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Full Length Research Paper

Microbial pretreatment of rice husk and groundnut shell for bioethanol production

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Bacterial species were isolated from the rumen of ruminants to test for their ability to hydrolyse rice husks and groundnut shells. The isolates from the rumen were characterized and identified as *Bacillus licheniformis, Bacillus lentus, Bacillus subtilis, Paenibacillus alvei, Yersinia enterocolitica* as well as *Salmonella* sp. Similarly, *Zymomonas mobilis* and *Saccharomyces cerevisiae* were isolated from rotten oranges and palm wine to test for their ability to ferment the hydrolysates. The highest yield of reducing sugar of 4.1 % was obtained from groundnut shell. Rice husk yielded the lowest quantity of reducing sugar with a total yield of 2.96 %. The highest concentration of bioethanol of 0.96 % was produced using a combination of *Saccharomyces cerevisiae* and *Zymomonas mobilis* from groundnut shell. Also, the lowest concentration of 0.11 % of bioethanol was obtained when *Zymomonas mobilis* was used on hydrolysates from groundnut shells. The combination of *Saccharomyces cerevisiae* and *Zymomonas mobilis* yielded the highest ethanol when compared with the yields obtained when the organisms were used seperately. The result reveals the potentials of the fermentative organisms to produce ethanol from agrowastes.

Keywords: Bioethanol, groundnut shell, rice husk, Saccharomyces cerevisiae, Zymomonas mobilis.

INTRODUCTION

The sharp increase in the price of petroleum products, the finite nature of fossil fuels, growing concerns especially related to greenhouse gas emissions and health and safety considerations are forcing the search for new energy sources and alternatives to power the world's motor vehicles as well as generating electricity. In the current time, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of limited fossil fuel stocks but also for safe and better environment (Chandel *et al*., 2007). Biomass is seen as an alternative energy source for several reasons. The main reason is that bioenergy can contribute to sustainable development. Resources are often locally available and conversion into secondary energy carriers is feasible without high capital investments. Furthermore, energy plantations may also create new employment opportunities in rural areas, it also contributes to the social aspect of sustainability. However, the use of food crops such as corn and sugarcane to produce biofuels is increasingly being discouraged due to the current worldwide rise in food prices. In order to minimize food-feed-fuel conflicts, it is necessary to integrate all kinds of biowastes into a biomass economy (Mahro and Timm, 2007).

The long-term benefits of using waste residues as lignocellulosic feedstocks will be to introduce a sustainable solid waste management strategy for a number of lignocellulosic waste materials, contribute to the mitigation in greenhouse gases through sustained carbon and nutrient recycling, reduce the potential for water, air, and soil contamination associated with the land application of organic waste materials, and to broaden the feedstock source of raw materials for the bioethanol production industry. In this research, rice husk and groundnut shell were utilized for the production of the

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bioethanol because of their abundance in this part of the country.

Bioethanol production from cellulose is a more difficult-and-expensive technical step. The enzymes involve in the hydrolysis of the cellulose are costly to make and current methods for cellulose break down require a lot of energy (Humphrey and Caritas, 2007). However, with the knowledge that ruminant livestocks (camel, cattle, sheep etc) that eat grass, then use slow enzymatic digestive processes to break it into glucose, there is growing interest in bioethanol laboratories worldwide to do the same thing, and subject the hydrolyzed products to fermentation, distillation, and drying to produce the bioethanol (Lynd et al., 2002). Production of bioethanol will go a long way to reduce green house gas effect and global warming. Therefore, the objective of this research is to screen rice husk and groundnut shell for their bioethanol production potentiality.

MATERIALS AND METHODS

Collection and processing of samples

The agrowastes (groundnut shell and rice husk) were collected from local milling centers in Sokoto metropolis of Sokoto State. Twenty grams of each agrowaste sample was collected in clean polythene bags and transported immediately to the laboratory. The wastes were powdered using pestle and mortar, sieved using a sieve with a mesh size of 0.5mm and used as carbon source. Different parts of the rumen of four different ruminant animals namely cattle, camel, sheep and goats were purchased from Sokoto central abattoir for the isolation of cellulolytic microorganisms. Samples of rotten oranges were collected from minor refuse dumps at the Sokoto vegetable market for the isolation of *Zymomonas mobilis* while samples of palm wine were purchased from Old airport mini market in Sokoto metropolis for the isolation of *Saccharomyces cerevisiae*. The rumen and rotten orange samples were placed in clean sterile polythene bags while the palm wine sample was placed in 2 litres capacity plastic container and transported immediately to the laboratory for analysis.

