Ire et al. Bioresour. Bioprocess. (2016) 3:26
DOI 10.1186/s40643-016-0104-x

Bioresources and Bioprocessing

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Production of bioethanol by bacterial co-culture from agro-waste-impacted soil through simultaneous saccharification and co-fermentation of steam-exploded bagasse

Francis Sopuruchukwu Ire, Victor Ezebuiro* and Chimezie Jason Ogugbue

Abstract

Background: The production of bioethanol by co-culture of cellulolytic and xylanolytic bacteria isolated from agro-waste-impacted soil through simultaneous saccharification and co-fermentation (SSCF) of steam-exploded bagasse was investigated.

Methods: The cellulolytic (VCE-19) and xylanolytic (VXE-41) isolates were screened using the Congo Red Plate Method. The DNS method was used in the determination of reducing sugar content. Chemical analysis of the sugarcane bagasse was determined using standard methods. The bagasse was subjected to steam explosion to reduce lignin content and enhance cellulose availability.

Results: Mean proximate composition analysis of the bagasse showed total carbohydrate and lignin content (% dry weight) of 70.3 ± 1.9 and 19.2 ± 1.2 before pretreatment and 85.4 ± 2.33 and 4.2 ± 0.44 after pretreatment, respectively. Phylogenetic analysis based on partial sequence of the 16S rRNA gene classified VCE-19 and VXE-41 as *Bacillus cereus* GBPS9 and *Bacillus thuringiensis* serovar kurstaki HD1, respectively. The sequences obtained from these isolates have been submitted to GenBank and accession numbers (KT350986.1 for VXE-41 and KT318371.1 for VCE-19) assigned to them. The result of the optimization of cultural conditions of the bacterial co-culture revealed optimum cellulase production at the following conditions: temperature, 40 °C; pH, 7; substrate concentration, 4.0 % (*w/v*); inoculum concentration, 4 % (*v/v*) and when yeast extract was used as nitrogen source. The gas chromatography–mass spectrometry (GC–MS) analysis of the fermentation broth detected the following components: acetone (3.49 g/L), ethylacetate (8.75 g/L), ethanol (19.08 g/L), *N*-propanol (4.96 g/L), isobutanol (3.73 g/L) and acetic acid (6.53 g/L).

Conclusions: This study has demonstrated the production of significant quantity of ethanol by a co-culture of *B. cereus* GBPS9 and *B. thuringiensis* serovar kurstaki HD1 through SSCF of steam-exploded bagasse. Efficient bioethanol production from bagasse can help solve the need for alternative source of energy and the crisis that results from bioethanol production from food and feed crops.

Keywords: Co-culture, Bioethanol, Bagasse, Simultaneous saccharification and co-fermentation (SSCF), *Bacillus cereus*, *Bacillus thuringiensis*

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Background

Fossil energy sources such as oil, coal, and natural gas have contributed to the drastic increase in the level of greenhouse gases in the Earth's atmosphere (Ballesteros et al. 2006). This problem has resulted in the search for alternative energy sources that are environmentally friendly. Bioethanol is one of these alternative energy sources. Bioethanol production from agricultural waste is a promising technology; however, the process has several challenges and limitations viz., biomass transport, biomass handling, efficient pretreatment methods for total delignification of lignocellulosics and appropriate fermentative organism (Sarkar et al. 2012).

In 2007, the federal government of Nigeria released an official gazette of the Nigerian biofuel policy and incentives. The gazette contains policies and approaches to be taken by the federal government alongside its partners for efficient blending of ethanol and petrol in Nigeria. This gazette calls on all stakeholders in the energy sector to research into ways of ensuring efficient indigenous production of bioethanol. The feedstocks recommended in the gazette include agricultural wastes as well as other sources in which the country has comparative advantage. Nigeria, from this vantage point, has large natural resources to support the development and even commercialization of bioethanol.

Traditionally, ethanol is produced from the processing of starch, utilizing enzymatic liquefaction and saccharification; leading to the production of a relatively clean glucose stream that is then fermented to ethanol by *Saccharomyces* (Sarkar et al. 2012). However, this yeast, *Saccharomyces cerevisiae*, cannot utilize the main C-5 sugar (xylose) of the hydrolysate resulting from agricultural waste hydrolysis (Xu et al. 1998; Talebnia et al. 2010). This limitation has prompted the need for microorganisms with the ability for enhanced sugar utilization; *Zymomonas mobilis* is one bacterium that has shown high potentials in this regard (Sarkar et al. 2012).

Sugarcane (*Saccharum officinarum*) is used worldwide as a feedstock for ethanol and sugar production (Rezende et al. 2011). Nigeria is one of the most important producers of the crop with a land potential of over 500,000 hectares of suitable cane field capable of producing more than 3.0 million metric tonnes of sugarcane (NSDC 2003). After sugarcane is milled for juice extraction, bagasse is obtained as a residue and corresponds to about 25 % of the total weight; containing 60–80 % of carbohydrates (Rezende et al. 2011). The plant cell wall of sugarcane bagasse is similar to those of other plants; it is formed by two carbohydrate fractions (cellulose and hemicellulose) embedded in a lignin matrix. Lignin is a phenolic macromolecule, resistant to enzyme attack and degradation, and thus its content and distribution are recognized as the most important factors determining cell wall recalcitrance to hydrolysis (Mosier et al. 2005; Himmel et al. 2007; Taherzadeh and Karimi 2008).

Processing of bagasse to ethanol follows the same procedure as the conversion of lignocellulosics to ethanol. The procedure involves three major operations: pretreatment for delignification, which is necessary to liberate cellulose and hemicellulose before hydrolysis; hydrolysis of cellulose and hemicellulose to produce fermentable sugars (glucose, xylose, arabinose, galactose and mannose) and fermentation of sugars to ethanol. The non-carbohydrate components of lignin also have value-added applications (Balat et al. 2008). Proper pretreatment methods can increase concentrations of fermentable sugars after enzymatic saccharification, thereby improving the efficiency of the whole process (Sarkar et al. 2012). Conversion of glucose as well as xylose to ethanol needs some new fermentation technologies and fermentative organisms, to make the whole process cost effective.

The main aim of this study was to produce ethanol through simultaneous saccharification and co-fermentation of pretreated sugarcane bagasse using a co-culture of *Bacillus* spp.

