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Bioethanol production potential of a cellulolytic and thermophilic *Geobacillus* species from compost sites

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Abstract

Screening of cellulolytic thermophilic bacteria from different compost sites in Ibadan for bio-ethanol production using readily available lignocellulosic wastes (corn cobs, cassava peels and saw dust) as substrate was carried out. Eleven out of 25 thermophilic isolates were cellulolytic. Out of these, two isolates (*Geobacillus* sp. OCO5 and *Geobacillus* sp. OCO1) with 15% ethanol tolerance were selected for further studies.The lignocellulosic wastes were biologically pretreated with *Pleurotus tuberregium* for 42 days. Statistical analysis showed that biological pretreatment of substrates with *P. tuber-regium* had a significant effect ($p<0.05$) on lignin reduction in the substrates. Percentage reduction of 36.21%, 16.82% and 1.13% was observed in pretreated corn cob, cassava peel and saw dust respectively. *Geobacillus* sp. OCO5 and *Geobacillus* sp. OCO1 on pretreated corn cobs respectively gave a reducing sugar yield of 68.5% and 18.75%, while pretreated cassava peels gave a yield of 35% and 25%. Thus, *Geobacillus* sp. OCO5 was chosen for bio-ethanol production. In single phase fermentation of pretreated corn cobs and cassava peels with this isolate, an ethanol yield of 62.13% and 45% was produced respectively. However, two phase fermentation of the same substrates using *Geobacillus* sp. OCO5 and *Saccharomyces cerevisiae* yielded 88.65% and 72.59% ethanol. These results show that the high recalcitrance of lignocellulosic wastes can be overcome by biological pretreatment using *Pleurotus tuber-regium* and corn cobs was the most suitable raw material for bio-ethanol production. The synergistic effect between the cellulolytic thermophile (*Geobacillus* sp. OCO5) and baker's yeast (*Saccharomyces cerevisiae*) also gave better ethanol yield compared to single phase fermentation. Thus this type of synergy can be employed in probable large scale bio-ethanol production.

Keywords: Bio-ethanol, Cellulolytic, Lignocellulosic wastes, Fermentation, Thermophilic.

Introduction

Cellulose is a linear crystalline macromolecule which makes up a major component of lignocellulosic materials (45% of the dry weight of wood). It's a polymer of β-1, 4-glucosidic bonds [1]. The composition and percentages of cellulose vary in different species of plant. Cellulose exists majorly as agricultural wastes and these include forestry waste, agroindustrial waste, wood wastes, corn cobs, sugarcane bagasse, paper waste, wheat straw to mention a few [2]. The increased concern over reduction in fossil fuel has generated a growing interest in nonconventional fuel derived from alternative bio-renewable sources such as lignocellulosic materials. Lignocellulosic materials are readily available and can be used as either varieties of residues of agricultural, industrial or domestic activities and production of a valuable biofuels [3].

Bio-ethanol is considered as an important renewable alternative energy that is environmentally friendly with economic benefits [4]. It is commonly used as a fuel additive [5]. There has been an increase in the production and utilization of biofuels due to reduced negative environmental impact. Ethanol production from biomass materials (such as sugar cane juice, corn starch and molasses) received

more attention in different parts of the world [6]. Brazil and the United States account for 89% of the recent global bioethanol production [7].

There are a number of challenges associated with the use of biomass materials (corn and sugar based materials) as a substitute for conventional fuel production. For instance, there are ethical concerns regarding the use of food and feeds as raw materials for biofuel production. There is also the concern of the inability of biomass to completely meet up with the supply of fossil fuels consumed yearly in different parts of the world. These concerns have led to research effort geared towards the use of agricultural residues/ waste/inedible feedstock alternatives [8]. Second generation ethanol production is a desirable alternative because it is produced from nonedible sources (lignocellulosic material) which contains mainly cellulose, hemicelluloses and lignin[9]. These long chain polymers can be hydrolysed into a mixture of hexoses (C_6) and pentoses (C_5). Lignin cannot be hydrolysed [4]. Despite the cheap source of raw materials for bio-ethanol production using lignocellulosic wastes, they are recalcitrant materials, thus the importance of generating a suitable pre-treatment method cannot be overlooked. Pre-treatment strategies that have been employed include acid hydrolysis, hot water treatment, dilute acid pretreatment and lime, enzymatic hydrolysis and mechanical pre-treatment [10,11]. The bioconversion of lignocellulosic biomass to ethanol requires hydrolysis of the two carbohydrate polymers to their constituent monomeric sugars prior to microbial fermentation [3].

There has been increased interest in screening for thermophiles/ thermo-tolerant microbes because the bioconversion of agricultural residues occurs at relatively high temperatures [12]. Microbes that withstand high temperature are of global significance in consolidated bio-production of ethanol [13]. Consolidated bioprocessing (CBP) is an approach integrating enzyme production, saccharification and fermentation into a single process. This is a strategy that is effective for ethanol production from lignocellulosic materials [14]. The CBP is a cost effective process due to substrate and raw material simplification during operation and reduction in utilities [13].This fact makes the search for more thermo-tolerant microbes as suitable bio-catalysts of CBP imperative [15].

A variety of thermophilic microbes such as *Geobacillus thermoglucosidasius, Clostridium thermocellum* and *Clostridium cellulolyticum*, that are capable of producing ethanol in low amounts have been isolated and characterized in past decades from diverse environments such as compost, landfills, farm soils, hot springs, sewage plants and river banks for their evaluation and development for large scale ethanol production[13,16]. The use of thermophilic bacteria for ethanol production using CBP have the following advantages; lower risk of contamination, saves cost in industrial scale, utilization of a wide range of sugars, fast growth rate and, increased bio-conversion rate and product recovery [13].