Isolation of cellulolytic microorganisms from rumen content of ruminants

The cellulolytic microorganisms were isolated in accordance with the method described by Oyeleke and Okusanmi (2008). The rumen of the ruminant animals was sliced and swabbed with a swab stick. The sample was then inoculated on Nutrient agar (NA) and MacConkey agar (MA) plates. The plates were incubated aerobically and anaerobically using candle jar for 24 hours at 37 ^{o}C. Colonies that developed after the incubation period were purified and maintained on agar slants for further characterization and identification. The pure isolates were then subcultured on cellulose agar plates and were incubated aerobically and anaerobically for seven days to test their ability to hydrolyze cellulose. Hydrolysis of cellulose was indicated by the appearance of clear zones around the colonies of the organisms. The bacterial isolates were characterized and identified using standard methods as described by Cheesebrough (2003) and Oyeleke and Manga (2008).

Isolation of *Zymomonas mobilis* from rotten orange

The isolation of *Zymomonas mobilis* was carried out according to the method described by Obire (2005). The rotten orange samples were washed and then squeezed to obtain the juice. The juice was serially diluted from tube 1 (10^1) to tube 5 (10^5). Then 0.1 ml aliquot from the 10^5 tubes was plated onto the MYPGA (malt yeast peptone glucose agar) medium using spread plate techniques. Each medium was treated with actidione (cycloheximide) to inhibit yeast growth. The plates were incubated in an anaerobic jar in which Gas pak sachet was placed to exhaust the oxygen in the jar and incubated at 37 ^{o}C for 2 days. Colonies suspected to be those of *Zymomonas* were characterized on the basis of their cultural and morphological characteristics.The isolates were purified by streaking on freshly prepared media and incubated for 2 days at 37 ^{o}C in an anaerobic jar. The ability of *Zymomonas mobilis* to ferment various carbohydrates using glucose, fructose, sucrose, maltose, lactose and arabinose was determined by growing the isolates in liquid standard medium (Yeast glucose broth pH 6.8) containing 1 % (w/v) of the particular carbohydrate. Durham tubes were inverted into culture tubes for gas collection. The tubes were incubated at 37 ^{o}C for 24 hours. Uninoculated broths were used as control.

Isolation of *Saccharomyces cerevisiae* from palm wine

The isolation of *Saccharomyces cerevisiae* was carried out using standard method as described by Brooks (2008). The isolates were identified based on morphology, surface characteristics, presence of pseudohyphae, ascospore formation and vegetative reproduction. The ability of isolates to ferment glucose, fructose, sucrose, maltose, lactose, mannitol, galactose

and arabinose was also tested.

Enzymatic hydrolysis

This was carried out according to the method described by Gupta et al. (2009). Four 500 ml capacity conical flasks of 500 ml capacity were used for the enzymatic hydrolysis of the agrowastes. The conical flasks were labelled A and B (A: Groundnut shell and B: Rice husks). Ten grams of each agrowaste was put in the flasks and 100 ml of distilled water was added. The flasks were plugged with cotton wool and aluminium foil and then sterilized at 121 ^{o}C for 30 minutes. Each flask was inoculated with 0.5 ml (1.3×10^{7}cfu/ml) suspension of the isolated cellulolytic microorganisms. The flasks were incubated at 37 ^{o}C for 5 days on an orbital shaker, and then the samples were filtered through Whatman filter paper No1. The filterate were then used for determination of reducing sugar and fermentation.

Determination of reducing sugar

The reducing sugar content following hydrolysis of the agrowastes was determined using the dinitrosalicylic acid colorimetric method of Miller (1959) with glucose as standard.

