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Saccharification and co-fermentation of lignocellulosic biomass by a cockroach-gut bacterial symbiont and yeast cocktail for bioethanol production

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Abstract

Background The eco-friendly transformation of agro-industrial wastes through microbial bioconversion could address sustainability challenges in line with the United Nations' Sustainable Development Goals. The bulk of agro-industrial waste consists of lignocellulosic materials with fermentable sugars, predominantly cellulose and hemicellulose. A number of pretreatment options have been employed for material saccharification toward successful fermentation into second-generation bioethanol. Biological and/or enzymatic pretreatment of lignocellulosic waste substrates offers eco-friendly and sustainable second-generation bioethanol production opportunities that may also contribute to waste management without affecting food security. In this study, we isolated a promising filamentous bacterium from the guts of cockroaches with commendable cellulolytic activity. The matrices of sequential statistics, from one-factor-at-a-time (OFAT) through significant variable screening by Placket-Burman design (PBD) to Box–Behnken design of a surface methodology (BBD-RSM), were employed for major medium variable modeling and optimization by solid-state fermentation. The optimized solutions were used to saccharify lignocellulose in real time, and the kinetics of reducing sugar accumulation were subsequently evaluated to determine the maximum concentration of sugars extracted from the lignocellulose. The hydrolysate with the highest reducing sugar concentration was subjected to fermentation by *Saccharomyces cerevisiae*, *Klyuveromyces marxianus* and a mixture of both, after which the ethanol yield, concentration and fermentation efficiency were determined.

Results Sequential statistics revealed that rice husk powder, corn cob powder, peptone and inoculum volume were significant variables for the bioprocess at 59.8% (w/w) rice husk powder, 17.8% (w/w) corn cob powder, 38.8% (v/w; 10^9 cfu/mL) inoculum volume, and 5.3% (w/w) peptone. These conditions mediated maximum cellulolytic and xylanolytic activities of 219.93 ± 18.64 FPU/mL and 333.44 ± 22.74 U/mL, respectively. The kinetics of saccharification of the lignocellulosic waste under optimized conditions revealed two peaks of reducing sugar accumulation between 16 and 32 h and another between 56 and 64 h.

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Conclusions Although *K. marxianus* had a significantly greater fermentation efficiency than *S. cerevisiae*, fermentation with a 50:50 (% v/v) mixture of both yeasts led to 88.32% fermentation efficiency with 55.56 ± 0.19 g/L crude bioethanol, suggesting that inexpensive, eco-friendly and sustainable bioethanol production could be obtained from renewable energy sources.

Keywords Optimized coproduction, *Thermobifida fusca*, Furniture-deteriorating cockroaches, Cellulosic biomass, Yeast cocktail, Sustainability

Background

One of the greatest problems facing urban cities in Sub-Saharan Africa in recent times is waste containment, disposal and/or management [1]. Waste consists of biomass, metals, combustibles and minerals, and its composition varies from community to community, depending on the dominant activities of the people, which are dictated by their living standards [2, 3]. Accumulated waste is often a source of disease outbreaks, and leachate can seep into underground water and pollute it [4, 5]. Proper waste disposal is essential from the viewpoints of public and/or environmental health and aesthetics. Where no standard disposal method is available, management becomes an alternative and may include reduction in production, bioconversion, recycling and reuse [6].

Traditional waste management options have revolved around landfilling, composting, recycling and incineration for decades, if not centuries now [6, 7]. However, the current trendy strategy of circular economy is a growing area that advocates for a regenerative approach to natural resource management, as opposed to the highly unsustainable linear method, owing to the finite availability of raw materials for production [7–9]. The circularity principle is further propelled by the overarching issue of sustainable development, which has attracted global attention and adoption.