Interest in bio-ethanol production has been stirred up due to diminishing fuel reserves in recent years [14]. This has necessitated the search for a sustainable, efficient, renewable and cost effective alternative source of energy which contributes economic benefits and reduction of negative environmental impact of the daily utilization of fossil fuels. The utilization of cellulolytic thermophilic bacteria in CBP for bioethanol production is cost effective and the process uses readily available bio renewable sources as raw materials, thus producing a type of nonconventional fuel that is environmentally friendly compared to presently available conventional fuel [17]. Hence the need for intensive research on the development of a suitable means to generate an alternative source of energy on a large scale.

In view of the above, this study aimed to produce bioethanol from lignocellulosic wastes using cellulolytic thermophiles isolated from different compost sites in Ibadan.

Materials and methods

Culture medium used

The media used for isolation was cellulase differential medium (Congo red CMC-Na medium). The medium contained: Mg SO $_4 7H_2O$ (0.5g), Carboxylmethylcellulose (CMC-Na) (5.0g), KH_2PO_4 (1.0g), $(NH_4)_2SO_4$ (2.0g), NaCl (0.5g), Congo-red (0.2g), agar (20g), and 1000ml distilled H_2O. The pH of the medium was adjusted to 7.0 with 1.0 N NaOH. The agar-agar and CMC-Na powder was dissolved in the medium using a magnetic stirrer-hot plate. The medium was then autoclaved for 15 min at 121^{o}C, cooled, dispensed into sterile petri dishes, air dried in a sterile laminar flow, and stored at 4^{o}C [18].

Sample collection

A total of 100 samples were randomly collected from different compost sites in Ibadan for this study. Compost samples were obtained from Onidundun, Iyanna Ofa, Moniya, Bodija, Saw mill, Iwo road and UI farm. The samples were obtained at a depth of 30-40 cm below the surface of a 3-week old composting heap [16]. The temperature at the point of sampling was at 50^{o}C. The samples were transported to the laboratory and processed immediately.

Selection and screening method

Ten grams of each sample was weighed aseptically into a clean Erlenmeyer flask containing 90mL of sterile nutrient broth and incubated at 55^{o}C for 3-5 days[18]. After incubation, the samples were spread on the cellulase differential medium using serial

dilution technique, and incubated at 55°C for 48 hours. Isolates with obvious hydrolyzed zones were selected and subcultured on a sterile nutrient agar plate and incubated at 55°C for 24-48 hours to obtain pure culture of each organism [18]. Each pure culture obtained was subcultured on a nutrient agar slant and stored at 4°C.

Determination of optimum growth temperature and pH

The optimum pH of the cellulolytic thermophiles was determined as described by Maney et al. [19] with some modifications. In this experiment, the pH of the nutrient medium (nutrient broth) was adjusted using sodium citrate buffer solutions. 1mL of standardized inoculum (3.5×10^5cfu/mL) was inoculated into 10mL of sterilized nutrient broth of varying pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) (Xuan, 2010). Each of the tubes was incubated for 2days at 55oC, the final optical density at 600nm wavelength (OD600) was measured and the corresponding number of cells was determined via plate count. To detect the optimum temperature, 1mL of standardized inoculum (3.5×10^5cfu/mL) was also inoculated into 10mL of sterilized nutrient broth and incubated at various temperatures (45°C, 50°C, 55°C, and 60°C).The final OD600 was measured and the corresponding number of cells was determined via plate count technique. The generation time was calculated based on the equation shown below [19].

$$\mu = \log Nt - \frac{\log N0}{0.301} \quad ; \quad \text{Generation time} = t/\mu$$

Screening for ethanol tolerance

The MIC (Minimum Inhibitory Concentration) determination was performed for the selected cellulolytic strains in order to determine their maximum ethanol tolerance. The medium used contained nutrient broth, 20 mM of glucose and 2 g $L^{-1}$of yeast extract. However, control samples did not contain any ethanol or glucose. Different tubes containing 10 mL of the medium had different concentrations of ethanol (0%, 2.5%, 5.0%, 10%, 15% and 20%). The optical densities were measured at 600nm in the beginning and at the end of the incubation period (120 h) to determine the MIC of ethanol for each isolate [19].

The cellulolytic thermophiles were identified using cultural, microscopic, biochemical and physiological properties [20].

Biological pretreatment of lignocellulosic wastes
The substrates used (corn cobs, saw dust and cassava peels) were grinded (to a size of approximately 0.5mm) to increase the surface area needed for enzyme activity. Fifteen grams of each of the substrates were weighed into a clean jar, and 45 mL of distilled water was added to the jar. The jars were covered with aluminium foil and sterilized in an autoclave at 121°C for 15 minutes. After cooling, the jars were inoculated at the centre with two agar blocks (5mm in diammeter) which contained an actively growing mycelium. The control jars were not inoculated. The jars were covered immediately and incubated at 30°C ± 2 for 42 days [21].

Analysis methods

Analysis of chemical composition

The amount of lignin present was determined by acid treatment. The chemical analysis of lignocellulosic biomass was carried out using the Technical Association of the Paper and Pulp Industry (TAPPI) test methods [22,23].

Determination of Reducing Sugar

Total reducing sugar was determined by the 3, 5-dinitrosalicylic acid (DNS) method using glucose as the standard as described by Miller [24] with some modifications. Phenol red and sodium meta-bisulphite was not used in the preparation of the DNS reagent. The samples were analysed using a spectrophotometer at 540 nm. The absorbance readings were then converted into equivalent sugar concentration (mg/mL) using a standard glucose solution curve. Reducing sugar yield was calculated using the following equation:

Reducing sugar yield (%) = mass of reducing sugar produced × V2 × 100
Mass of biomass × V1
V1= volume of analysis medium
V2= volume of hydrolysis medium

Determination of the physical composition of pretreated lignocellulosic substrates

The determination of the physical composition of pretreated lignocellulosic substrates was carried out using Fourier Transform Infra-Red (FT-IR) spectroscopy. The FT-IR spectra of untreated and treated lignocellulosic substrates were obtained by direct transmittance using the KBr pellet technique. Spectra were recorded with a Perkin Elmer 1650 FT-IR spectrometer (Perkin Elmer, Waltham, MA, USA). The spectra (4000– 500 cm^{-1}) were measured at a spectral resolution of 4 cm−1and 64 scans per sample [23].

