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RESEARCH ARTICLE

Alcohol production from tapioca waste of the sago industry

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Abstract

An investigation was carried out at Tamil Nadu Agricultural University to utilize tapioca waste from the sago industry, a major waste left unutilized, which may be effectively used for bio ethanol production. Sago industry waste is abundantly available in Tamil Nadu, India, especially in the southern belt comprising of Salem, Erode and Bhavani. In the present study, amylase and the chemical agents HCl and H_2SO_4 were used separately to determine the most effective saccharifying agent. In comparison with different concentrations (0.1 to 0.8 N) of HCl and H_2SO_4 treatments, the amylase enzyme at a concentration of 67.5 Ug^{-1} of 0.6 mL for 60 hr at a temperature of 45 °C served as effective saccharifying agent. It effectively recovered reducing sugar level of 80.2 % from sago industry wastes. The saccharified substrate was subjected to fermentation with an effective yeast strain *Saccharomyces cerevisiae* YS10 isolated from sugarcane molasses. In the cell free extracts, alcohol recovery of 16.8 % was noticed in sago industry wastes after 15 days of fermentation. Thus, proving its potentiality in the current scenario of recycling the sago industry wastes into bioethanol as a gift to biofuel ultimately to mankind by which the current alarming problem of price rise and demand in the fuel sector can be minimized.

Keywords

ethanol; saccharification; saccharifying agent; sago industry; tapioca waste

Introduction

Ethanol, a versatile fuel reduces global dependence on fossil fuels by providing a renewable alternative, enhancing energy security. It lowers greenhouse gas emissions by up to 50 %, supporting international climate goals. Additionally, ethanol boosts agricultural economies by increasing demand for crops like corn and sugarcane. Ethanol and biodiesel, the predominant biofuels, can substitute for gasoline and diesel or be blended with them to reduce greenhouse gas emissions. Biofuels can help communities to improve air and water quality by reducing pollutants, generate revenue for the agriculture sector and support the rural economy (1). Ethanol and methanol can be green fuels if they are derived from sustainable energy crops. Despite their lower energy densities, they hold

significant potential in reduced pollution rate globally. There are many opportunities for producing fuel from agricultural crops and crop residues. Ethanol can be produced from any grain, tuber crops, fruit or juice crop containing fermentable carbohydrates. Ethanol can be produced from three main types of biomass sources: (a) sugar-bearing materials (such as sugarcane, molasses and sweet sorghum) which contain carbohydrates in sugar form; (b) starches (such as corn, cassava and potatoes) that contain carbohydrates in starch form; and (c) celluloses (such as wood and agricultural residues) whose carbohydrate form is more complex (2).

Cassava waste is a viable alternative feedstock for ethanol production (3). Cassava yields a higher ethanol output per ton (150 liters per ton of fresh root) compared to sugarcane (48 liters per ton) (4). Cassava starch, which has lower swelling and gelatinization temperature can be easily saccharified to simple sugars by amylase enzyme. The main advantage of cassava over any other crop in ethanol production is the presence of high quantity of fermentable sugars after saccharification. The fresh roots contain about 30 % starch and 5 % sugars and the dried roots contain about 80 % fermentable substances which are equivalent to rice as a source for alcohol production.

Different techniques are available for the conversion of starch and cellulosic materials into fermentable sugars. Some methods rely on acid hydrolysis, using either dilute or concentrated sulfuric or hydrochloric acid. Other techniques involve pre-treating raw materials with alkali, steam explosion, or enzymatic hydrolysis. Though production of ethanol from starch is not new in fermentation technology, few attempts have been made in the past to standardize the technologies for the recovery of ethanol from above mentioned agricultural wastes in India. Modern biotechnological tools like immobilization of enzymes and live yeast cells were also employed to reduce the cost of production and make the process more economically viable.

Materials and Methods

Isolation of fermentative yeasts

One gram of the sample from each source *viz.,* Molasses, Fruit wastes, Tapioca wastes and soil was inoculated into 5 ml sterile Yeast extract Malt extract (YM) broth in test tubes. To prevent the contaminants and to establish the fermentative ability, surface of the broth in the tubes was sealed with 1 cm thick sterile paraffin layer. The tubes were incubated at 28 ± 1 ° C for 48 hr (5). After incubation, yeast cultures were isolated from YM broth by dilution plate technique. Eleven isolates were isolated from different sources and the selected colonies were grown in Yeast Extract Peptone Dextrose (YEPD) agar slants and stored at 4 °C for further study. The type of strain *Saccharomyces cerevisiae* (strain MTCC 189) was obtained from the microbial type culture collection, IMTECH, Chandigarh and used as a reference.