Methods

Biomass collection, processing and comminution

The agricultural biomass used in this study was sugarcane bagasse, obtained from local sugarcane sellers in Choba, Rivers State, Nigeria. The sugarcane bagasse was washed and dried at atmospheric temperature (28 ± 2 °C) for 3 days. The dry biomass was further ground with an electric blender (Philips blender HR2001, Japan), filtered with a 60-Mesh (0.250 mm) sieve and stored under dry conditions until use.

Chemical analysis of sugarcane bagasse

The method described by Milne et al. (1992) was used to determine the dry matter, acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents of the sugarcane bagasse. Crude protein was determined by Kjeldahl method and total carbohydrate by Clegg Anthone method as described by Sluiter et al. (2011). The method described by Sluiter et al. (2008) was used to determine crude fibre and total ash.

Cellulose

The ADF was used for the estimation of cellulose employing the method described by Sluiter et al. (2011). The contents of the crucible were covered with cooled (15 °C) 75 % or 24 N H_2SO_4 and stirred with glass rod to a smooth paste, breaking all lumps. Thereafter, the crucible was filled about half-full with acid, left to stand for 1 h after which the acid was drained away. The crucible

was then refilled with 72 % acid and left to stand for 3 h. The acid was filtered off as much as possible with vacuum and the content dried at 100 °C overnight and the weight determined thereafter. The loss in weight was taken as cellulose and it was calculated by the following formula:

$$\text{ADF}\ (\%) = \frac{\text{Weight of ADF} - \text{Weight of dried residue after acid treatment}}{\text{Weight of sample}} \times 100$$

Hemicellulose

Hemicellulose was determined as the difference between neutral detergent fibre NDF (%) and ADF (%).

$$\text{Hemicellulose}\ (\%) = \text{NDF}\ (\%) - \text{ADF}(\%)$$

Lignin

The residue that remained after the determination of cellulose was treated with phosphate-buffered solution (0.1 M, KH_2PO_4:K_2HPO_4, pH = 8) of 25 mM $KMnO_4$ for 90 min at 20–25 °C. Lignin was dissolved leaving cutin and silica as insoluble materials. The contents were then filtered through tarred sintered crucible using gentle suction and the residue obtained, washed with distilled water and then with acetone. The crucible and residue were dried in an oven at 100 °C

$$\text{Lignin}\ (\%) = \frac{\text{Wt. after acid treatment} - \text{Wt. after } KMnO_4 \text{ treatment}}{\text{Weight of sample}} \times 100$$

where Wt = weight of dried residue.

Sugarcane bagasse pretreatment

The sugarcane bagasse was pretreated using the steam explosion (SE), acid and alkali pretreatment methods. The pretreatment was performed to delignify the bagasse, a necessary procedure towards liberating cellulose and hemicellulose prior to hydrolysis (Ezebuiro et al. 2015). SE pretreatment method described by Sharma et al. (2007) was employed for the pretreatment of the sugarcane bagasse. Ten grams of each biomass was suspended in 90 mL of distilled water in a conical flask and placed in an autoclave for 45 min at 121 °C. After 45 min, the autoclave was depressurized by suddenly fully opening the valve. The solid residue remaining was collected and extensively washed with tap water until neutral pH was reached prior to simultaneous saccharification and fermentation (SSF). After the solid residue was washed, drying was achieved at 60 °C overnight using the method described by Fan et al. (1980). Thereafter, the dry hydrolysate was analysed for cellulose, hemicellulose and lignin content and stored in sterile polypropylene bags for further use.

The acid and alkali pretreatment methods described by Olanbiwoninu and Odunfa (2012) were employed in the pretreatment of the sugarcane bagasse.

Isolation of cellulolytic and xylanolytic bacteria

To isolate cellulolytic and xylanolytic bacteria from agricultural waste-impacted soils, aliquots from various dilutions (10^{-3}–10^{-6}) were plated in duplicate on carboxymethyl cellulose (CMC) agar (Apun et al. 2000) and xylan agar (Khasin et al. 1993; Agustini et al. 2012), respectively. The CMC agar [comprising (g/L) carboxymethyl cellulose (CMC), 5; $NaNO_3$, 1; K_2HPO_4, 1; KCl, 1; $MgSO_4$, 0.5; yeast extract, 0.5; glucose, 1 and agar powder, 17] and xylan agar (containing in 1 L of distilled water: 5 g Beechwood xylan; 1 g yeast extract; 0.2 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.25 g $CaCl_2 \cdot 2H_2O$; 0.6 g KH_2PO_4 and solidified with 16 g agar powder) were prepared by dissolving their respective ingredients in 1L distilled water. The mixtures were heated to a boil in order to homogenize the sample and sterilized in an autoclave at 121 °C for 15 min at 15 psi. The sterile molten CMC and xylan media were thereafter maintained at 45 °C in a water bath. Fifteen to twenty millilitres of the molten media was dispensed into sterile petri dishes and allowed to solidify. The inoculated CMC and xylan agar plates were incubated at 40 °C for 48 h (Apun et al. 2000; Behera et al. 2014).

Screening for cellulolytic and xylanolytic bacteria

After 48 h of incubation, each of the duplicate plates was screened for cellulase and xylanase activities by flooding the plates with 0.1 % Congo red solution, left undisturbed for 15–20 min and then destained with 1 M NaCl (Apun et al. 2000; Behera et al. 2014). Halo zones around the growing cellulolytic and xylanolytic bacterial isolates confirmed positive isolates. The ratio of the clear zone diameter to colony diameter was measured and the highest cellulase and xylanase producers were selected. The largest ratio was assumed to exhibit the highest activity; the selected isolates were transferred into minimal CMC and xylan agar slants for cellulase- and xylanase-producing bacteria, respectively. The slants were maintained at 4 °C for further analysis (Behera et al. 2014).