Statistical analysis

Chemical compositions analysis was carried out in triplicate and the values presented were average of the three values obtained within a 95% confidence level. The effects of biological pretreatment on lignin, hemicellulose, and cellulose reduction as well as the effect of the different types of fermentations

on ethanol yield were analyzed using the Statistical Analysis Software (SAS) program, version 21 [25].

Fermentation of pretreated lignocellulosic wastes

Inoculum preparation

The selected cellulolytic isolate was subcultured from the slant to a sterile nutrient agar plate, incubated for 24 h at 55^{o}C. Discrete colonies were selected and inoculated into 50 mL sterile nutrient broth supplemented with 20 g/L glucose in 250 mL Erlenmeyer flasks previously sterilized by autoclaving (Autoclave model number-RAU 123) at 121^{o}C for 15 min. Each inoculated flask was incubated at 55^{o}C with agitation of 150 rpm for 10-14hours. After incubation, each culture flask was harvested by centrifugation at 2,000 rpm for 5 min. The supernatant was removed, and the cell pellets were re-suspended in 5mL of distilled water. The cells harvested from the Erlenmeyer flasks were combined and used as the inoculum for the experimental flasks. A similar procedure was used to prepare the inoculum for baker's yeast Saccharomycescerevisiae except that sterile yeast peptone (YP) medium supplemented with 50 g/L glucose was used [26].

Fermentation of the lignocellulosic substrates

Fermentation experiments were each performed using 100 mL of production medium (10g peptone, 5g yeast extract, and 10g NaCl) in 250mL Erlenmeyer flasks plugged with cotton wool. The initial substrate loading for the experiments was approximately 6% w/v of pretreated biomass. In single phase fermentation, the flasks were inoculated with the cellulolytic thermophile and incubated at 55^{o}C with agitation of 150 rpm for 120h. In two phase fermentation, the flasks were inoculated with the cellulolytic thermophile and incubated at 55^{o}C for 48 hours followed by fermentation by baker's yeast (Saccharomyces cerevisiae) at 37^{o}C with agitation of 150 rpmfor 120h. After fermentation, samples were taken for GC analysis to determine the amount of ethanol produced [26].

Analysis of products

The fermented samples were analysed for residual sugars, ethanol and other byproducts by gas chromatography (GC) using FID detectors [27]. The gas chromatogram was HP 6890 powered with HP Chemstation Rev. A 09.01 (1206) software. The split ratio used for this analysis was 20:1, carrier gas was nitrogen, and inlet temperature was 200^{o}C. The column type used was HP INNO Wax (Dimensions 30cm×0.25mm×0.25µm). The initial temperature of the oven program was at 110^{o}C, ramping at 6^{o}C/min, and maintained for 1 minute. The detector temperature was 240^{o}C, hydrogen pressure was 20psi and compressed air pressure was 31psi. The samples (fermented medium) were recovered and stored in an air tight bottle at 4^{o}C until ready for further analysis. The mixture was placed in the round bottom flask of the rotary evaporator. The flask and the control were coupled and temperature of system was set to ethanol boiling point for proper distillation. The recovered distillate was kept at 4^{o}C for gas chromatography analysis. Initial and final concentrations of monomeric sugars present in the fermentation medium were also determined by gas chromatography. Ethanol yields were calculated as follows:

Ethanol yield (YE) [%] = Ethanol produced [g] in vessel × 100%
Initial sugars in vessel [g] × 0.511
Note. Sugar is interpreted as glucose plus xylose in fermentation experiments [26].

Results

A total of 25 thermophilic organisms were isolated, of which 11 isolates were cellulolytic thermophiles. Eleven percent of the compost samples analyzed contained thermophilic cellulolytic isolates. The highest number of thermophilic organisms (18) was isolated from Onidundun compost site while the least number of thermophilic organisms (2) was isolated from Moniya compost site. No growth was observed in samples from Bodija, Sawmill, U.I. farm and Iwo road compost sites. Cellulolytic thermophiles isolated from Onidundun compost site was 73% and the remaining 27% were from Iyanna ofa compost site. The two thermophiles isolated from Moniya compost site were not cellulolytic. The data is shown in Table 1.

Table 1. Total number of cellulolytic thermophiles isolated from compost sites studied

Sample collection site	Total number of samples	Total number of thermophiles	Total number of cellulolytic thermophiles
Bodija	20	0	0
Moniya	15	2	0
Saw mill	20	0	0
Onidundu	20	18	8
UI farm	5	0	0
Iyanna ofa	10	5	3
Iwo road	10	0	0
Total	100	25	11

The highest diameter of cellulolytic zones of clearance observed on the cellulase differential medium was with isolate OCO5 (34mm), followed by isolate OCO1 (31mm) and the least zone of clearance (10mm)were from Onidundun compost sites.

Growth at optimum pH and temperature

Out of eight isolates from Onidundun compost sites, isolates OCO7 and OCO14 had an optimum growth pH of 6.5, isolates OCO1, OCO5, OCO8, OCO11, and OCO12 had an optimum growth pH of 7, while only isolate OCO15 had an optimum growth pH of 8. All isolates from Iyanna Ofa compost sites (IOC6, IOC4 and IOC9) had an optimum growth pH of 6.5 (Table 2). At the optimum growth pH, the least generation time varied from 0.49h (isolate OCO9) to 0.61h (isolate OCO12).

From table 2 , out of a total of eight isolates from Onidundun compost sites, 4 isolates (OCO7, OCO11, OCO12, and OCO14) had an optimum growth temperature of 50^{o}C, 3 isolates (OCO1, OCO5, and OCO8) had an optimum growth temperature of 55^{o}C, while only isolate OCO15 had an optimum growth temperature of 60^{o}C. Out of three isolates from Iyanna Ofa compost sites, isolates IOC6 and IOC9 had an optimum growth temperature of 60^{o}C while only isolate IOC4 had an optimum growth temperature of 55^{o}C. At the optimum growth temperature, the least generation time for the isolates varied from 0.49h (isolate OCO11) to 0.73h (isolate IOC9).