Waste can be valorized into green fuels and other value-added products through biological conversion processes [10–13]. Ubi et al. [14] used waste cassava peels to produce an assortment of raw-starch digesting amylases by *Priestia flexa* strain UCCM 00132, and the hydrolysate was converted to good yields of bioethanol by *Saccharomyces cerevisiae*. The production of bioethanol from agro-industrial materials has been heralded as a dependable alternative to fossil fuel. The driving advantage of fossil fuel replacement by lignocellulosic bioethanol is low CO_2 emissions, which will lead to a significant reduction in greenhouse gas emissions in the near future [15]. Currently, global bioethanol production is obtained from food-grade (first-generation) crops, including cassava, rice, corn and sugarcane. In Nigeria, 90% of bioethanol is produced by three companies, which rely on the importation of crude anhydrous bioethanol (feedstock) from Brazil to be refined into ethanol for specific applications beyond fuel, such as in the food and pharmaceutical industries [16]. The 123 million liters of bioethanol imported from Brazil in 2007 met only 2% of the national ethanol demand, suggesting gross insufficiency of the product and calling for immediate remedial approaches to bioethanol production. This situation seems to have worsened as Nigeria had to import between 300 and 350 million liters of anhydrous bioethanol for industrial purposes alone in 2019 [17]. A major reason for this importation is the country's inability to grapple with the huge food crises attendant on use of food-grade crops as feedstock for bioethanol production as observed in other countries. An alternative in lignocellulosic biomass (feedstock) has been recommended as renewable and sustainable [18].

Second-generation bioethanol production, which proceeds from lignocellulosic waste bioconversion, is still in its infancy but is surely the way to go, as it utilizes waste agro-industrial materials, solving environmental waste problems without infringing on food security [17–19]. The major advantage of lignocellulosic biomass ethanol is its non-competitiveness for arable land and food crops for human and livestock consumption [20]. Lignocellulosic bioethanol production has been reported from laboratories with many microorganisms successfully utilizing lignocellulosic wastes, such as switchgrass, corn cob, corn stalk, sugarcane bagasse, rice straw, rice and groundnut husks, cassava, yam and potato peels [20–23]. Apart from soil, sediment and water column sources of these microorganisms, recent reports support the presence of a significant population of lignocellulosic biomass degrading microorganisms in the guts of plant-eating insects such as termites, cockroaches and beetles. These gut microbiomes exist as symbionts in the host insects and provide reducing sugars and amino acids required for nutrition through hydrolysis of requisite lignocellulosic biomass using relevant enzymes [24–26].

The inherent ability of microorganisms to carry out bioconversion of waste lignocellulosic substrates into value-added products may not be significantly economical in a biotechnological sense [15]. Therefore, a number of approaches are available to improve metabolite production, including strain improvement; bioprocess upstream optimization, including media, environmental conditions, fermentation type and mode; and optimization of recovery and other downstream requirements [27–29]. The concept of optimization ensures that levels of significant bioconversion factors are carefully adjusted

to meet specific needs of the bio-converting microorganism. A typical optimization approach is response surface methodology (RSM), a stepwise statistical method that allows the building of mathematical models, especially of the second-order type, to simultaneously adjust the levels of significant influencing variables of a bioprocess [30, 31]. Fermentation type is also a factor considered in lignocellulose bioconversion [32]. Several reports support the production of lignocellulosic enzymes by submerged fermentation [33]. However, solid-state fermentation (SSF) may offer a rapid solution to waste problems while ultimately producing large amounts of value-added metabolites at low cost with little or no waste stream [34–36].

In the present study, we report the efficient RSM-optimized solid-state saccharification of rice husk and corn cob waste lignocellulosic biomass by an actinomycete isolated from the gut of furniture-deteriorating cockroaches. The obtained hydrolysate was fermented into bioethanol by a mixture of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* in keeping with the circular economy and sustainable renewable energy actualization. The lignocellulosic bioethanol potential of rice husk by solid-state fermentation is reported for the first time.