Ethanol tolerance test for the bacterial isolates

The isolates that showed high cellulolytic and xylanolytic activities (indicated by the high zone of hydrolysis after the cellulolytic and xylanolytic screening procedures using Congo Red) were subjected to ethanol tolerance test. CMC and xylan broths amended with varying concentrations of ethanol ranging from 0 (the control) to 10 % (*v/v*) were used in the screening procedure. Ten microliter (10 μl) of inoculum from a 24-h broth culture of each isolate was used to inoculate the test tubes containing the sterile CMC and xylan broths with various ethanol concentrations. The inoculated test tubes were incubated for 48 h. After 48 h of incubation, the optical density (OD) reading at 600 nm, cellulase and xylanase activities were determined. Isolates with the highest OD reading as well as cellulase and xylanase activity at elevated ethanol concentrations were taken for further analysis.

Estimation of enzyme activity

Cellulase and xylanase activities were assayed using dinitrosalicylic acid (DNS) reagent (Lab M, India) by estimation of reducing sugars released from CMC and xylan solubilized in 0.05 M phosphate buffer at pH 8, respectively (Bailey et al. 1992). The culture broths were filtered using Whatman™ Qualitative filter paper and the clear supernatant served as crude enzyme source. Crude enzyme was added to 0.5 mL of 1 % CMC for cellulase and xylan for xylanase in 0.05 M phosphate buffer and incubated at 50 °C for 30 min. After incubation, the reaction was stopped by the addition of 3 mL of DNS reagent for cellulase activity and 2 mL DNS reagent for xylanase activity and boiled at 100 °C in water bath for 5 min. Development of colour was observed after boiling and sugars liberated were determined by measuring absorbance at 540 nm. Respective cellulase and xylanase productions were estimated using glucose and xylose calibration curves. One unit of cellulase activity was expressed as the quantity of enzyme, required to release 1 μmol of glucose per min per mL under standard assay conditions (Behera et al. 2014) whilst one unit of xylanase activity was defined as the amount of enzyme that liberated 1 μmol of xylose equivalents per min per mL under the assay conditions (Coughlan and Hazlewood 1997).

Inoculum development

The best cellulase- (VCE-19) and xylanase-producing (VXE-41) bacteria were selected based on their enzyme activities (cellulase and xylanase), their ability to ferment sugar to ethanol and their tolerance to ethanol concentration of up to 6 % (*v/v*). Isolates with the highest cellulase and xylanase activities as well as highest ethanol tolerance were selected for further studies.

Pure cultures of VCE-19 and VXE-41 were individually maintained on CMC and xylan supplemented minimal agar slants in a refrigerator for further use. Pure cultures of VCE-19 and VXE-41 were inoculated in broth medium containing in 1 L of distilled water: 7 g K_2HPO_4; 0.1 g $MgSO_4$; 2 g KH_2PO_4; 1 g yeast extract; 0.5 g sodium citrate; 10 g glucose (pH 7) for the cellulase-producing bacterium (VCE-19) and 1 g yeast extract; 2.5 g $(NH_4)_2SO_4$; 2 g $KHPO_4$; 0.5 g $MgSO_4 \cdot 7H_2O$; 6 g K_2HPO_4 for the xylanase-producing bacterium (VXE-41) and incubated for 24 h. After incubation, these vegetative cells were used as inoculum source.

The effect of different parameters on the production of enzymes and reducing sugars by the co-culture of VCE-19 and VXE-41

Optimum inoculum concentration for the cellulase- and xylanase-producing bacterial (VCE-19 and VXE-41) co-culture was determined by varying the inoculum concentrations in the culture media. Each culture broth was inoculated with 0.5, 1, 2, 3, 4, 5 and 6 % (*v/v*) of inoculum obtained from 24-h culture broth of the co-culture, containing a total viable cell count of 5.4×10^8 cfu/mL. The culture broths were incubated at 37 °C for 48 h. After incubation, the cellulase activity was determined from the supernatants obtained from the fermentation broth by the DNS methods described earlier.

The effect of substrate loading on the production of cellulase and reducing sugars by the bacterial co-culture was determined by varying the substrate concentrations [1, 2, 3, 4, 5 and 6 % (*w/v*)] whilst leaving other parameters constant.

The effect of different incubation temperatures (25, 30, 35, 40, 45, 50 and 60 °C) on the production of cellulase and reducing sugar by the bacterial co-culture was studied by varying the incubation temperature of the culture media whilst other parameters were kept constant.

To determine the effect of different pH (5, 6, 7, 8, 9, 10 and 11) on the production of cellulase and reducing sugar by the bacterial co-culture, the pH of the culture medium was adjusted with 0.1 M HCl and 0.1 M NaOH whilst other parameters were kept constant.

Determination of the effect of different nitrogen sources on the production of cellulase and reducing sugar by the bacterial co-culture was studied by supplementing the culture media with 1 % (*w/v*) of each of the following nitrogen sources: $NaNO_3$, casein, NH_4NO_3, peptone, urea and yeast extract.

Phenotypic and biochemical characterization of selected bacteria

The selected bacterial isolates were subjected to several biochemical tests as described by Holt et al. (1994) and Madigan et al. (2012).

Molecular identification of isolates

DNA extractions, PCR amplification of the partial bacterial 16S rRNA genes and gel electrophoresis of the isolates were carried out at the Molecular Biology Laboratory of National Institute for Medical Research (NIMR), Yaba, Lagos, Nigeria. The PCR products were sent to GATC Biotech AG (European Genome and Diagnostics Centre) Jakob-Stadier-Platz 7, 78467 Constance, Germany for 16S rRNA sequencing.

DNA extraction was carried out directly from the samples using a QIAGEN QIAamp DNA extraction kit according to the manufacturer's instruction.

The PCR amplification of the partial 16S rRNA genes was carried out using the primer set 27F-5′-AGA GTTTG ATYMTGG CTC AG-3′, and 515R 5′-TACCGCGGCKG CTGGCA C-3′. The reaction was carried out according to the method described by Ezebuiro et al. (2015). Twenty microlitres containing 1× PCR buffer (Solis Biodyne), 1.5 mM magnesium chloride (Solis Biodyne), 0.2 mM of each dNTP (Solis Biodyne), 2 U Taq DNA Polymerase (Solis Biodyne), 20 pMol of each primer and sterile water was used to make up the reaction mixture. PCR was carried out in an Eppendorf Nexus thermal cycler with the following cycling parameters: an initial denaturation step at 95 °C for 5 min, followed by 30 consecutive cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. After this, a final extension at 72 °C for 10 min was carried out.

