

REGULAR ARTICLE

POTENTIAL IN BIOETHANOL PRODUCTION FROM VARIOUS AGRO WASTES FERMENTING BY MICROORGANISMS USING CARROT PEEL, ONION PEEL, POTATO PEEL AND SUGAR BEET PEEL AS SUBSTRATES

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ABSTRACT

Large amount of agro wastes are produced in Rwanda each year. The global annual potential bioethanol production from the major vegetables wastes such as carrot peel, onion peel, potato peel and sugar beet peel was estimated. Those wastes processing were successfully used as raw materials for the production of bioethanol, employing by cellulase produced from various filamentous fungi including *Cladosporium cladosporioides* was used for hydrolysis and the fermentation of the hydrolyzed samples was done using *Sacharomyces cerevisiae*. The fermented product was purified by primary distillation process at 79 °C and the fraction was collected. The ethanol is then determined by specific dichromate method and Gas Chromatography. Instantaneous saccharification and fermentation process yielded maximum ethanol in the substrate of carrot peel was 16.9 % at 21st day and further confirmed by Gas chromatography and the yield of ethanol obtained was 15.8 %.

Keywords: Agro wastes, Bioethanol, *Cladosporium cladosporioides*, *Saccharomyces cerevisiae*, Gas Chromatography

INTRODUCTION

During oil crisis in 1970s a rising crude oil price, political instability and unstable oil market in countries producing oil and climatic changes, biomass has high potential to replace the supply of energy (Nagashima *et al.*, 1984). The amount of wastes produced by society each day is increasing in line with increasing populations worldwide and Rwanda is no exception. Agro wastes are an important part of total solid wastes produced nationally; they have potential to become an environmental pollution or more logically, to be utilized for the production of energy and other products.

Production of bioethanol from lignocelluloses materials such as agro wastes can substitute fossil oil production. Today, raw materials producing bioethanol by fermentation are classified as sugars, starches and cellulosic materials because fermentation is cheaper and easily than other fermentation. The potential of bioethanol production from agro wastes of four crops which are carrot, onion, potato and sugar beet have been investigated. Currently, agro wastes are burnt by the rural farmers as cookers in households. Production of bioethanol from agro wastes have been attempted with enzymes from different sources for hydrolysis of biomasses and with different organisms for fermentation (Ohgren *et al.*, 2006; Eken-Saracoglu and Arslan 2000).

The demand for bioethanol is expected to increase dramatically until 2020 where there is an increase in the world population with expected 9 billion in the year 2050 increasing the need for food and energy. *S. cerevisiae*, also known as brewer's yeast, is the most commonly used fermentation microbe because of the baking and beer brewing industries (Michilka, 2007; Roehr, 2001). Many of the sugar crops that would be suitable for industrial fermentation include sugar cane, sugar beet, fruits, sweet potato, sweet sorghum, Jerusalem artichokes and agro wastes (Atiyeh and Duvnjak, 2002; Pramanik, 2003).

The objective of this study was producing bioethanol from carrot peel, onion peel, potato peel and sugar beet peel for submerged fermentation and management system to maximize economic benefits at the same time protection of the environment.

MATERIALS AND METHODS

Raw materials

Carrot peels, onion peels, potato peels and sugar beet peels (Fig. 1), were collected from the local restaurant in volcanic region at early morning. They were clean to make free from sand, stone and dust by washing it twice in water. They were sun dried then each raw material was grinded and sieved into a 1mm. Those agro wastes are favorable for bioethanol production due to their availability and cheapest throughout the year.



Figure 1 Raw materials: agro wastes

Microorganisms producing ethanol

Several bacteria, yeasts, and fungi have been used for bioethanol production. *S. cerevisiae*, is the most yeast, which can produce ethanol of the fermentation broth. The yeast *S. cerevisiae* can produce bioethanol up to 18% of the fermentation broth (Pretorius, 2000).

Sources of microorganism

The isolated fungi were done from the rhizosphere of strawberry fields of College of Agriculture, Animal Science and Veterinary Medicine (Busogo) identified by serial dilution and wet mount technique (Aneja, 2005).

Culture medium chemical

The fermentation used was 0.2% yeast extract, 0.2% (NH₄) NO₃, 0.1% MgSO₄·7H₂O, 0.2% KH₂PO₄ (El-Gendy *et al.*, 2013) and 5 g powdered of each substrate has been added.