Materials and methods

Sample descriptions and bacterial isolation

The flow chart of the study is shown as Fig. 1 to clarify and guide the navigation of the various bioprocess steps. Five samples were analyzed for the isolation of cellulolytic bacteria, including decaying lawn grass (*Cynodon dactylon*), decaying sawdust, cow dung, and gut contents of cockroaches (*Periplanata americana*) and termites (*Microcerotermes diversus* [Silvestri] Isoptera: Termitidae). The decaying lawn grass samples were cut grass heaped on garden soils and allowed to decay for 6–9 months without recourse to sun or rain. Sawdust samples were obtained from a saw mill heap after monitoring for 6–9 months without recourse to sunlight or rain. Cow dung samples were obtained from abattoir cow dung heaps. The gut contents of 500 furniture-deteriorating cockroaches were emptied into 5 mL of sterile normal saline (0.85% w/v NaCl). Prior to this, cockroach surfaces were washed with 95% ethanol, immobilized at 4 °C for 20 min and each aseptically dissected with a pair of sterile scissors to extract the gut [37]. Termites (workers).

numbering ~ 1,000 were collected from ant-hills and surfaces washed with 95% ethanol for surface sterilization before gently macerating in a laboratory mortar with 5 mL of sterile normal saline [38]. Ten grams of decaying grass or decaying sawdust was soaked in 90 mL of sterile normal saline. Samples were plated in triplicate, from 10-fold serial dilutions, onto freshly prepared nutrient agar (Sigma–Aldrich, MA, USA) for isolation of aerobic and/or facultative anaerobic bacterial strains. The plates were incubated at room temperature (28 ± 2 °C) for 24–36 h. Morphologically-distinct colonies were isolated on the basis of colony pigmentation, elevation, form, margin and consistency and subsequently purified by repeated subculturing using the quadrant-streak plate technique.

Primary screening of pure bacteria for cellulolytic potential

All the purified isolates were screened for cellulolytic potential on minimal medium supplemented with carboxy-methyl cellulose (CMC – 0.1% w/v) as a carbon source. The minimal medium contained (g/L) $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, $MgSO_4.7H_2O$ 0.3, $CaCl_2$ 0.3, $FeSO_4.7H_2O$ 0.005, $ZnSO_4.7H_2O$ 0.0014, $CoCl_2.6H_2O$ 0.002, and $MnSO_4.5H_2O$ 0.0016 [39]. The medium was supplemented with 2% agar–agar (Sigma–Aldrich, MA, USA), and the pH was adjusted to 7.0 before sterilization (121 °C for 15 min). In brief, the plates were inoculated by single-line streaking of pure bacteria and incubated at room temperature (28 ± 2 °C) for 48–120 h. The cellulolytic potential was evaluated by flooding the plates with 1% (w/v) Congo red solution, allowing them to stand for 5 min and wash excess stain with 1 M NaCl solution for 20 min. The plates were also flooded with a solution of acetic acid (5% v/v) to improve contrast and enhance the clarity of the zone diameters. Potential cellulase-producing bacteria were selected on the basis of the diameter of the Congo red/NaCl/acetic acid clear zone.

Secondary screening of bacteria for cellulase production potential

Successful bacterial isolates from primary screening were subjected to shake flask studies in 250 mL Erlenmeyer flasks. The minimal medium (50 mL) was prepared for primary screening without agar or agar, with 0.1% (w/v) CMC serving as the carbon source. The flasks were incubated on a shaker (150 rpm) at room temperature for 120 h. The fermentation broth (4.0 mL) was centrifuged at 12,000 × *g* for 10 min. The proteins were detected and quantified in the supernatants by the Bradford protein-dye technique [40] using Coomassie brilliant blue dye (G-240) as the protein reagent and bovine serum albumin as the standard protein at a wavelength of 595 nm. Cellulolytic activity was assayed by the filter paper assay method [41, 42], and activity was reported as filter paper units per milliliter (FPU/mL). Briefly, 1 mL of sodium citrate buffer (0.05 M, pH 5.0 ± 0.2) was added to 0.5 mL of sterile crude enzyme solution appropriately diluted in citrate buffer and placed in a warm water bath at 50 °C. One strip (1 × 6 cm) of filter paper (Whatman No. 1) was then added, mixed and held at that temperature for 60 min. Afterwards, 3 mL of 3,5-dinitrosalicylic (DNS) acid solution was added, mixed and boiled in a vigorously