After the PCR, PCR products were separated on a 1.5 % agarose gel. One hundred base-pair (100 bp) DNA ladder (Solis Biodyne) was used as DNA molecular weight marker. Electrophoresis was done at 80 V for 1 h 30 min, and the gel was viewed under UV light after staining with ethidium bromide.

Sequence analysis

The sequences generated by the sequencer were visualized using Chromaslite for base calling. BioEdit was used for sequence editing, before performing a Basic Local Alignment Search Tool (BLAST) using NCBI (National Centre for Biotechnology Information) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Similar sequences were downloaded and aligned with ClustalW and a phylogenetic tree was drawn with MEGA 6 software (Tamura et al. 2013).

Bioethanol production through SSFC of steam-exploded sugarcane bagasse

SSFC was carried out on pretreated bagasse by VCE-19 and VXE-41 singly and in consortium. The fermentation protocol was carried out under optimal conditions. Four percent (*w/v*) (i.e. 8 g of each sugarcane bagasse in 200 mL) of the pretreated bagasse in 250-mL Erlenmeyer flask containing 200 mL of the fermentation medium was used. The medium was sterilized at 121 °C for 20 min at 15 psi. After cooling to room temperature, 4 % (*v/v*) of each of the inoculum from a 24-h broth culture was added to the suspension of the biomass and incubated at 37 °C. The fermentation broth was monitored daily for 7 days for pH changes, enzyme activity and bacterial growth.

Estimation of fermentation products using gas chromatography–mass spectrometry (GC–MS)

The fermentation broth samples were centrifuged (Centrifuge 5804R, Eppendorf, Hamburg, Germany) at 13,000*g* at 4 °C for 3 min to separate suspended particles and the clear liquid was analysed for the presence of fermentation products. Before injection into the GC instrument, clarified samples and standards were filtered through 0.45-mm Whatman nylon filter (Whatman, UK) to remove insoluble materials that could block the column. All clear filtrate samples were kept frozen in sealed vials to maintain the stability of volatile components until they were analysed. Six grams per litre (6 g/L) of chromatographic samples was prepared with isobutanol as the IS (internal standard) in 2-mL screw-cap septum vials, which were then loaded into the autosampler.

Chromatographic conditions

The experiments were performed as described by Lin et al. (2013) using a GC system (Agilent 7890, Santa Clara, CA) equipped with an MS. Separation of compounds was conducted on a 60-m HP-INNOWAX capillary column of 0.25 mm i.d., coated with polyethylene glycol 0.25 mm film thickness using nitrogen as the carrier gas. The flow rate was 2 mL/min, the injector temperature kept at 180 °C with a split ratio of 90:1 whilst the GC–MS temperature was 220 °C. The oven temperature was programmed as follows: the column was held initially at 70 °C for 0.5 min, then increased to 190 °C at 20 °C/min and held at that temperature for 14 min. As the sample was injected into the GC, the sample first underwent gasification in the injection port liner, and then entered the capillary column with a split ratio of 90:1. The actual injection volume was 0.011 mL, so there was no column damage. Chromatographic data were recorded and integrated using Agilent Chemstation software.

Statistical analysis

The results obtained in the study were compared by one-way analysis of variance (one-way ANOVA) and multiple range tests to find the differences between the measurement means at 5 % (0.05) significance level. The data were analysed using IBM® SPSS® Statistics Version 20.0 (Gailly and Adler, US) (Ezebuiro et al. 2015).

Results

Composition analysis and pretreatment

The total carbohydrate and lignin composition of the sugarcane bagasse before and after pretreatment is shown in Table 1. The result indicated that the total available carbohydrate and lignin before pretreatment were 70.3 ± 1.9 and 19.2 ± 1.2 % (*w/w*), respectively. The best pretreatment method (steam explosion) achieved increase in total carbohydrate of 85.6 ± 2.33 % dry weight whilst the lignin was reduced to 4.2 ± 0.44.

Screening for cellulolytic and xylanolytic bacteria

Table 2 depicts the zone of clearance (mm) and ethanol tolerance level of the selected isolates. Out of the 84 bacterial isolates from agricultural waste soils screened for cellulase and xylanase production, 46 showed zones of clearance. One cellulase and one xylanase were selected based on their enzyme productivity, ethanol tolerance and ability to primarily ferment sugar to ethanol.

Optimization of cultural conditions for enzymatic hydrolysis by the bacteria

Effect of inoculum concentration on cellulase and reducing sugar production

The effect of inoculum concentration on cellulase and reducing sugar production is depicted in Fig. 1a and Table 3. The result showed that optimum cellulase and reducing sugar production was achieved at 3 % (*v/v*) of the inoculum and that their production declined thereafter.

Effect of substrate concentrations on cellulase and reducing sugar production

Figure 1b and Table 3 show the effect of substrate concentrations on enzyme and reducing sugar production. There was steady increase in cellulase and reducing sugar production with increase in substrate concentration up to 4 % (*w/v*) of the substrate after which there was observed decrease in production. The result also indicated that the optimum cellulase and reducing sugar produced was obtained at substrate loading of 4 % (*w/v*).

Effect of incubation temperature on cellulase and reducing sugar production

Figure 1c and Table 3 show the effect of incubation temperature on cellulase and reducing sugar production. The result revealed that optimum cellulase and reducing sugar production was obtained at temperature of 40 °C. Beyond 40 °C there was noticeable decline in both cellulase and reducing sugar production.

Effect of different pH on cellulase and reducing sugar production

The effect of medium pH on cellulase and reducing sugar production is shown in Fig. 1d and Table 3. The result revealed that optimum amount of reducing sugar and cellulase production was obtained at pH of 7. From the result obtained, it was observed that neither acidic nor alkaline pH enhanced the production of cellulase and reducing sugar.

Effect of nitrogen source on cellulase and reducing sugar production

Figure 2 and Table 3 depict the effect of nitrogen source on cellulase and reducing sugar production. The best nitrogen source for both cellulase and reducing sugar production was yeast extract followed by sodium nitrate, whilst ammonium nitrate yielded the least cellulase and reducing sugar.