Enzyme molecular weights

Poly-Acrylamide Gel Electrophoresis (PAGE) of the partial purified cellulase enzyme was performed according to (Lemml, 1970). After electrophoresis, the gel was immersed in fixing solution. Staining of the band was done with Coomassie brilliant blue, R-250 (CBB) for 2 h and later de-stained. The molecular weight of the cellulase was estimated using standard protein molecular weight marker consisting of Bovine Serum Albumin.

Protein estimation

The protein from partially purified samples of carrot peel, onion peel, potato peel and sugar beet peel were estimated (Bradford, 1976) method. Optical density of the reaction mixture was observed at 660 nm against a blank prepared with 0.1 mL buffer.

Fermentation

Culture filtrate was further inoculated with *S. cerevisiae* and allowed for fermentation for 14th, 21st and 28th days. After fermentation, it was filtered and ethanol content was determined (Caputi et al., 1968). As part of this study, we have reported a process for producing ethanol from agro wastes pre-hydrolysed by alkali followed by saccharification carried by co-cultivation of *C. cladosporioides* and fermentation of the released sugars to ethanol, using *S. cerevisiae* for ethanol production

Distillation process

Distillation was carried in rotary vacuum flask at 80 °C (boiling point of ethanol) and fraction is collected as shown on Fig 2.

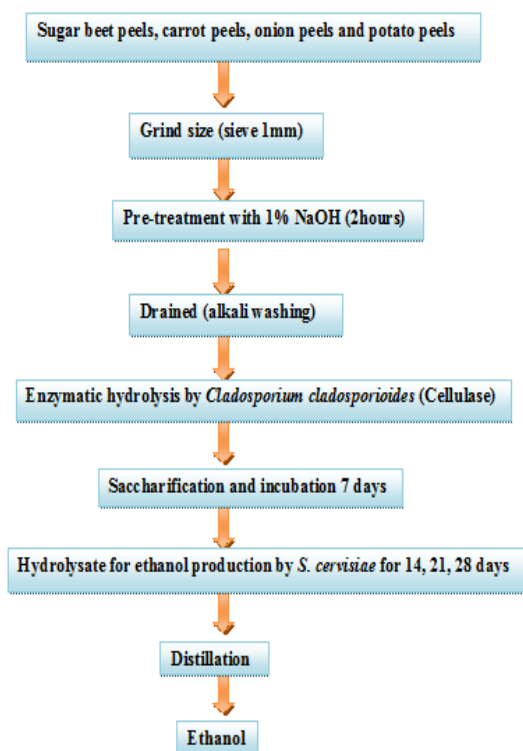


Figure 2 Bioethanol production from onion peel, sugar beet peel, carrot peel and potato peel

Bioethanol estimation by potassium dichromate method

Standard ethanol was prepared from concentrations of 2% to 10% with blank. 2.5 ml of freshly prepared potassium dichromate solution (1g of potassium dichromate in 100 ml of pre-chilled H_2SO_4) was mixed with 15ml of distillates and standards (2%, 4%, 6%, 8% and 10%) taken in separate test tubes and were incubated at 60 °C for 30 minutes (for color appearance) (Caputi et al., 1968). Tubes were allowed to cool to room temperature and absorbance was estimated at 600 nm (William and Reese, 1950).

Determination of quantity of ethanol produced

The distillate collected was measured using a measuring cylinder and expressed as quantity of ethanol produced in g/l by multiplying the volume of the distillate by the density of ethanol (0.8033g/cm³).

Statistical Analysis

MS Excel version 2007 was employed for all statistical analysis. Data was recorded in triplicates and represented as a mean value.

RESULTS AND DISCUSSION

Currently bioethanol is produced from alcoholic fermentation of molasses or simple sugar, which are produced from crops generating starch or sugar. While technologies to produce ethanol from simple carbohydrates are well established, the technologies to produce bioethanol from agro wastes are still under development. It is possible that agro waste products may be economically converted to bioethanol. We used agro wastes peel as a source of lignocellulosic substrate for ethanol production (Figure1 and 2).

Enzyme molecular weights

The protein present in various agro wastes substrates showed several bands ranged from 30 to 130 kDa. The crude protein extract of carrot peel which contains maximum yield concentration of bioethanol confirmed its homogeneity and protein was resolved on 5% stacking and 12% running gel. The molecular weight of the protein bands were 30 kDa and 130 kDa for carrot peel (Figure 3).