Fermentation

The fermentation of the hydrolysed samples was carried out in accordance with the methods described by Brooks (2008) and Oyeleke *et al.* (2009). Ten milliliters (10 ml) of the rice husks hydrolysates was dispensed into twelve different 100 ml capacity conical flasks. Each conical flask was replicated three times. The flasks were then covered with cotton wool, wrapped in aluminium foil and autoclaved at 121 ^{o}C for 15 minutes. The tubes were allowed to cool at room temperature and aseptically inoculated with the fermentative organisms. Conical flask A is inoculated with *Saccharomyces cerevisiae*; B inoculated with *Zymomonas mobilis* and C with *Saccharomyces cerevisiae* and *Zymomonas mobilis*. All the flasks were incubated anaerobically at 37 ^{o}C. The same procedure was repeated for the groundnut shell hydrolysates. The hydrolysates were then distillated according to standard method.

Determination of concentration of bioethanol produced

Determination of concentration of bioethanol produced was carried out using the method described by Oyeleke and Jibrin (2009). 1ml of standard ethanol was diluted with 100 ml of distilled water to give a concentration of 1 %. From this stock solution 0, 0.2, 0.4, 0.6 and 0.8 % of the ethanol was prepared by diluting it with distilled water. To each of the varying ethanol concentrations 2 mls of chromium reagent was added and allowed to stand for an hour for colour development. The absorbance of each concentration was measured at 588 nm using UV-VIS spectrophotometer and the readings used to developed standard ethanol curve. Then 5 mls of each bioethanol samples were put in test tubes and treated with 2 mls of the chromium reagent. The mixture was allowed to stand for an hour and the absorbance was measured as for standard curve.

RESULTS

The results of the characterization and identification of cellulose utilizing bacteria is presented in Table 1. The bacteria were characterized and identified as *Bacillus licheniformis, B. lentus, B. subtilis, Paenibacillus alvei, Yersinia enterocolitica* and *Salmonella* sp. The result of the isolation and identification of *Zymomonas mobilis* is presented in Table 2. The result of the isolation and identification of *Saccharomyces cerevisiae* is presented in Table 3. The results of the reducing sugar yield of the hydrolysed agrowastes is presented in Table 4. The highest yield of reducing sugar of 4.096 % was obtained from groundnut shell, whereas 2.962 % was obtained from rice husk. The results of the concentration of the bioethanol produced from fermentation of the agrowastes using *Saccharomyces cerevisiae, Zymomonas mobilis* and a combination of *Saccharomyces cerevisiae* and *Zymomonas mobilis* is presented in Table 5. The highest concentration of bioethanol of 0.955 % was produced using a mixture of *Saccharomyces cerevisiae* and *Zymomonas mobilis* from groundnut shell, while the lowest concentration of 0.105 % was obtained when *Zymomonas mobilis* was used alone. The combination of *Saccharomyces cerevisiae* and *Zymomonas mobilis* yielded the highest ethanol yield as when compared with the yields obtained from the individual organisms.

DISCUSSION

The production of bioethanol from agro-waste was examined. The result reveals that *Bacillus* species account for 58 % of the total isolates that was isolated from the rumen of the ruminants along with some members of the coliform bacteria. This agrees with Oyeleke and Okusanmi (2008) who reported the isolation of about 37.8 % of *Bacillus* species from the rumens of cow, goat and sheep. Lynd et al. (2002) also isolated these organisms from the rumen and were implicated in

Table 1. Cellulose-utilizing bacteria isolated from the rumen of ruminants

Isolates	Gram	Mot	Cat	Glu	Lac	Suc	H_2S	Gas	MR	VP	Cit	Ind	Ure	Oxi	CH	Organism
1	+Rod	+	+	+	-	-	-	-	-	+	+	-	+	-	+	*Bacillus licheniformis*
2	+Rod	+	+	+	-	+	+	-	+	-	-	-	+	+	+	*Bacillus lentus*
3	+Rod	+	+	+	-	-	-	+	-	+	+	-	-	-	+	*Bacillus subtilis*
4	+Rod	+	+	+	-	-	-	-	-	+	-	+	+	+	+	*Paenibacillus alvei*
5	-Rod	+	-	+	-	+	-	-	+	-	-	-	+	-	+	*Yersinia enterocolitica*
6	-Rod	+	+	+	-	-	+	-	+	-	-	-	-	-	+	*Salmonella* sp

KEY: + positive, - negative, Mot- motility, Cat- catalase, Glu- glucose, Lac- lactose, Suc- sucrose, MR- methyl red, VP- voges proskauer, Ind- indole, Cit- citrate, Oxi- oxidase, Ure- urease, CH- cellulose hydrolysis