Production of bioethanol by simultaneous saccharification and fermentation

Table 4 shows the summary of the GC–MS estimation of the three set-ups containing the steam-exploded

Table 1 Total carbohydrate and lignin composition of the sugarcane bagasse before and after pretreatment

Biomass	Total carbohydrate (% dry weight)	Cellulose	Hemicellulose	Lignin (% dry weight)
Untreated bagasse	70.3 ± 1.9	38.2 ± 1.3	31.4 ± 1.1	19.2 ± 1.2
SE pretreated bagasse	85.4 ± 2.33	79.5 ± 0.7	5.9 ± 0.4	4.2 ± 0.44
Acid pretreated bagasse	79.8 ± 4.6	72.3 ± 1.1	7.5 ± 0.6	6.9 ± 1.6
Alkali pretreated bagasse	80.21 ± 3.0	74.1 ± 0.8	6.1 ± 0.9	6.82 ± 0.83

All values are mean ± SD for triplicate cultures

Table 2 Enzyme productivity and ethanol tolerance of selected bacterial isolates during screening

Isolate code	Type of screening	Zone of clearance	Ethanol tolerance at 6 % *v/v* ethanol ($A_{600\ nm}$)
VCE-19	Congo red (cellulase)	2.75 ± 0.02	0.4729 ± 0.03
VXE-41	Congo red (xylanase)	2.5 ± 0.07	0.2784 ± 0.06

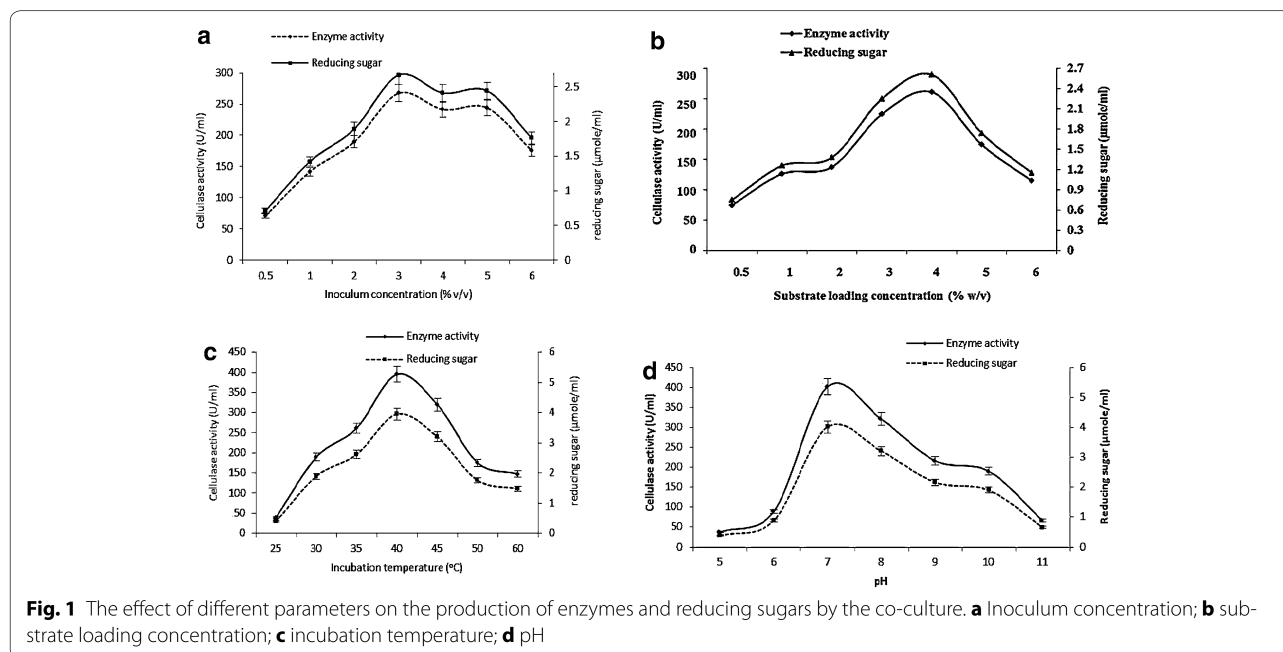

Fig. 1 The effect of different parameters on the production of enzymes and reducing sugars by the co-culture. **a** Inoculum concentration; **b** substrate loading concentration; **c** incubation temperature; **d** pH

Table 3 Effect of cultural conditions on enzymatic hydrolysis of sugarcane bagasse

Inoculum concentrations (IC)		Substrate concentration (SC)		Incubation temperature (T)		PH		Nitrogen sources (NS)	
IC (%)	Cellulase (U/mL)	SC (%)	Cellulase (U/mL)	T (°C)	Cellulase (U/mL)	PH	Cellulase (U/mL)	NS (%)	Cellulase (U/mL)
0.5	71 ± 7.4	0.5	75 ± 6.2	25	41 ± 3.7	5	38 ± 0.9	NH_4NO_3	62 ± 2.8
1	142 ± 6.2	1	126 ± 3.9	30	189 ± 8.3	6	90 ± 8.3	Casein	104 ± 1.9
2	189 ± 10.2	2	138 ± 0.7	35	262 ± 9.3	7	402 ± 22.2	Urea	228 ± 0.9
3	267 ± 1.5	3	225 ± 4.9	40	395 ± 11.1	8	321 ± 0.0	Peptone	237 ± 6.5
4	241 ± 0.9	4	261 ± 3.6	45	320 ± 1.9	9	216 ± 7.4	$NaNO_3$	289 ± 4.6
5	244 ± 0.9	5	174 ± 4.3	50	176 ± 16.7	10	190 ± 0.9	YE	374 ± 1.9
6	181 ± 9.3	6	115 ± 3.1	60	213 ± 1.9	11	66 ± 6.5		

All values are mean ± SD for duplicate cultures; *YE* = yeast extract

sugarcane bagasse and the fermentative bacteria singly and in consortium. GC–MS chromatogram of the fermentation broth is given in Fig. 3. An ethanol content of 18.40 g/L was obtained in the set-up containing bagasse with *Bacillus cereus*, whereas 15.27 g/L of ethanol was obtained in the set-up containing bagasse with *B. thuringiensis* whilst the set-up containing a co-culture of the two *Bacillus* spp. had an ethanol content of 19.08 g/L.