Morphological and physiological characterization of selected yeast isolates

Eleven yeast isolates were identified based on morphological characters (6, 7) and screened for alcohol production and fermentation rate that is assessing the quantity of alcohol produced after 15 days of fermentation under different treatments with acids and enzymes.

Cell morphology

Twenty-four-hour old yeast cultures were inoculated at 1 % level in 100 mL of sterile glucose yeast extract peptone broth in 250 Ml conical flask and incubated at 28±1 °C for 48 hr and examined for (cell) shape and budding pattern under microscope after staining with lactophenol cotton blue.

Ascospore formation

Ascospore formation was studied using potato dextrose agar medium (8). Ascospore formation was verified by spore staining (6).

For physiological characterization, selected yeast isolates were compared with standard *Saccharomyces cerevisiae* strain (MTCC 189) were screened for their growth in different carbon sources, sugar concentration, alcohol, temperature and pH as per the standard protocols (9).

Growth at different concentrations of glucose

Growth of yeast isolates at different levels of glucose concentration was assessed. Different glucose concentration (10, 15, 20, 25 and 30 %) were added to YM broth and sterilized. The flasks were then cooled to room temperature and 1 mL of 24 hr old yeast culture (approximately 10^5 cells/mL) inoculated aseptically. These flasks were then incubated at 28 ± 1 °C for 7 days. After 7 days, the population was estimated by serial dilution and plating (10).

Growth at different concentrations of alcohol

Growth of yeast cultures at different concentrations of alcohol was tested in comparison with the standard strain *S. cerevisiae* MTCC 189 using serial dilution and plating count method. One mL of 24 hr old culture (approximately 10^5 cells/ml) was inoculated in 100 mL YM broth having 4 ^{0}BRIX was adjusted to 20^0 BRIX with glucose to have uniform growth and subjected to different concentrations of alcohol (0, 8, 10, 12, 14, 16 and 18 % v/v) by addition of ethanol to YM broth and incubated at 28 ± 1- °C for 7 days. After one week, the population was estimated by serial dilution and plating (10).

Growth at different temperature

Yeast growth at different temperature level was tested in comparison with the standard *S. cerevisiae* MTCC 189. One mL of 24 hr old yeast culture was inoculated aseptically into 100 mL YM broth of 4 ^{0}BRIX was adjusted to 20 ^{0} BRIX with glucose and incubated at 10, 15, 20, 25, 30, 35 and 40 °C for 7 days period in BOD incubator. After one week the population was counted after serial dilution and plating as described earlier.

Testing the tolerance to different pH levels

Tolerance to different pH levels was assessed relative to *S. cerevisiae* MTCC 189. In 100 ml YM broth adjusted to 20 ^{0} BRIX with glucose. The pH was adjusted to 2, 2.5, 3, 3.5, 4, 4.5, 5 and 5.5 using 5 % citric acid and 0.1 N sodium hydroxide before sterilization. One mL of 24 hr old yeast culture (10^{-5}) was inoculated in the pH adjusted, sterilized YM medium and incubated for 7 days. After incubation, the population was estimated by serial dilution and plating.

Physio-chemical characterization of the substrate

The physio-chemical characters *viz.,* pH, oxidation-reduction potential (O/R), total soluble sugars (TSS) and acidity of the tapioca waste were analysed. Ten grams of the sample were ground using pestle and mortar with water to form paste, which was then transferred to 100 mL beaker. 50 mL sterile distilled water was added to it and the oxidation-reduction potential and pH were recorded using cyber scan pH meter 510 and TSS using brix meter. The protein content in the substrate was estimated as per the previously mentioned protocol (11). The ash content was determined based on the gravimetric loss by heating the substrate to 150 °C for a period of 24 hr (12). The total fibre content of the substrate was estimated according to the method proposed previously (13). The volatile acidity of substrate was estimated as described elsewhere (14).

Substrate preparation for alcoholic fermentation

Saccharification of tapioca waste: Saccharification of the waste materials was done as per the procedure described earlier (15). Tapioca wastes collected from the sago industry wastes were weighed to 25 g in a 250 mL Erlenmeyer flask and substrate were saccharified with acids and amylase enzymes either alone or in various combinations and allowed for fermentation with yeast *Saccharomyces cerevisiae* YS10 as free cells. The following are the details of the treatment used for the saccharification; it includes:T1- HCl at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 N); T2- H_2SO_4 at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 N); T3- Alpha amylase enzyme (67.5 Ug^{-1}) (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mL); T4- HCl (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 N) + Alpha amylase enzymes (67.5 U g^{-1}) 0.6ml T5- H_2SO_4 (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 N)+ Alpha amylase (67.5 U g^{-1}) 0.6 mL .