Table 2. Optimum growth pH and temperature for the isolated cellulolytic thermophiles.

Isolate code	pH Optimum pH	Generation time (h)	Optimum Temperature (oC)	Generation time (h)
OCO1	7	0.59	55	0.60
IOC4	6.5	0.60	55	0.61
OCO5	7	0.59	55	0.59
IOC6	6.5	0.58	60	0.58
OCO7	6.5	0.59	50	0.61
OCO8	7	0.60	55	0.93
IOC9	6.5	0.49	60	0.73
OCO11	7	0.58	50	0.61
OCO12	7	0.61	50	0.61
OCO14	6.5	0.59	50	0.60
OCO15	8	0.49	60	0.59

Isolation pH- 7; Isolation temperature- 55^{o}C
Key. OCO- Onidundun Compost site
IOC- Iyanna Ofa Compost site

Table 3. Ethanol tolerance profile of the cellulolytic thermophiles (OD_{600}).

Isolate code	Ethanol concentration (%)					
	0	2.5	5.0	10	15	20
OCO1	++++	+++	++	++	+	-
ICO4	++++	+++	++	+	-	-
OCO5	++++	+++	+++	++	+	-
ICO6	++++	+++	++	++	-	-
OCO7	++++	++	++	+	-	-
OCO8	++++	++	++	+	-	-
IOC9	++++	++	++	++	-	-
OCO11	++++	+++	++	++	+	-
OCO12	++++	++++	++	+	-	-
OCO14	++++	++++	++	++	-	-
OCO15	++++	++++	+++	+	-	-

Key.
OD_{600} = Optical density read at 600nm wavelength
OD > 1.0 = ++++
OD between 0.7 and 1.0 = +++
OD between 0.3 and 0.7 = ++
OD below 0.3 but above control = +
OD below or the same as control = -

The ethanol tolerance of the isolated cellulolytic thermophiles is shown on Table 3. From the table, as the ethanol concentration increased, the survival tendency of the cellulolytic thermophiles decreased. All the isolates tolerated up to 10% ethanol concentration. Three of the cellulolytic thermophiles from Onidundun compost sites (OCO1, OCO5, and OCO11) tolerated 15% ethanol concentration while none of the cellulolytic thermophiles showed tolerance to 20% ethanol concentration.

Two isolates (OCO5 and OCO1) were selected for further study based on the result of the high ethanol tolerance and high zone of cellulolytic clearance observed after the screening processes. They were identified as *Geobacillus* sp. OCO5 and *Geobacillus* sp. OCO1 based on their colonial, morphological, microscopic and biochemical characteristics.Morphologically, the two isolates were cream color, rough and friable surface with lobed edge. Microscopically, they are Gram positive, diplobacilli with sub-terminal endospore formation. Also, they are positive to most biochemical tests and ferment most of the sugars tested except isolate OCO1 which did not ferment maltose.

Effect of biological pretreatment on lignocellulosic substrates

Percentage chemical composition of the three untreated lignocellulosic substrates (corn cobs, cassava peels, and saw dust) using TAPPI methods is shown in Table 4. Saw dust contained the highest percentage lignin and cellulose content while cassava peels contained the highest hemicellulose content after pretreatment. The mean value of the percentage lignin and hemicellulose contents of the pretreated substrates were not significantly different ($P<0.05$) from each other.

Table 4. Percentage chemical composition of untreated and pretreated lignocellulosic substrates

Treatment	Lignocellulosic substrate	Lignin content (%)	Cellulose content (%)	Hemicellulose content (%)
Untreated	Corn cobs	16.48 (0.02)a	40.01 (0.03)a	30.60 (0.07)a
	Cassava peels	22.25 (0.08)a	48.01 (0.02)a	37.05 (0.07)a
	Saw dust	23.23 (0.09)a	48.15 (0.03)a	28.10 (0.08)a
Pre-treated	Corn cobs	10.39 (0.01)a	38.09 (0.16)a	22.63 (0.03)a
	Cassava peels	18.68 (0.09)a	42.92 (0.98)a	29.38 (0.30)a
	Saw dust	22.97 (0.06)a	47.90 (0.25)a	26.99 (0.06)a

The standard deviation between treatments is indicated in parenthesis. Letters on the right side of the values in the same column indicated significant levels (ANOVA at $\alpha_{0.05}$, F (5, 3)).

The percentage weight loss of the lignocellulosic substrates after 42 days pretreatment is shown in Table 5. Corn cobs showed the highest percentage weight loss of lignin (36.21%) and hemicellulose (26.04%) compared to other pretreated lignocellulosic substrates, while saw dust showed the least percentage weight loss of lignin (1.13%), hemicellulose (3.96%) and cellulose (0.53%) as shown on the table. Statistical analysis showed that biological pre-treatment had a significant effect on the reduction of lignin, cellulose and hemicellulose at 5% level of significance ANOVA at $\alpha_{0.05}$, F (5, 3). The mean value of the percentage weight loss of lignin and hemicellulose in corn cobs after pretreatment was significantly different ($P<0.05$) from that of cassava peels and saw dust, while the mean value of the percentage weight loss of cellulose in cassava peels was significantly different ($P<0.05$) from that of corn cobs and saw dust.