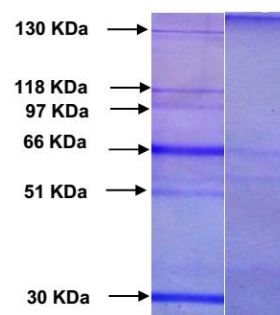


Figure 3 Molecular weight and cellulase activity from *Cladosporium cladosporioides*

Our results are close to the findings of Bai et al. (2013). They reported that the molecular weight of cellulase produced by different fungal species may vary from 12 kDa to 126 kDa. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used method for judging the apparent molecular weight of enzymes (Ramani et al., 2012). Cellulase produced by *Trichoderma viride* was purified to homogeneity using DEAE-sepharose column and the molecular weight was estimated at 87 kDa by SDS-PAGE (Yasmin et al., 2013). *Penicillium pinophilum* MS 20 produced a monomeric cellulase with molecular weight of 42 kDa, which appeared as a single band on SDS-PAGE gel (Laxman et al., 2012). The cellulase produced by *Aspergillus niger* revealed a molecular weight of 60 kDa on SDS-PAGE gel (Barlado et al., 2014).

Protein estimation

The protein content with *C. cladosporioides* was observed in carrot peel 643.48 µg/ml, onion peel 1336.5 µg/ml, potato peel 1318.76 µg/ml and sugar beet peel 1101.12 µg/ml (Table 1). Ado and Yabaya (2008) reported the mycelial protein production by *Aspergillus niger* using banana peel. The protein content obtained by *Cladosporium* sp. with lignocellulosic biomass was about 0.224 mg/g and mycelial protein about 60.6±1.12 mg/g, reported by Mohan et al. (2013).

Table 1 Substrates protein content (µg/ml) with *Cladosporium cladosporioides*

Carrot peel protein content (µg/ml)	Onion peel protein content (µg/ml)	Potato peel protein content µg/ml	Sugar beet peel protein content µg/ml
643.48 µg/ml	1336.5 µg/ml	1318.76 µg/ml	1101.12 µg/ml

Mango peels, ranged from 1.2258–13.8715 mg/ml in which *Aspergillus tamarii* produced the maximum protein concentration released on day 12 of cultivation. Watermelon peels, it ranged from 1.8926–5.2474 mg/ml in which *Aspergillus terreus* gave the maximum biosynthesis potential on day 3 of fermentation. The yield of extracellular protein on the rampage on medium containing banana peels ranged from 0.9247–4.0108 mg/ml in which *Mucor piriformis* had the maximum biosynthesis potential on day 3 of submerged cultivation. Furthermore, on medium with plantain peels, it ranged from 1.1725–8.3441 mg/ml in which *Aspergillus sclerotium* had the maximum biosynthesis potential on day 3 of

cultivation. *Aspergillus* sp. take over *Fusarium* sp. and *Mucor* sp. in polygalacturonase (PG) production.

Bioethanol obtained by dichromate method

Lignocellulosic materials and various agro wastes with different methods have been employed 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 to 28th day (Figure 4). The maximum concentration of ethanol was achieved on 28th day 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 carrot peel was a very promising raw material for bioethanol production with *C. cladosporioides*. Mishra et al. (2012) founded increase in quantity of ethanol produced in sub-merged state fermentation as compared to the produced by solid state fermentation and founded optimal incubation period 72 hours for bioethanol production by orange peel using *S. cerevisiae*. Senthilkumar and Gunasekaran (2005) reported that some Gram-positive bacteria *Clostridium cellulolyticum*, *Lactobacillus casei* have been engineered for bioethanol production. Dien et al. (2003) worked on Gram-negative bacteria *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis*. *E. coli* and *K. oxytoca* are naturally able to use a wide spectrum of sugars, and work has concentrated on engineering these strains to produce ethanol selectively.

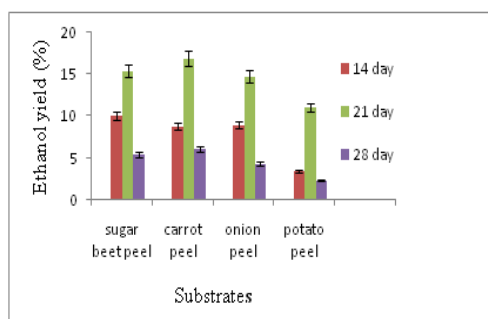


Figure 4 Ethanol obtained by dichromate method bioethanol yield (%) with agro wastes peel after 14th, 21st and 28th days of incubation of *Cladosporium cladosporioides*

Gas Chromatography and Bioethanol concentration

The Purity level for the ethanol through Gas Chromatography for the sample carrot peel was found to be 15.85 %, the Retention Time [min] was 1.06, and Area [mV.s] was 2400, Height [mV] 168.364 and Area [%] 93.4 (Figure 5).