Characterization of the bacterial isolates

Morphological and biochemical features classified the isolates as belonging to the genus *Bacillus.* Further molecular characterization based on phylogenetic analysis of their partial 16S rRNA genes classified them as *Bacillus cereus* GBPS9 for VCE-19 and *Bacillus thuringiensis* serovar kurstaki HD1 for VXE-41 (Figs. 4, 5). The isolates have been deposited at the Gen Bank and the respective accession numbers KT318371.1 and KT350986.1 have been assigned to them.

Discussion

This study was carried out to produce bioethanol from sugarcane bagasse using bacterial isolates from agro-waste-impacted soils. The bagasse served as substrates for the bacterial cultures used in the fermentation process. Sugarcane bagasse is an example of lignocellulosics and can be converted to bioethanol following pretreatment, hydrolysis and fermentation by appropriate microorganisms (Sarkar et al. 2012).

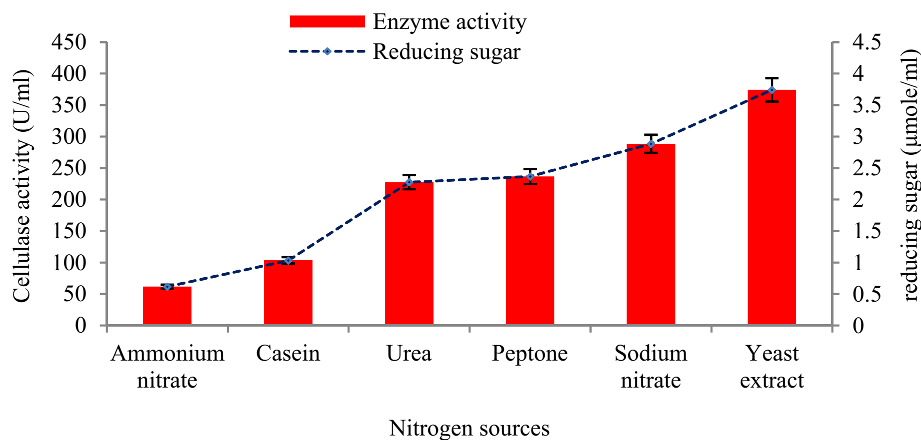

Fig. 2 Effect of different nitrogen sources on cellulase and reducing sugar production

Table 4 Products of SSF of bagasse as obtained from GC–MS analysis of fermentation broth

Sample Code	Acetone (g/L)	Ethyl acetate (g/L)	Ethanol (g/L)	*N*-propanol (g/L)	Isobutanol (g/L)	Acetic acid (g/L)
BA(A)	1.72	3.76	18.40	4.43	4.04	3.32
BA(B)	3.91	2.35	15.27	5.14	2.13	5.99
BA(A + B)	3.49	8.75	19.08	4.96	3.73	6.66

BA(A) = VCE-19; *BA(B)* = Bagasse with VXE-41 and *B(A + B)* = Bagasse with co-culture

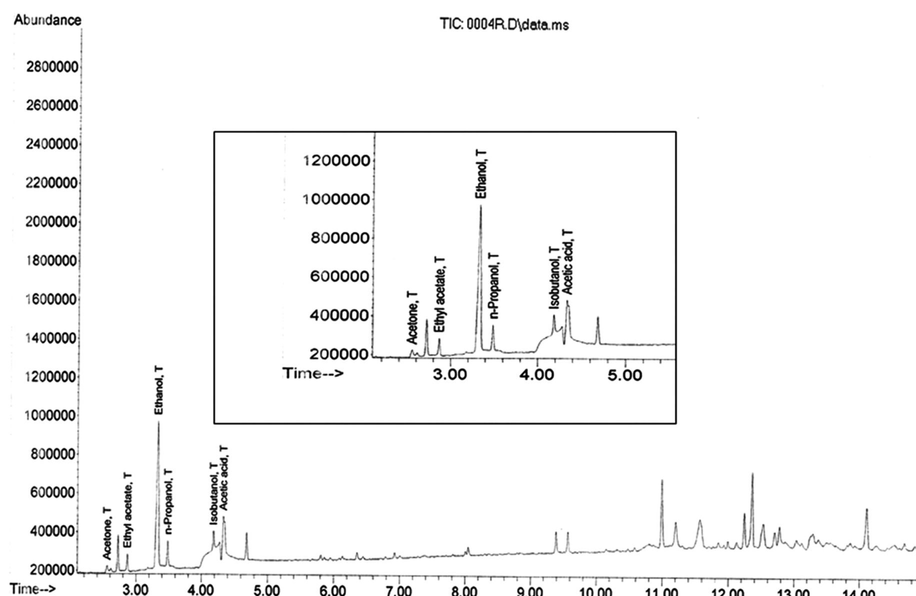

Fig. 3 Chromatogram of fermentation broth showing ethanol production by co-culture of *B. cereus* and *B. thuringiensis* with bagasse as substrate

Chemical analysis of the sugarcane bagasse after comminution showed that total available carbohydrate before pretreatment was 70.3 ± 1.9. It has been reported that minor differences in the chemical composition of bagasse between the different varieties of sugarcane exist (Gastón et al. 2000). This can be considered an advantage, given that the composition is to some extent homogeneous when using bagasse from different sources. Amores et al.

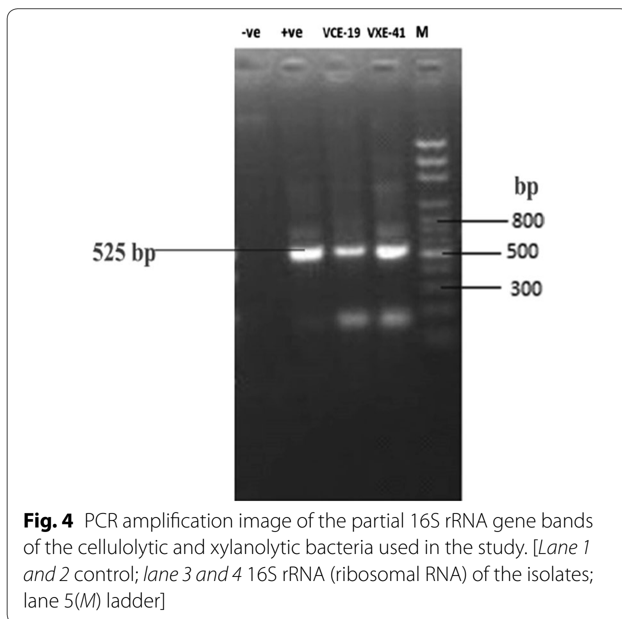

Fig. 4 PCR amplification image of the partial 16S rRNA gene bands of the cellulolytic and xylanolytic bacteria used in the study. [*Lane 1 and 2* control; *lane 3 and 4* 16S rRNA (ribosomal RNA) of the isolates; lane 5(*M*) ladder]

(2013) reported a total carbohydrate of 65 % dry weight for sugarcane bagasse used in ethanol production. Meanwhile, El-Tayeb et al. (2012) reported total carbohydrate composition of 86.9 % *w/w* for bagasse.