Table 5. Percentage weight losses of lignocellulosic wastes pre-treated by *Pleurotus tuber-regium* for 42 days

Lignocellulosic substrate	Weight loss (%)		
	Lignin	Cellulose	Hemicellulose
Corn cobs	36.21 (0.04)a	4.80 (0.40)b	26.04 (0.08)a
Cassava peels	16.82 (0.40)b	10.61 (2.03)a	20.71 (0.75)b
Saw dust	1.13 (0.25)c	0.53 (0.51)c	3.96 (0.21)c

The standard deviation of the replicates are indicated in parenthesis. Letters on the right side of the data in the same column indicated significant levels (ANOVA at $\alpha_{0.05}$, F (5, 3))

Results of the physical composition of untreated and pretreated lignocellulosic substrates

In untreated cassava peels, the FT-IR spectrum showed the various bonds that make up the lignin polymer before degradation occurs. The band absorption at 1639.46cm^{-1} represents the aromatic groups present in lignin, the band absorption at 1507.69cm^{-1} represents the C=C alkenes present in the lignin molecule, lignin attached to the CH_2 cellulose molecule was responsible for the band absorption at 1454.54cm^{-1}. The band absorption observed at 1250.34cm^{-1} and 1035.48cm^{-1} represents the phenolic groups, alcohols, and aliphatic esters. After 42 days pre-treatment of cassava peels, there was a general increase in the intensity and subsequent percentage transmittance of the FT-IR spectrum. There was a notable shift in the band absorption from 1639.46cm^{-1} to 1638.66cm^{-1}, 1247.55cm^{-1} to 1250.34cm^{-1} and at 1454.54cm^{-1} to 1448.95cm^{-1}. The band absorption at 1507.69cm-1 was no longer visible in the spectrum.

The FT-IR spectrum of untreated corn cobs shows band absorptions at 1640cm $^{-1}$ to 1044cm^{-1} caused by the lignin molecule. The band absorption at 1387.41 cm^{-1} is caused by the attachment of lignin to the $–CH_2$ of cellulose. The absorbance observed at 1253.14cm^{-1} and 1044.00cm^{-1} corresponds to the O-H phenolic groups and O-H primary and secondary alcohols present in the lignin molecule. After 42 days pretreatment, there was a general increase in percentage transmittance as well as a shift in band absorption from 1640cm^{-1} to 1641cm^{-1}, 1387.41cm^{-1} to 1376.22cm^{-1}, 1253.14cm^{-1} to 1250.34cm^{-1} and 1044.00cm^{-1} to 1039.66cm^{-1}.

The FT-IR spectrum of untreated saw dust shows band absorptions at 1640cm-1 (presence of aromatic groups), 1399.46cm-1 (lignin bonded to CH_2 of cellulose), 1323.07cm-1 (cellulose and hemicellulose molecules), 1250.34cm-1 (O-H phenolic groups) and 1043.22cm-1 (O-H groups). The absorption band at 1640.08cm-1 to 1043.22cm-1corresponds to the presence of lignin molecule. After 42 days, the spectrum showed a slight increase in percentage transmittance and shift in absorption from 1640.08cm^{-1} to 1642.57cm^{-1} , 1399.46cm^{-1} to 1389.45cm^{-1}, 1323.07cm^{-1} to 1320.27cm^{-1}, 1250.34cm^{-1} to 1253.14cm^{-1}, and 1043.22cm^{-1} to 1037.30cm^{-1}.

Reducing sugar yield

Table 6 shows the sugar concentration and reducing sugar yield from corn cob and cassava peels by the selected isolates. Selection of the substrates was based on the results obtained from table 5. The action of the two cellulolytic thermophiles on pretreated corn cobs generated sugar concentrations of 2.2mg/mL (*Geobacillus* sp. OCO5) and 0.8mg/mL (*Geobacillus* sp. OCO1) respectively while their action on pretreated cassava peels produced sugar concentrations of 1.12mg/mL (*Geobacillus* sp. OCO5) and 0.6mg/mL (*Geobacillus* sp. OCO1). The corresponding calculated reducing sugar yield for corn cobs was 68.75% (*Geobacillus* sp. OCO5) and 35% (*Geobacillus* sp. OCO1), while for cassava peels it was 25% (*Geobacillus* sp. OCO5) and 18.75% (*Geobacillus* sp. OCO1). For both isolates, corn cobs gave a higher reducing sugar yield.

Table 6. Sugar concentration generated and reducing sugar yield by cellulolytic thermophiles on selected substrates.

Cellulolytic thermophile	Lignocellulosic substrate	Sugar concentration (mg/mL)	Reducing sugar Yield (%)
Geobacillus sp.OCO5	Cassava peels	1.12 (0.14)	35.00 (0.35)
	Corn cobs	2.20 (0.42)	68.50 (0.69)
Geobacillus sp.OCO1	Cassava peels	0.60 (0.28)	18.75 (0.53)
	Corn cobs	0.80 (0.13)	25.00 (0.38)

Standard deviation of the replicates are indicated in parenthesis

Gas chromatogram analysis for bio-ethanol production

In single phase fermentation, a high ethanol concentration (1.2718g/mL) and yield (62.13%) was obtained using pretreated corn cobs with a final glucose concentration of 0.041g/mL and xylose concentration of 0.383g/mL compared with pretreated cassava peels which gave an ethanol concentration of 0.9223g/mL and yield of 45% with a final glucose concentration of 0.122g/mL and xylose concentration of 0.561g/mL (Table 7). Other by-products of the fermentation include methanol and methanoic acid.

In the two phase fermentation, pretreated corn cobs gave the highest ethanol concentration (1.6843g/mL) and yield (88.65%) with a final glucose concentration of 0.031g/mL and xylose concentration of 0.424g/mL while pretreated cassava peels also gave a high ethanol concentration (1.4838g/mL) and yield (72.59%) with final glucose concentration of 0.031g/mL and xylose concentration of 0.424g/mL respectively (Table 7). Other by-products of fermentation observed include methanol and methanoic acid.

Statistical analysis showed that the different types of fermentation had a significant effect ($P<0.05$) on the different ethanol concentrations, ethanol yields and final glucose and xylose concentrations observed.