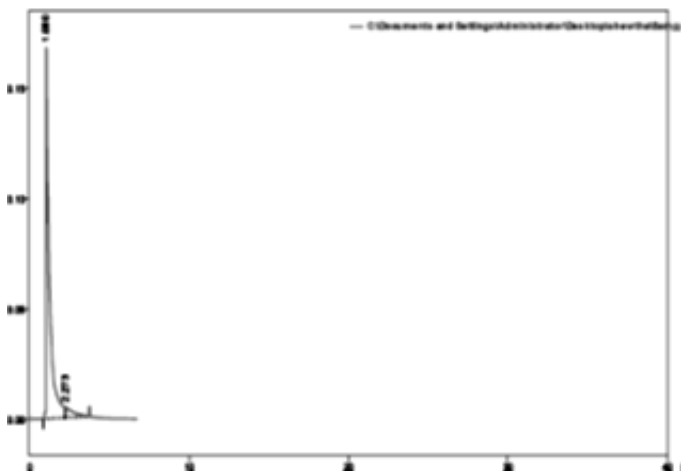


Figure 5 Gas Chromatogram of *Cladosporium cladosporioides* with carrot peel at 28 days.

Isaie and Padmavathi (2016) reported that agro 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 *S. cerevisiae* for the production of alcohol which was fermented at 14, 21, 28 days to produce alcohol. 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 %. Muchtaridi et al. (2012) determined alcohol contents of fermented

black tape ketan based on different fermentation time with three different methods. Methods used are specific gravity, refractive index and GC-MS. Alcohol concentration obtained by using specific gravity method at 3, 10, 17, 24, and 31 days of fermentation, respectively, are 3.17 % v/v; 3.02 % v/v; 3.63 % v/v; 3.12 % v/v; and 4.47 % v/v, using the method of refractive index is 3.90 % v/v; 3.69 % v/v; 4.31 % v/v; 3.80 % v/v and 5.04 % v/v, and using GC-MS method was 4.30 % v/v; 4.23 % v/v; 5.01 % v/v; 4.75 % v/v; and 5.34 % v/v. The variation of fermentation time obviously did not influence the produce of alcohol contents statistically.

From the results obtained on bioethanol production potential of various lignocellulosic wastes varied, and can be concluded that carrot peel was a very promising raw material for bioethanol production with *C. cladosporioides*. The maximum bioethanol concentration obtained in carrot peel at 21st day by *C. cladosporioides* was 133.341 g/l (Table 2).

Table 2 Bioethanol concentration by *Cladosporium cladosporioides* (g/l)

Substrates + <i>Cladosporium cladosporioides</i>	Bioethanol yield (g/l) at 14 th day	Bioethanol yield (g/l) at 21 st day	Bioethanol yield (g/l) at 28 th day
Sugar beet peel	79.136	121.506	42.606
Carrot peel	69.353	133.341	47.734
Onion peel	7.101	115.983	34.400
Potato peel	27.615	87.026	18.620

From the results obtained on bioethanol production potential of various lignocellulosic wastes varied, and can be concluded that carrot peel was a very promising raw material for bioethanol production with *C. cladosporioides*. The maximum bioethanol concentration obtained in carrot peel at 21st day by *C. cladosporioides* was 133.341 g/l (Table 2). Oyeleke et al. (2009) reported that the maximum volume of ethanol (27.10 g/l) produced from guinea com husk and millet husk (18.24 g/l) at the 120th hours with *Zymomonas mobilis*. Agulejika et al. (2005) reported maximum ethanol yield at 120th hour from fresh fruit (64.01 g/l) and waste fruits (21.14 g/l) using *Zymomonas mobilis*. Ismail et al. (2012) has reported yields of bioethanol 0.475 g/g to 0.51 g/g of the Wheat Straw and com cobs, and hulls acid hydrolysate respectively. Using green algae (Trivedi et al., 2013; Ge et al., 2011). Wu et al. (2014) obtained an ethanol yield of 0.45 g/g from *U. fasciata*, 0.44 g/g from *Laminaria japonica* and of 0.47 g/g from hydrolysate *Gracilaria* sp..

CONCLUSION

The present study examined the influences of fermentation period on ethanol production ability of *S. cerevisiae* using the carrot peel, onion peel, potato peel and sugar beet peel as substrates. The results of this study indicate incubation time for fermentation using *S. cerevisiae* which may enhance ethanol yield and minimize the cost of production could be obtained from agro wastes as substrates. Bioethanol production by *S. cerevisiae* may be used as successful alternative of *S. cerevisiae* in bioethanol production.

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Conflict of Interest Statement: The authors declare that there is no conflict of interest.

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