The processed bagasse was subjected to three different pretreatments, namely: steam explosion, acid and alkali pretreatments. The choice of the final pretreatment method used for the fermentation process was based on the analysis of the total carbohydrate content and the enzyme productivity obtained after the different pretreatments. The pretreatment method that gave the highest total carbohydrate content as well as enzyme production was steam explosion. The highest carbohydrate content obtained after the pretreatments of the bagasse was 85.4 ± 2.33. Ferreira-Leitão et al. (2010) and Amores et al. (2013) have reported different pretreatment methods for sugarcane bagasse. Ferreira-Leitão et al. (2010) and Amores et al. (2013) also reported steam explosion pretreatment for bagasse used as feedstock for ethanol production. Likewise, Martin et al. (2002) reported bagasse pretreatment by steam explosion using different impregnating agents.

Pretreatment is a necessary step in the use of lignocellulosics for bioethanol production. Joshi et al. (2011) described pretreatment as the most important rate limiting step in the overall bioethanol production process. Pretreatment was carried out to break the lignin–hemicellulose–pectin complex, disrupt/loosen-up the crystalline structure of cellulose and increase the porosity of the biomass used in the study. When these changes are achieved enzymatic saccharification becomes easier, resulting in higher fermentable sugar levels (Mosier et al. 2005; Sun and Cheng 2007; Yang and Wyman 2008). The pretreatment methods employed achieved high delignification of the different agricultural biomass.

The two bacterial co-cultures (VCE-19 and VXE-41) used in the study were classified as *Bacillus cereus* GBPS9 and *B. thuringiensis* serovar kurstaki HD 1 based on the phylogenetic tree analysis (Tamura et al. 2013) of their partial 16S rRNA genes sequences. The nucleotides of the partial sequence of 16S rRNA of the two isolates have been sent to the GenBank and accession numbers KT318371.1 and KT350986.1, assigned to them.

There are reports on the production of cellulase by *Bacillus cereus* strains and their potential in the production of bioethanol (Yan et al. 2011; Behera et al. 2014).

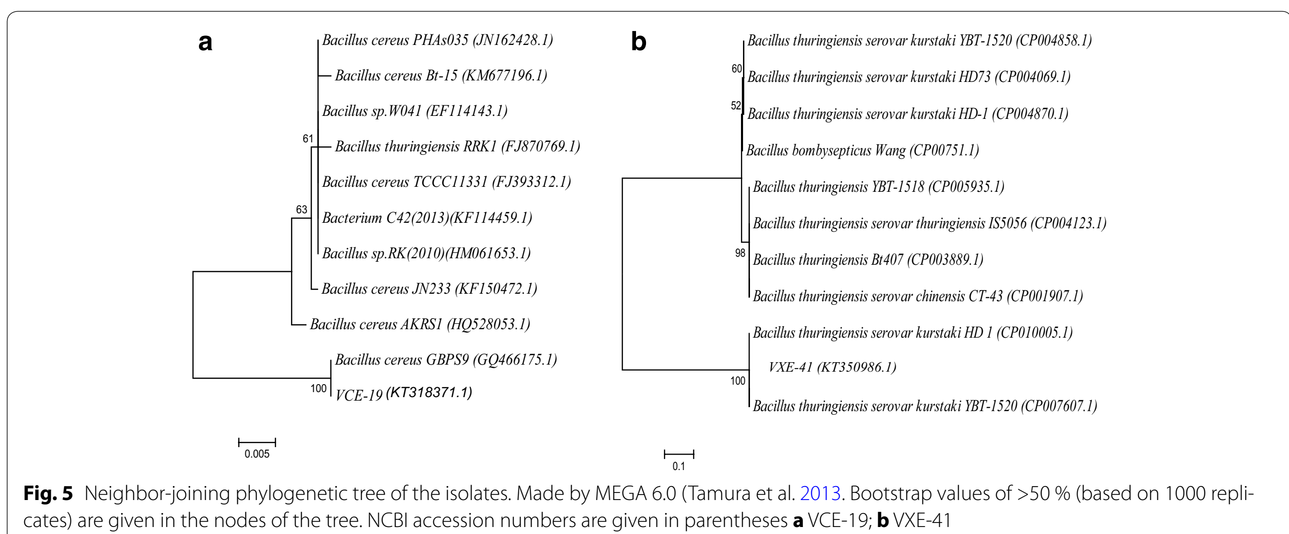

Fig. 5 Neighbor-joining phylogenetic tree of the isolates. Made by MEGA 6.0 (Tamura et al. 2013. Bootstrap values of >50 % (based on 1000 replicates) are given in the nodes of the tree. NCBI accession numbers are given in parentheses **a** VCE-19; **b** VXE-41

In this study, this organism showed high potentials for cellulase and bioethanol production. To achieve maximum cellulase production, the cultural conditions of the incubation medium of the bacterial co-culture were optimized by studying the effects of pH, temperature, substrate loading, inoculum concentration and nitrogen on the production of cellulase. The results obtained showed marked effects of all these parameters on enzyme production. Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives pH of the medium and presence of inducers are important parameters for the optimized production of cellulase enzymes. Other researchers have also reported cellulase production enhancement by the optimization of cultural conditions (Ray et al. 2007; Abou-Taleb et al. 2009; Behera et al. 2014; Ladeira et al. 2015).

Investigation of the effect of inoculum concentrations of the bacterial co-culture to produce cellulase and reducing sugar revealed maximum cellulase production of 267.4 U/mL with 3 % *v/v* inoculum concentration. However, Subramaniyan and Prema (2002) reported the use of 1.0–5.0 % (*v/v*) inoculum for hyper-production of xylanase.