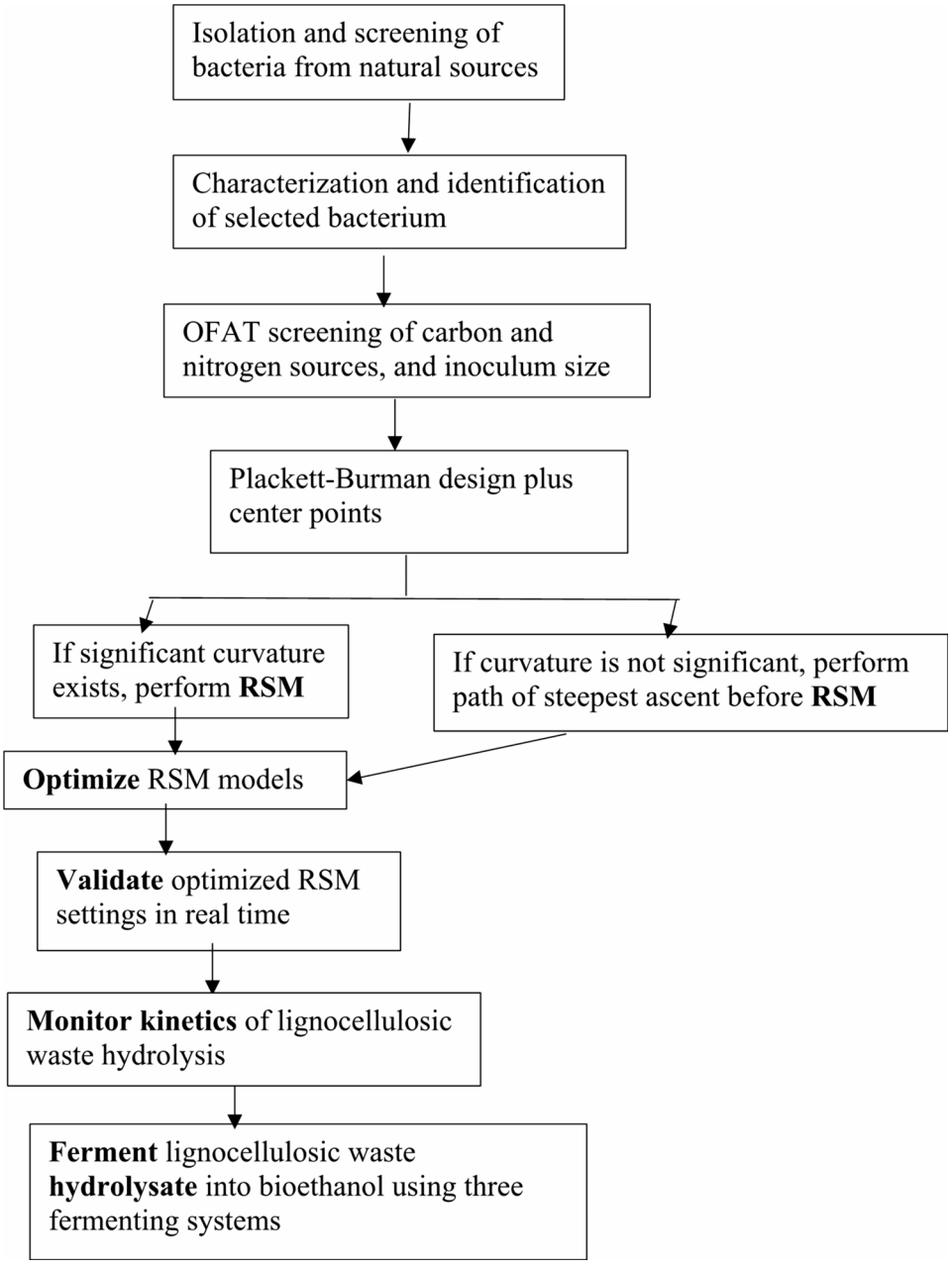

Fig. 1 Flow chart of lignocellulosic waste biomass saccharification and fermentation of hydrolysate into bioethanol