Table 2. Morphological and biochemical characterization of isolates from rotten orange

Isolates	Gram reaction	Motility	Catalase	Glucose	Fructose	Sucrose	Maltose	Arabinose	Urease	Oxidase	Lactose	Organism
1	-Rod	+	+	+	+	+	-	-	-	-	-	*Zymomonas mobilis*

Key: +: fermentation; -: no fermentation/negative

Table 3. Morphological and biochemical characterization of isolates from palm wine

Isolates	Colonial characteritics	Cell shape	Man	Glu	Fru	Suc	Mal	Ara	Lac	Gal	Cat	Organism
1	Smooth creamish	Spherical	-	AG	AG	AG	AG	-	-	AG	+	*Saccharomyces cerevisiae*

Key: Man: mannitol; Glu: glucose; Fru: fructose; Suc:sucrose; Mal: maltose; Ara: arabinose; Lac: lactose; Gal: galactose; Cat: catalase; AG: acid/gas production, -: no fermentation; +: positive

Table 4. Glucose yield of the hydrolysed agro-wastes (%)

	Total	*Mean±SD
Rice husk	2.962	0.246±0.01
Groundnut shell	4.096	0.341±0.08

** Values are mean ± standard deviations of three replications*

Table 5. Bioethanol yield of the different agro-wastes (%)

Agrowastes	Fermentative organism	Total	*Mean±SD
Rice husk	*Saccharomyces cerevisiae*	0.404	0.034±0.009
	Zymomonas mobilis	0.378	0.032±0.010
	S. cerevisiae + *Z. mobilis*	0.416	0.035±0.013
Groundnut shell	*Saccharomyces cerevisiae*	0.912	0.076±0.015
	Zymomonas mobilis	0.105	0.009±0.004
	S. cerevisiae + *Z. mobilis*	0.955	0.079±0.016

** Values are mean ± standard deviations of three replications*

the hydrolysis of cellulose. Evidences based on zones of clearing in cellulose agar led to the conclusion that *Bacillus* possesses firmly bound cellulase. This is in agreement with Colombattoo et al. (2002) who described the cell morphology of *Bacillus* species as having a thin cell coat and which adhered tightly to the plant cell wall. *Zymomonas mobilis* and *Saccharomyces cerevisiae* were isolated from rotten orange and palm wine, respectively. These organisms being sugar-loving are found to thrive in such substrates as they receive available juices that they utilize as their growth factor. *Zymomonas mobilis* was identified as gram-negative, catalase positive, motile, anaerobic plump rods with an unusual width. The species were heterofermentative, producing gas from glucose, fructose and sucrose. However, they were oxidase and urease negative. These findings were in conformity with that of Obire (2005) who reported the isolation of *Zymomonas mobilis* from fresh palm wine saps. *Saccharomyces cerevisiae* was identified as having smooth creamish colonies with spherical cell shape. The organism was able to ferment glucose, fructose, sucrose, maltose and galactose producing acid and gas. The result is in conformity with that of Elijah et al. (2010) who reported the isolation of *S. cerevisiae* among other yeasts from palm wine. Ezeogu and Emeruwa (1993) reported the isolation of *S. cerevisiae* from palm wine juice in a sake-type fermentation. The highest yield of reducing sugar and bioethanol was obtained from the groundnut shell when compared with that of rice husk. The high lignin content of the rice husk may inhibit enzymatic hydrolysis of the cellulose in the lignocellulose biomass.These findings were in agreement with the study of Epstein et al. (2010) who reported an ethanol volume as low as 0.06 % from apple and grape juices. However, the findings were not in agreement with that of Salvi et al. (2010) who reported an ethanol yield of 24.53 g/l from dilute ammonia treated sorghum. The study reveals the potentiality of the agrowastes cellulose-utilizing bacteria as well as the fermentative organisms for bioethanol production as being very promising.

CONCLUSION

Rumen can serve as a cheap source of cellulolytic microorganisms for hydrolysis of different industrial raw materials. Rice husk and groundnut shell wastes can be exploited as cheap carbon source for industrial production of bioethanol. However, there is the need to optimize the processes for higher yields of both reducing sugar and bioethanol.

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