The effect of different concentrations of the pretreated bagasse on the production of cellulase and reducing sugar by the bacterial co-culture revealed maximum cellulase and reducing sugar yield with substrate concentration of 4 % (*w/v*). This finding is similar to Sharma et al. (2007), who reported that amongst the various substrate concentrations used to enhance xylanase production, 2.25 % had a stimulatory effect and exhibited maximum enzyme production of 72.450 IU/mL. Additionally, the finding of this study is supported by Ashfaque et al. (2014) who reported maximum cellulase activity with 5 % bagasse concentration. Furthermore, Ojumu et al. (2003) reported high cellulase activity when 3 % pretreated saw dust substrate was used. However, Acharya et al. (2008) reported maximum cellulase activity with 9.6 % pretreated saw dust. Substrate concentration is an influencing factor that affects the yield and initial rate of hydrolysis of cellulose (da Silva et al. 2005). Very low substrate concentration fails to trigger enzyme production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. Optimum substrate concentration normally results in an increase in the yield and reaction rate of the hydrolysis (Regina et al. 2008). However, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme production (Liu and Yang 2007; Singhania et al. 2007). In this study, substrate concentrations in the excess of 5 % *w/v* were not favourable for enzyme production.

The bacterial co-culture yielded maximum amount of cellulase and reducing sugar when incubated at 40 °C. This shows that the isolates preferred temperature of 40 °C for cellulase production. This result is similar to other reports; Fagade and Bamigboye (2012) reported optimum cellulase activity for three *Bacillus* species when incubated at temperature of 40 °C. Incubation temperature is a critical factor in enzymatic productivity (Seyis and Aksoz 2003). Maximum enzyme production is obtained at optimal temperature and the decrease in enzyme production at lower or higher temperatures may be due to the fact that at these temperatures, growth of the organisms was inhibited, causing a decrease in the synthesis of the enzymes as suggested by Simões et al. (2009). In addition, production of more activity at optimum temperature may be due to faster metabolic activity, increase in protein content and extracellular enzyme production in culture. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity (Willey et al. 2008).

The optimal pH for the production of cellulase and reducing sugar by the bacterial co-culture was seven. Das et al. (2004) obtained optimum enzyme production at pH 7. Several other researchers (Subramaniyan and Prema 2002; Fagade and Bamigboye 2012) have reported pH 7 as the optimal pH for many bacterial xylanases and cellulases. Precisely, Fagade and Bamigboye (2012) reported optimum cellulase activity at pH of 7 for *Pseudomonas putida*, *Bacillus subtilis* and *B. lichenformis* I grown on corn cob.

Maximum cellulase production by the bacterial co-culture of *B. cereus* and *B. thuringiensis* was observed with yeast extract as the nitrogen source. This result shows that yeast extract was the preferred nitrogen source for cellulase and reducing sugar production.

Gas chromatography–mass spectrometry estimation of the fermentation broth showed that a higher ethanol production (19.08 g/L) was obtained in the set-up containing steam explosion pretreated bagasse fermented by the co-culture of *B. cereus* and *B. thuringiensis* when compared to ethanol production of 18.40 and 15.27 g/L obtained in fermentation by *B. cereus* and *B. thuringiensis*, respectively singly. This shows that the co-culture produced higher yield of ethanol when used to ferment sugarcane bagasse after pretreatment and enzyme hydrolysis than the individual isolates. The high ethanol yield observed particularly for the fermentation of bagasse by co-culture of *B. cereus* and *B. thuringiensis* suggests higher substrate conversion to reducing sugars by the enzymes produced by the co-culture. Taherzadeh and Karimi (2008) and Banerjee et al. (2010) explained that enzymatic hydrolysis is carried out by cellulase enzymes that are highly substrate specific. This

yield obtained can be compared with the yield by other wild-type bacteria. Svetlitchnyi et al. (2013) reported maximum ethanol yield of 3.5 g/L from the wild-type bacterium *Caldicellulosiruptor* DIB 004C. Sato et al. (1993) reported ethanol production of 4 g/L by wild-type *Clostridum thermocellum* strain I-1-B and an improved 23.6 g/L ethanol yield by the same strain when grown in optimized medium. The ethanol yield obtain in this study is higher than the yield (7.5 g/L) obtained from the fermentation of sugarcane bagasse hydrolysate using *Pichia stipitis* DSM 3651 as reported by Canilha et al. (2010) and a yield of 17.1 g/L as reported by Ingale et al. (2014) from banana pseudo stem. However, the yield by the co-culture is lower than the report of Sequeira et al. (2007) who achieved ethanol maximum yield of 58.6 g/L from soybean molasses by *Saccharomyces cerevisiae*. The high ethanol yield obtained with the co-culture shows the potentials of exploiting pentose (C_5) and hexose (C_6) sugar conversion to ethanol by simultaneous saccharification and co-fermentation of agricultural wastes biomass.

Conclusion

The cultural conditions (substrate loading, inoculum concentration, pH, temperature and nitrogen source) of the bacterial culture media used were optimized to enhance the enzyme production. It was observed that cellulase activity was enhanced by optimizing cultural parameters in the fermentation media.

A maximum ethanol yield (19.08 g/L) determined by GC–MS analysis of the fermentation broth after 7 days was obtained in the set-up containing co-culture of *B. cereus and B. thuringiensis* with sugarcane bagasse as the substrate. This study is novel as it has demonstrated efficient ethanol production by co-culture of *Bacillus cereus* GBPS9 and *B. thuringiensis* serovar kurstaki HD1 through simultaneous saccharification and co-fermentation of steam-exploded bagasse.

Authors' contributions

FSI and CJO designed the experiment. VE planned and conducted all the experiments, collected and calculated all data and drafted the manuscript under FSI's and CJO's supervision. FSI and CJO proofread and corrected the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors wish to acknowledge Mrs. Fuwora Adenike of Nigeria Institute of Medical Research, Yaba Lagos, Nigeria for her assistance with the molecular identification of the isolates. In addition, the authors wish to thank Mike Ochoche of Halden Nig. Ltd Port Harcourt, Nigeria, for carrying out the GC–MS analysis of the fermentation products.

Competing interests

The authors declare that they have no competing interests.

Received: 17 February 2016 Accepted: 11 May 2016
Published online: 26 May 2016

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