AND DISCUSSION

Bacteria mediated saccharification

In this process, the bacterial inoculum was added to the previously processed substrate with the initial glucose concentration as 1 mg/ml and incubated for 72 hours. Meanwhile glucose concentrations were taken at regular intervals. After 42After 72 hours, the final glucose concentration was 28.5 mg/ml (200 grams in the broth). Results were shown in Fig. 1. Glucose utilization in ethanol fermentation shown in Fig.2.

Enzyme mediated saccharification

This process showed a dominant role through out the incubation time over the bacteria mediated saccharification in terms of glucose produced per time. After addition of enzyme to the substrate, with initial glucose concentration of 1 mg/ml, the broth was incubated for 72 hours. Glucose concentrations were taken time to time and the final concentration was found to be 35.7 mg/ml (250 grams in total broth). Results were shown in Fig. 1. Glucose utilization in ethanol fermentation shown in Fig. 2.

Yeast fermentation of bacteria saccharified substrate

The process of alcohol fermentation was carried by employing *Saccharomyces cerevisiae for proper* saccherification. After the fermentation period of 42 hours, the concentration of ethanol in the broth was found to be 6.4 mg/ml. Results were shown in Fig. 3.

Yeast fermentation of enzyme saccharified substrate

Enzyme saccharified substrate was subjected to the process of alcohol fermentation by employing *Saccharomyces cerevisiae*. Ethanol concentration obtained from this process was found to be 6.6 mg/ml after a fermentation period o f 42 hrs at 30C. Results were shown in Fig. 3.

Immobilized yeast fermentation of bacteria saccharified substrate

Immobilized yeast cells were added to bacteria saccharified substrate and maintained the temperature at 30°C for overall period of 42 hrs and no further increase in ethanol concentration was observed. Final ethanol concentration was found to be 7.2 mg/ml. Results were shown in Fig. 3.

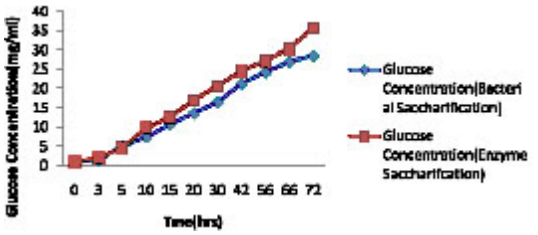

Fig. 1. Represents concentration of glucose in saccharification process

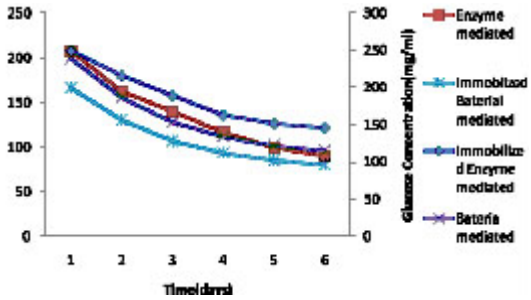

Fig. 2. Represents concentration of glucose profile in ethanol production

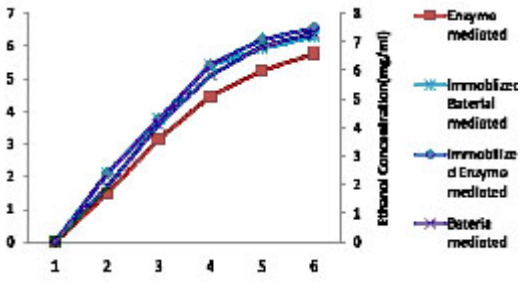

Fig. 3. Represents concentration of ethanol produced in various processes

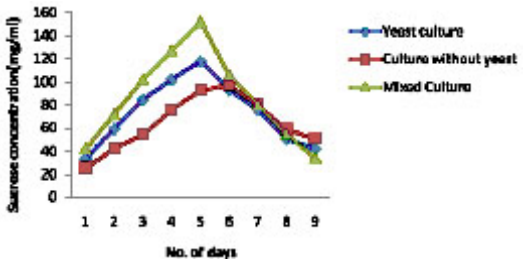

Fig. 4. Represents saccharification profile with pure and mixed cultures.(for all vegetable waste)