boiling water (100 °C) bath for 5 min. Samples, enzyme blanks, and glucose standards were all boiled together and subsequently transferred to a cold-water bath (4 °C). Finally, sterile distilled water (20 mL) was added to the tube, and the contents were mixed by continuous inversion for 20 min until the paper pulp settled. The color formed was measured by means of a UV−Vis's spectrophotometer (DR6000, HACH, Loveland, CO) at 540 nm. The amount of reducing sugar was quantified by the DNS method [43] using glucose as a standard. One filter paper unit of cellulase activity was defined as the amount of cellulase that released 2.0 mg of glucose from 50 mg of filter paper (1×6 cm) substrate in one minute under the assay conditions. All the determinations were made in triplicate, and the results are reported as the means±standard deviations. The isolate with the highest cellulolytic activity was selected for further studies.

Characterization and identification of selected cellulolytic bacterium

The selected bacterium was characterized by biochemical, physiological and molecular methods. Molecular characterization was conducted by DNA isolation, quantification, amplification, purification and sequencing of

the 16 S rRNA gene using reverse and forward primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [44]. The obtained sequences were analyzed via comparison with validated sequences in the GenBank database at the National Center for Biotechnology Institute (NCBI) using the basic local alignment search tool (BLAST) in Molecular Evolutionary Genetic Analysis (MEGA) software, version 11. A phylogenetic tree was constructed using the neighbor–joining approach to determine the closest relative of the isolate in the GenBank database.

Medium optimization for improved lignocellulosic bioconversion

One-factor-at-a-time screening of carbon sources

Carbon sources were screened by the one-factor-at-a-time (OFAT) method for improved lignocellulolysis by the selected bacterial strain. The carbon sources, including rice husk powder (RHP), corn cob powder (CCP), sugarcane bagasse (SCB), grass (lawn) biomass (GBM), sawdust powder (SDP), and carboxy-methyl cellulose (CMC) as control, were tested at a 0.1% (w/v) concentration. Lignocellulosic waste was pretreated by allowing each material to dry in air to a constant weight, followed by milling into powder with a diameter ≤ 3.0 mm. The amounts of cellulose, hemicellulose and lignin in each lignocellulosic material were quantified by wet chemistry as described by Zhang et al. [45]. The respective cellulose, hemicellulose and lignin contents of the lignocellulosic substrates were obtained as follows: RHB 37.74%, 33.85%, and 9.67%; CCP 36.84%, 24.61%, and 13.73%; SCB 33.22%, 24.93%, and 22.47%; GBM 32.29%, 29.34%, and 9.78%; and SDP 34.94%, 23.63%, and 21.83%.

The minimal medium was prepared for primary screening, and fermentation was conducted as described for secondary screening. The cellulolytic activity was measured in triplicate using a filter paper assay (FPU/mL). The results were compared using one-way analysis of variance (one-way ANOVA) in GraphPad Prism 8 at the 5% significance level, and significant means were separated by the Tukey HSD post hoc test. The best lignocellulosic biomass was used for further studies.

OFAT screening of nitrogen sources

The nitrogen sources screened included yeast extract, corn steep liquor, casamino acid, proteose peptone, NH_4NO_3, urea, KNO_3 and NH_4Cl as controls. The nitrogen sources were tested at 1% (w/v). The best waste lignocellulosic biomass served as a carbon source. The medium composition, incubation conditions, harvesting and determination were performed as described for carbon source screening, and the results were reported and compared as described for carbon source screening.