Estimation of reducing sugars in substrate: The reducing sugar concentration in the samples during saccharification of the substrate was estimated as per method described earlier (16).

Ethanol production using yeast broth culture

Yeast isolates for alcohol production*:* The yeast isolate *Saccharomyces cerevisiae* YS10, obtained from molasses, was selected based on its ability to grow in varying pH levels, temperatures, alcohol and glucose concentrations in YM medium. It was subsequently used for the fermentation process.

Fermentation using yeast broth culture : Fermentation using the yeast broth culture was carried out as per an earlier adopted protocol (17). After saccharification, the substrates were allowed for fermentation in 250 mL Erlenmeyer flasks. To 100 mL of the saccharified substrate, 3 mL of a 48-hour-old culture (10^6 cells/ml) of *Saccharomyces cerevisiae* (YS10) was inoculated. The mixture was kept under aerobic conditions at 28 ± 1 °C for two days to allow cell multiplication. After this period, anaerobic conditions were established by sealing the flask with a cork and a water-sealed special valve. Fermentation was then carried out for 16-18 days at 28 ± 1 °C.

During fermentation, the samples were withdrawn at a regular interval of 0, 5, 10, 15 and 20 days and the yeast population was counted by serial dilution method. The substrate which was saccharified with different time periods and kept for different period of fermentation *viz*., 5, 10, 15 and 20 days in replications were taken and filtered through muslin clothes, the extracts (100 mL) were distilled at 79 °C and the alcohol content was estimated by alcohol meter and expressed in % (v/v) of substrate.

Results

Morphological and physiological characterization of yeast isolates

The yeasts isolated from different sources were identified based on their colony morphology, vegetative cell structure, budding position and ascospore production, along with their ability to utilize different sugars. Most of the isolated yeast colonies were butyrous, raised, smooth and glossy. A few isolates were cream coloured, while others were brown. Yeasts colonies isolated from soil (YS1) were brownish and raised with light striations. Yeast colonies isolated from banana cultivar-Poovan (YS2) were creamy, raised and glossy. Yeast cultures isolated from banana cultivar - Nendhran (YS3), banana cultivar - Karpooravalli (YS4), banana cultivar- green banana (YS5), mixed fruits (YS9), sweet sorghum (YS11) were butyrous and creamy colonies. Tapioca cultivar - Mulluvadi (YS6), tapioca cultivar - Burma (YS7), grapes (YS8), molasses (YS10) and standard strain *Saccharomyces cerevisiae* (MTCC189) from IMTECH, Chandigarh were butyrous, brownish and raised with light striations (Plate 1a and 1b) all the isolates produced ascospores when examined after spore staining, they appeared as bluish green. All the isolates were able to utilize various sugars like glucose, sucrose, maltose but none of them utilized lactose resembling the standard strain *Saccharomyces cerevisiae* (MTCC189) among the 10 isolates the isolate YS10 exhibit maximum population by proving its capability of growth in glucose concentration at 20 %, alcohol tolerance at 18 %, temperature tolerance up to 45 °C and a pH tolerance of 5.

The physico chemical properties of tapioca wastes used for fermentation were analysed and results are presented in Table 1. The results indicate the potential platform of the substrate for alcohol production by yeast fermentation.

Effect of different concentrations of HCl, H_2SO_4 and Alpha-amylase enzymes

Among the different saccharifying agent used, amylase enzyme at a concentration of (67.5 Ug^{-1}) of 0.6 mL for 60-hr saccharification at a temperature of 45 °C performed better in recovering reducing sugar level of 80.2 % (Table 2) from sago industry wastes.

Table 1. Physico-chemical properties of tapioca wastes used for alcohol production (dry weight basis)

Substrates	pH	O/R potential (Me v)	Volatile acids (gl^{-1} acetic acid)	TSS ((BRIX))	Starch content %	Reducing sugar %	Protein %	Ash %	Cellulose content %	Total fibre %	Moisture content %
Tapioca wastes	5.2	107.7	0.62	14	34.5	10.5	2.7	0.21	12.6	2.4	28.52

Table 2. Effect of different concentrations of amylase enzyme (67.5 U g^{-1}) on saccharification of tapioca wastes at 45 °C

Substrates	Reducing sugar (%) Quantity of enzymes (ml) at different time periods 60h							
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Tapioca wastes	74.4	76.5	78.6	79.3	80.2	82.4	78.4	76.9

Effect of amylase enzyme on alcohol yield

Alcohol production from tapioca wastes saccharified with amylase enzyme (67.5 Ug^{-1}) at a concentration of 0.6mL and at a temperature of 45 °C was found higher in the tapioca wastes of about 16.8 % is given in Table 3, Fig. 1 with a fermentation period of 15 days.