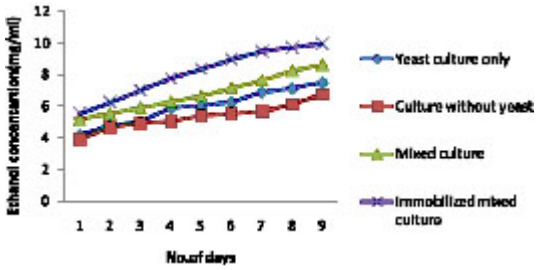

Fig. 5. Represents Ethanol production profile with different cultures (for all vegetable waste)

Immobilized yeast fermentation of enzyme saccharified substrate

Immobilized yeast cells were added to enzyme saccharified starch and maintained at temperature 30°C for overall period of 42 hrs and no further increase in ethanol concentration was observed. Final ethanol concentration was found to be 7.5 mg/ml. Results were shown in Fig. 3.

Ethanol production from all vegetable waste by using mixed microbial culture

In this process ethanol production is carried out by employing a wide variety of substrates like corn starch, potato starch, wheat, cassava, malt, cellulose and hemi cellulose etc. Studies on Simultaneous Saccharification and fermentation of vegetable waste, a major environmental solid effluent as the substrate using starch digesting glucoamylase enzyme derived from *Aspergillus niger,* starch digesting amylase enzyme derived from *bacillus subtillis* and non starch digesting and sugar fermenting *Saccharomyces cerevisiae* in a batch fermentation. This method comprehends four different protocols for the production of ethanol. Two different methods were employed in each step of saccharification and fermentation. In the saccharification step, when mixed cultures (*Bacillus subtils, Aspergillus niger, sacvharomyces cervassia*) were used more amount of glucose is produced than the single cultures. Results were shown in the Fig.4. The ethanol production was even more in the immobilized mixed cultures than in the normal mixed culture. When the alcohol fermentation was carried out with mixed culture was found to be 9.98 mg/ml. So the ethanol produced by immobilized mixed cultures is more than the normal mixed cultures. Results were shown in Fig. 5.

DISCUSSION

In the saccharification procedure, when amylase producing bacteria was employed, 200 grams of glucose was obtained. As the crude enzyme was added to the substrate, it was observed that rate of saccharification was more when compared to the bacterial mediated one. The final glucose concentration by this process was found to be 250 grams within the same incubation time. In the alcohol fermentation step, when yeast *(Saccharomyces cerevisiae)* was employed directly the ethanol production was less. As immobilized yeast was employed, it was observed that ethanol concentration was high when compared to the direct yeast fermentation. The yields of ethanol in the four types of protocols were measured. When the alcohol fermentation was carried with direct yeast on bacteria mediated and enzyme mediated saccharified substrate, it was found to be 6.4 mg/ml and 6.6 mg/ml concentrations respectively. Where as in the immobilized yeast fermentation of bacteria saccharified and enzyme saccharified substrate yielded 7.2 mg/ml and 7.5 mg/ml of ethanol concentration respectively. Thus, employing enzyme for the process of saccharification was found to be quite efficient over employing bacteria as saccharifying agent. In the same way Immobilized mixed culture has shown more sustainability towards the ethanol concentration than the direct yeast and increased ethanol productivity (in terms of concentration) by 2.5%. The comparison of the above methodologies was shown in Fig. 5. In the summary, for the production of ethanol from spoiled starch rich vegetables, Enzyme mediated saccharification' and 'Fermentation by Immobilized yeast' are the efficient methods to be followed and their combination will provide 9.98 mg/ml concentrated ethanol.

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