Table 7. Ethanol yield of pretreated substrates at different phases of fermentation by isolates

Fermentation type	Pre-treated Substrate	Ethanol concentration (g/mL)	Ethanol yield (%)	Glucose concentration (g/mL)	Xylose concentration (g/mL)
One phase	Corn cobs	1.2718 (0.25)	62.13 (0.12)	0.041 (0.05)	0.383 (0.13)
	Cassava peels	0.9223 (0.14)	45.00 (0.11)	0.122 (0.12)	0.561 (0.19)
Two phase	Corn cobs	1.6843 (0.22)	88.65 (0.23)	0.031 (0.01)	0.424 (0.11)
	Cassava peels	1.4838 (0.16)	72.59 (0.05)	0.062 (0.01)	0.162 (0.16)

Standard deviation of the replicates are in parenthesis

Discussion

The percentage of thermophilic cellulolytic bacteria isolated from different compost sites in this study was low (11%). This is similar to the report by Yang et al. [18] in which 6% of cellulolytic thermophilic bacteria was isolated from different compost sites in India. This observation may be due to variable optimal conditions needed for the proliferation of the organism at the site of isolation. This validates the need for pre-enrichment culture step under optimal conditions prior to isolation as stated by Abdulnasser and Ahmed [28]. In this study, the isolation temperature used was 55^{o}C.

All the cellulolytic thermophilic bacteria showed tolerance to 10% ethanol concentration while only three of the isolates showed tolerance to 15% ethanol concentration. This result is in contrast to the study by Jiunnet al. [16] in which only two thermophilic facultative anaerobic bacteria showed tolerance to 10% ethanol tolerance amongst other anaerobic bacterial isolates. The increased tolerance to ethanol observed in this study may be due to the possible presence of ethanol and chemical inhibitors produced as by-products of metabolism at the site of isolation. The two cellulolytic thermophiles selected for further studies (*Geobacillus* sp. OCO5 and *Geobacillus* sp. OCO1) produced the largest zones of cellulolytic clearance and could tolerate 15% ethanol concentration. These organisms also grew at an optimum pH of 7 and temperature of 55^{o}C. In a study by Hansunuma and Kondo [14]; and Bashir and Mohd [13] and it was documented that such features are a suitable advantage for industrial scale production of bio-ethanol.

The most degraded lignocellulosic substrate by Pleurotus tuber-regium after 42 days pretreatment was corn cobs (36.21% lignin loss), followed by cassava peels (16.82% lignin loss) and saw dust (1.13% lignin loss). This is similar to a study by Kai et al. [29] in which a lesser percentage weight loss of lignin (i.e. initial lignin content of 17.1% and final lignin content of 13.1%) was observed in the dilute acid pretreatment of corn cobs. The observation in this result may be due to the use of an effective lignin degrading white rot fungi in this study[21]. The low percentage weight loss of lignin in this study is probably due to the reduced incubation period for saw dust pretreatment carried out in this experiment (42 days incubation period)[21,23]. In a study by Forough et al. [23], it was reported that the longer the incubation period, the better the biological pretreatment of lignocellulosic wastes.

The FT-IR spectra showed that biological pretreatment using *Pleurotus tuber-regium*, resulted in lignin reduction in lignocellulosic wastes used. This was deduced from an increase in intensity and percentage transmittance of peaks for pretreated cassava peels, corn cobs and saw dust. This result is similar to that reported by Forough et al. [23] in which an increase in intensity of lignin peaks suggested lignin degradation and shifts in wave numbers suggesting the distortion of the carbon skeleton. The absence of band absorptions at 1700cm^{-1}in this study also suggested the absence of toxic inhibitory by-products such as carboxylic acids and anhydrides during the depolymerisation of the lignin polymer.The result also suggests that biological pretreatment increased the porosity of the molecule, increased the surface area and accessibility of enzymes for subsequent hydrolysis of cellulose.

The result of the statistical analysis carried out using SPSS version 21 shows that biological pretreatment using *Pleurotus tuber-regium* had a significant effect ($P<0.05$) on the lignin reduction observed in the different lignocellulosic wastes. The significant lignin reduction observed after biological pretreatment is similar to that reported by Jae et al. [30]; and Forough et al. [23]. These authors documented that the use of white-rot fungi had a significant effect ($P<0.05$) on lignin reduction.

Geobacillus sp. OCO5 produced the highest reducing sugar yield from pretreated corn cobs (68.75%) and cassava peels (35%) compared to *Geobacillus* sp. OCO1 which produced a lower reducing sugar yield from pretreated corn cobs (25%) and cassava peels (18.75%). This result may be because *Geobacillus* sp. OCO5 could produce more cellulolytic enzymes that could liberate reducing sugars compared to *Geobacillus* sp. OCO1. This result is in contrast to

that reported by Olanbiwoninu and Odunfa [31] in which a higher reducing sugar yield of 88% was produced from the dilute acid pretreatment of cassava peels. This difference may be due to the reduced percentage of lignin degradation after biological pretreatment of cassava peels subsequent to enzymatic hydrolysis in this study.

Single phase fermentation of pretreated cassava peels using *Geobacillus* sp. OCO5 gave an ethanol concentration of (0.9223g/mL) and an ethanol yield of 45%. This result is similar to that reported by Oyeleke *et al*. [17] in which a lesser ethanol yield of 23% was reported from the separate hydrolysis and fermentation of pretreated cassava peels. The high yield reported in this study may be due to the use of a cellulolytic thermophile with a high tolerance to ethanol and the simultaneous saccharification and fermentation of glucose sugars produced during consolidated bio-ethanol processing. Result of this study is in contrast to that reported by Jirasak [32], in which a higher ethanol yield of 84.34% was reported from the fermentation of pretreated cassava peels by *Saccharomyces cerevisiae*. The lesser yield obtained in this study may be due to the production of other by-products of fermentation such as methanol and methanoic acid by the cellulolytic thermophile.