Discussion

Isolation and selection of yeast isolates

Alcohol fermentation and recovery depend not only on the substrate but also on the efficiency of the yeast strain in converting reducing sugars to alcohol. The best performing yeast isolate *Saccharomyces cerevisiae* obtained from molasses (YS10) was selected for further studies. In an earlier observation *Saccharomyces cerevisiae* isolated from molasses showed higher ethanol production rate at different dilutions of sugar-cane molasses than the other strains of *S. cerevisiae* (18). Previously, the sugar concentration of 25 ^{0} BRIX for the fermentation of yeast cells showed 71 % viability with the ethanol content of 9.5 % (w/ v) (19). Further addition of sugar incrementally at 15 °C anaerobically, accounted for a lower rate of metabolic activity and at higher concentrations of sugar, substrate inhibition may affect the rate of glycolysis restricting the energy supply for the survival of yeast cells. This deficiency with respect to energy may ultimately cause death of the cell. The present results revealed the high glucose tolerance of the *Saccharomyces cerevisiae* isolate YS10 and YS8 and may be attributed to the high concentration of the sugar in substrates (molasses and grape) from which they were isolated which is in accordance with characters of *Saccharomyces cerevisiae* isolate YS10. The isolate YS10 (98 cfu x 10^{5} mL^{-1}) recorded higher population than other isolates at pH 5. With increase in pH, population increased with all the isolates and maximum was recorded at pH 5 indicating the pH optima for growth. A previous study documented that *S. cerevisiae* at pH 4.5, 5, 5.5, 6, 7 and 8 and found that the optimal pH for ethanol production was around 5.5 and also, they concluded that the production of xylitol, arabitol, glycerol and acetic acid increased with increasing pH, which lowered the ethanol yield (20).

Enzyme saccharification of agricultural wastes

Agricultural wastes were treated with different concentration of α -amylase enzymes at 45 °C over different time exposures. Maximum amount of reducing sugar was recovered from cassava waste and enzymes treatment after 60 hr incubation. More concentration of enzymes is needed to break the granular molecules of starch to linear ones and the maximum recovery was with 0.6 mL of enzymes of 67.5 Ug^{-1}. Previously, the degree of hydrolysis was shown to be strongly dependent on the concentration of enzyme, hydrolysis time and incubation temperature (21). After fermentation the available starch began to gelatinize, where upon it was attacked by the alpha enzyme or alpha-amylase and reduced to a simpler carbohydrate which is in line with our results obtained by amylase enzymes saccharification tapioca that have more content of starch (22).

Table 3. Effect of amylase enzyme (67.5U g^{-1}) at a concentration of 0.6 ml on the alcohol yield of tapioca wastes inoculated with yeast isolate YS10 at different time period of fermentation

Substrates	Alcohol yield in percentage (v/v) Sachharification period (60 hr)			
	5th day	10th day	15th day	20th day
Tapioca wastes	15.4	16.0	16.8	16.4