Single phase fermentation of pretreated corn cobs using *Geobacillus* sp. OCO5 gave an ethanol concentration of 1.2718g/mL and an ethanol yield of 62.13%. This result is similar to that reported by Davinia et al. [27] in which a higher ethanol yield of 90% was produced from the fermentation of pretreated lignocellulosic waste using *Saccharomyces cerevisiae*. The difference may be due to the production of other by-products of fermentation such as methanol and methanoic acid by the cellulolytic thermophile. However, the result of this study is in contrast to that reported by Kai et al. [29] in which a lesser ethanol yield was produced from the fermentation of dilute acid pretreated corn cobs. In addition, this result is similar to that reported by Nan et al. [33] in which an ethanol yield of 62% was reported from the fermentation of pretreated corn cobs.

In two phase fermentation of pretreated corn cobs and pretreated cassava peels using *Geobacillus* sp. OCO5 and *Saccharomyces cerevisiae*, a better ethanol yield of 88.65% (1.6843g/mL) and 72.59% (1.4838g/mL) was observed respectively compared to single phase fermentation. The result of this study is in agreement with that reported by Xuan [26], in which a better ethanol yield (73%) was produced from the two phase fermentation of pretreated corn stover compared to the single phase fermentation of corn stover (ethanol yield of 60%). The ability of the cellulolytic thermophile to hydrolyse cellulose and provide glucose sugars needed by the baker's yeast for subsequent fermentation as well as also fermenting the glucose sugars may have contributed to the better yield in this study. However, the result of this study is in contrast to that reported by Sheelandra and Shilpa [34], where an ethanol yield of 32.72% was produced from the two phase fermentation of pretreated corn cobs under optimal fermentation conditions. The better yield in this study may be due to the successful synergy between the cellulolytic thermophile (*Geobacillus* sp. OCO5) and the yeast (*Saccharomyces cerevisiae*) compared to single phase fermentation. The result of this study is also similar to that reported by Farook and Mohammed [35] in which a lesser ethanol yield of 63% was produced from the fermentation of pretreated corn cobs using a co-culture of *C. tropicalis* and *Saccharomyces cerevisiae*. The better yield in this study might be attributed to the availability of cellulose and the possible production of cellulolytic enzymes which yielded minimal amounts of cellobiose and more glucose sugars that could be readily fermented. Statistical analysis of the results shows that the different type of fermentations had a significant effect (ANOVA at $\alpha_{0.05}$) on the residual concentration of monomeric sugars (glucose and xylose), ethanol concentration, and ethanol yield after the fermentation.

The residual amount of glucose after the single phase fermentation of pretreated corn cobs and cassava peels was 0.041g/mL and 0.122g/mL while after two phase fermentation, glucose concentrations of 0.031g/mL and 0.062g/mL was reported. The result of this study suggests that the bacteria (*Geobacillus* sp. OCO5) and yeast inocula (*Saccharomyces cerevisiae*) readily used up the glucose sugars during fermentation. This observation is similar to the study reported by Daviniaet al. [27] in which a residual glucose concentration of 0.1g/mL was recorded.

In two phase fermentation of pretreated corn cobs and cassava peels, the pH of the experiment was not controlled. The successful synergy between the bacteria (*Geobacillus* sp. OCO5) and yeast inocula (*Saccharomyces cerevisiae*) suggests that the bacterial inocula provided the required carbon source needed by the yeast. The result of this study is in consonance with the report documented by Xuan [26].The author study documented a successful synergy between bacteria and yeast inocula in two phase fermentation.

Conclusion

The most suitable lignocellulosic substrate for biological pretreatment in this study was corn cobs which resulted in a relatively high lignin loss and minimal cellulose loss. The cellulolytic thermophile *Geobacillus* sp. OCO5 isolated from compost heap

is a promising candidate for bio-ethanol production due to its relatively high generation of reducing sugars that can be utilized during fermentation, its capability to give a good ethanol yield, ability to tolerate 15% ethanol, growth at an optimum pH of 7 and temperature of 55^{o}C. The synergistic effect between the cellulolytic thermophile (*Geobacillus* sp. OCO5) and baker's yeast (*Saccharomyces cerevisiae*) produced the best ethanol yield compared to the single phase fermentation experiments in this study.Thus, this type of synergy can be employed in the fermentation of lignocellulosic biomass for probable large scale bio-ethanol production.

References

[1] Perez, J., Munoz-Dorado, T., Rubia, J., and Martınez, J. 2005: Biodegradation and biological treatments of cellulose, hemicellulose and lignin: An over view of International Microbiology 5: 53–63.

[2] Reshamwala, S., Shawky, B., and Dale, B. 1995: Ethanol production from enzymatic hydrolysates of AFEX-treated coastal Bermuda grass and switchgrass. Applied Biochemistry and Biotechnology 51: 43–55.

[3] Waleed, K., Maha, M., Ibrahim, Y., Abdel, F., Nadia, A., Soliman, M., and Mahmoud, M. 2010: Laboratory investigation of ethanol production from agricultural residues. 18th European Biomass Conference and Exhibition (May 3-7, Lyon, France).

[4] Badger, C. 2007: An overview of ethanol from cellulose. Appalachian woody biomass to ethanol conference. Available at: www.wdo.org/ community/Badger2.pdf>2007

[5] Alya, L., and Ricke, S. 2012: Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Progress in Energy and Combustion Science 38(4): 449-467.

[6] Renewable Energy Network for the 21st Century (REN-21), 2006: Global Status Report. Paris: REN-21 Secretariat and Washington, DC: Worldwatch Institute.

[7] Renewable Fuels Association (RFA), 2010: US fuel ethanol industry biorefineries and capacity. Washington, DC: Renewable Fuels Association. Available at: http://www.ethanolrfa.Org/ industry/locations/

[8] Taherzadeh, M. 1999: Ethanol from lignocellulose-physiological effects of inhibitors and fermentation strategies. Chemical reaction engineering. Chalmers University of Technology. Göteborg, Sweden. Doctoral thesis Nr. 1247.

[9] Olsson, L., and Hahn-Hagerdal, B. 1996: Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microbiology and Biotechnology 18: 312-331.

[10] Wyman, C., Dale, B., Elander, R., Holtzapple, M., Ladisch, M., and Lee, Y. 2005: Coordinated development of leading biomass pretreatment technologies. Bioresources Technology 96: 1959-1966.