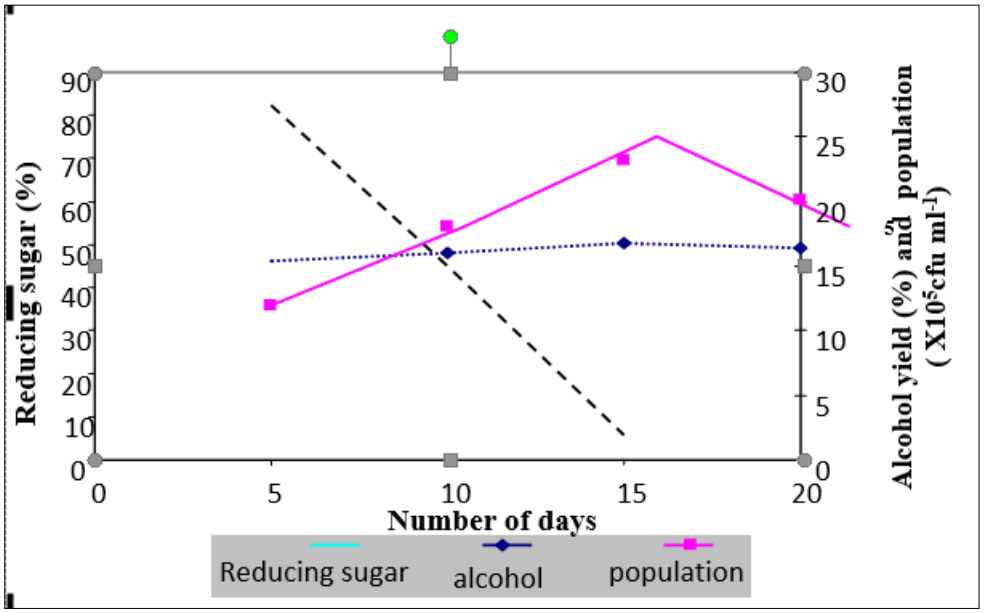

Fig. 1. Changes in alcohol content, reducing sugar and yeast population of 60h enzyme saccharified tapioca waste after yeast (YS10) fermentation.

An earlier study preferred enzymatic methods over chemical saccharification due to their lower energy requirements and elimination of neutralization steps. Moreover, undesirable products like branched oligosaccharides, panose, isopanose and isomaltose, which form during the starch-conversion process, were prevented by alpha amylase. This enzyme is highly thermostable, functions in an acidic to neutral pH range and is independent of cations such as Ca^{2+} for stability and activity (23). The alpha amylase converts starch into 60 % maltose, which is utilized by yeast. The enzyme breaks down complex molecules into simpler glucose molecules, which can be directly used by yeast. When increasing the time of saccharification from 48 to 60 hr the reducing sugar content increased, this clearly indicates the saccharification with enzymes also depends on the time interval and temperature (24). These earlier findings clearly reflect the importance of time period, reducing sugar content in the substrates and the temperature for saccharification. The maximum reducing sugar recovery from tapioca (82.4 %) was possible when saccharifed with increased enzyme concentration 67.5 Ug^{-1} (0.6 mL) with an incubation period of 60 hr at 45 °C.

Alcohol recovery from the agricultural wastes

The alcohol after fermentation was distilled and collected. The alcohol recovery was more in the substrate saccharified with amylase enzyme at a concentration of 67.5 Ug^{-1} (0.6 mL) tapioca gave more alcohol after saccharification and fermentation. The alcohol recovery is more in tapioca wastes which may be due to the higher percent of reducing sugars, whereas for acid treated substrates it was less and this may be attributed to the formation of compounds capable of inhibiting alcohol production by the yeast (25).

Previous studies reported that alcohol recovery in tuber crop fermentation is highly dependent on the reducing sugar content and yeast biomass (26). The higher yield of alcohol depends mainly on the substrate reducing sugar content and cell biomass present (27). In another study on simultaneous saccharification and fermentation (SSF) recorded a maximum alcohol yield at 37 °C in free cells when compared to immobilized inoculum. The reduction in the yield of the alcohol may be due to the presence of the inhibitory compounds such as furfural and phenolic compounds and due to the low population and poor dispersion of the immobilized cells in the media (28). Normally tapioca wastes fetch lower price and the use of tapioca wastes for production of alcohol will help poor farmer because they can get good price for their products when converted into biofuel and the present study indicates the profitable use tapioca for ethanol production.

Conclusion

The findings of this study highlight the potential of tapioca waste from the sago industry as an effective substrate for bioethanol production. The use of amylase enzyme as a saccharifying agent proved to be the most effective in recovering reducing sugars, with an efficiency of 80.2 %, outperforming the chemical treatments with HCl and H_2SO_4. Furthermore, fermentation using *Saccharomyces cerevisiae* YS10 resulted in a notable alcohol recovery of 16.8 % after 15 days of fermentation. The study demonstrates that tapioca waste, a currently underutilized by-product, can be transformed into a valuable resource for bioethanol production. This process not only offers a sustainable method of waste management but also contributes to the renewable energy sector, offering a cost-effective and eco-friendly alternative to fossil fuels. The successful conversion of tapioca waste into bioethanol could help mitigate the rising demand and price volatility of conventional fuels, thus providing economic and environmental benefits.

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Authors' contributions

JE conceived and designed the study, supervised the research. RJ, DR, KC contributed to conducting the experiments and KGS and MG performed the data analysis. RR, JNP contributed to manuscript editing. All authors contributed to the final version of the manuscript and approved its submission.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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