[11] Bunnell, K., Martin, E., Lau, C., Pelkki, M., Patterson, D., and Clausen, E. 2010: Hot water and dilute acid pretreatment from high and low specific gravity Populus sp. Bioengineering Research Lab. University of Arkansas. Poster Biofuels conference, Tampa, Florida.

[12] Perez, J., Touzel, M., Philippe, D., Eric, S., and Christelle, B. 2000: Thermobacillusxy-lanilyticusgen. nov., sp. a new aerobic thermophilic xylan-degrading bacterium isolated from farm soil. International Journal of Systematic and Evolutionary Microbiology 50: 315–320.

[13] Bashir, S., and Mohd S. 2013: Thermotolerant micro-organisms in Consolidated Bioprocessing for ethanol production: A review 1 & 2. Research in Biotechnology 4(4): 01-06.

[14] Hasunuma, T., and Kondo, A. 2012: Consolidated bioprocessing and Simultaneous Saccharification and Fermentation of lignocellulose to ethanol with thermotolerant yeast strains. Process Biochemistry 47(9): 1287-1294.

[15] Taylor, M., Eley, K., Martin, S., Tuffin, M., Burton, S., and Cowan, D. 2008: Thermophilic ethanologenesis: future prospects for second-generation bioethanol production. Trends in Biotechnology 27: 398-405.

[16] Jiunn, C., Fong, A., Charles, J., Svenson, K., Caine, T., Leong, J., Bowman B., Dianne, R., Glenn-Brett, A., and Neilan, P. 2006: Isolation and characterization of two novel ethanol-tolerant facultative-anaerobic thermophilic bacteria strains from waste compost. *Extremophiles* 10: 363–372.

[17] Oyeleke, S., Dauda, B., Oyewole, O., Okoliegbe, I., and Ojebode, T. 2012: Production of bio-ethanol from cassava and sweet potato peels. Advances in Environmental Biology6(1): 241-245.

[18] Yang, M., Zeng X., Tianjia, T., and Zhang, X. 2011: Screening of complex thermophilic microbial community and application during municipal solid waste aerobic composting. African Journal of Biotechnology10(67): 15163-15169

[19] Maney, S., Steinarrafn, B., and Johann, O. 2009: Ethanol production from mono-sugars and lignocellulosic biomass by thermophilic bacteria isolated from Icelandic hot springs. Journal of Agricultural science 22: 45-58

[20] Olutiola, P., Famurewa, O., and Sontag, H. 1991: An introduction to general microbiology-A practical approach. ISBN 3-89426-042-4.

[21] Adenipekun, C., and Fasidi, I. 2005: Bioremediation of oil-polluted soil by Lentinussubnudus, a Nigerian white-rot fungus. African Journal of Biotechnology 4 (8): 796-798

[22] Ehrman, T. 1994: Standard Method for Determination of Total Solids in Biomass. National Renewable Energy Laboratory: Golden, CO, USA.

[23] Forough, N., Dzulkefly, K., Abdullah, N., and Reza, A. 2013: Evaluation of Biological Pretreatment of Rubberwood with White Rot Fungi for Enzymatic Hydrolysis. Materials6: 2059-2073.

[24] Miller, G.L. 1959: Use of dinitrosalycilic acid reagent for determination of reducing sugar. Analytical Chemistry 31: 426-428.

[25] Statistical Analysis Software (SAS), 1988: Program, version 6.12; SAS Institute Inc.: Cary, NC, USA.

[26] Xuan, L. 2010: Bioethanol production from lignocellulosic feedstock using aqueous ammonia pretreatment and simultaneous saccharification and fermentation (SSF): process development and optimization. Graduate Theses and Dissertations. Paper 11300.

[27] Davinia, S., Alicia, P., Maria, L., Thelmo, L., Angel, T., and Maria, J. 2011: Fungal pretreatment-An alternative in second generation ethanol from wheat straw. Bioresources Technology102: 7500-7506.

[28] Abdulnasser, S., and Ahmed, E. 2007: Isolation and identification of new cellulase producing thermophilic bacteria from an Egyptian hot spring and some properties of crude emzyme. Australian Journal of Basic and Applied Sciences1(4): 473-478.

[29] Kai, L., Xiaohui, L., Jun, Y., Xueahi, L., Xu, F., Minghan, Z., Jianqiang, L., Yinbo, Q., and Lin, X. 2010: High concentration ethanol production from corn cob residues by fed batch strategy. Bioresources Technology101: 4952-4958.

[30] Jae, W.L., Ki-seob, G., Jun-Yeong, P., Mi-Jin, P., Don-Ha, C., and In-Gyu, C. 2007: Biological pretreatment of soft wood Pinusdensiflora by three white rot fungi. Journal of Microbiology1: 485-491

[31] Olanbiwoninu, A., and Odunfa, S. 2010: Enhancing the production of reducing sugars from cassava peels by pretreatment methods. International Journal of Science and Technology 2:9

[32] Jirask, K. 2012: Ethanol production from dilute acid pretreated cassava peel by FED batch Simultaneous Saccharification and Fermentation. International Journal of the Computer, the Internet and Management 20(2): 22-27.

[33] Nan, X., Nan, J., Mingjia, Z., Wei, Q., Rongxin, S., and Zhimin, H. 2014: Effect of different pretreatment methods of corncobs on bio-ethanol production and enzyme recovery. Journal of Cellulose Chemistry and Technology 48(3-4): 313-319.

[34] Sheelandra, M.B., and Shilpa, B. 2014: Bio-ethanol production from economical agro-waste (Groundnut Shell) in Simultaneous Saccharification and Fermentation mode. Research Journal of Pharmaceutical, Biological, and Chemical Sciences 5(6): 1211.

[35] Farook, L., and Mohammed, I. 2001: Production of ethanol and xylitol from corn cobs by yeasts. Bioresources Technology 77: 57-63.

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