OFAT screening of inoculum sizes

The inoculum size was screened within the range of 10^4 to 10^{10} cfu/mL. The sizes were determined by serial 10-fold dilutions of twice-washed cells, and the absorbance of the dilutions was measured spectrophotometrically at a wavelength of 600 nm. The dilutions were adjusted with culture suspension until a regression curve between the optical density at 600 nm (OD_{600}) and the CFU/mL was obtained at $p=0.01$ and adjusted $r^2=0.99$. The obtained dilutions (inoculum sizes) were used to inoculate fermentation media in 500 mL Erlenmeyer flasks at 2% (v/v) for determination of cellulase activity on selected carbon and nitrogen sources. The results were reported and compared as described earlier.

Placket–Burman (2k) design screening for significant bioprocess parameters

The design matrix of Placket-Burman (PBD) in Design Expert software version 13.0 (Stat Ease, Inc., Minneapolis, USA) was used to screen for significant influencing bioprocess parameters among RHP, CCP, peptone, KNO_3, SCB, inoculum volume (INV - % v/w), Mn^{2+}, water content (% v/w), Ca^{2+}, Mg^{2+}, and Zn^{2+}. Cellulase activity (FPU/mL) was the response variable, *y*. The variables were tested at 2 levels, high (+1) and low (-), as per Table S3 (Supplementary material). To determine if a factorial model would suffice or not, 5 center points were included in the design, and a total of 17 experimental runs were obtained. Lignocellulosic waste degradation was performed by batch mode solid-state fermentation (SSF) in 500 mL Erlenmeyer flasks. All the experiments were performed in triplicate, and the flasks were incubated at 50 °C for 120 h. The obtained data were analyzed via ANOVA and a first-order model built with coefficients of significant main effect terms using the general form below [14].

$$y = b_0 + \sum b_i x_i + \sum b_{ij} \sum x_i x_j + \varepsilon \qquad (1)$$

where y is the response variable (cellulase activity), b_0 is the coefficient of the constant term, b_i is the coefficient of the linear term, b_{ij} is the coefficient of the interaction terms and e is the error term.

Response surface modeling and optimization

Four of the 11 variables screened by PBD were significantly associated with improved cellulolytic activity. The Box–Behnken design (BBD) of RSM in Design Expert software version 13.0 (Stat-Ease, Inc., Minneapolis, USA) was used to model the bioprocess (Table S5; Supplementary material). The design matrix consisted of RHP (X_1 - % w/w), CCP (X_2 - % w/w), INV (X_3 - % v/w) and temperature (X_4 - °C), with 5 center points and 24

factorial points in 29 experimental runs. Bioconversion was conducted by batch mode SSF in 500 mL Erlenmeyer flasks for 120 h at 50 °C. Cellulase (Y_1) and xylanase (Y_2) activities were the main responses. Cellulase activity was quantified as previously described. Xylanase activity was determined by measuring the release of reducing sugars from sterile supernatant from 1% (w/v) beechwood xylan (Sigma–Aldrich, MA, USA) prepared in 50 mM acetate buffer at pH 5 [46]. The reducing equivalent was determined via the DNS method. One unit of xylanase activity was defined as the amount of enzyme that liberated 1 µmol of xylose under the assay conditions. The data obtained were subjected to multiple regression analysis, and models were built by the least squares' method using the general quadratic form below [14].

$$\beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j=2}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where β_0 denotes a constant coefficient, k=the k^{th} factor, β_{ii}=quadratic effect of the i^{th} factor and β_{ij}=effect of the interaction between the i^{th} and j^{th} factors, where x_1, x_2 ……., and x_k are the independent variables and ε is the error arising from the computation of the response variable Y.

The quadratic models for the two response variables Y_{1-2} were subjected to multiobjective optimization to find a set of operating conditions for the input variables that would optimize both responses simultaneously. All the input variables were left at the 'in range' level with 'moderate importance' (***), while the response variables were left at the 'maximum' and 'highest importance' (*****). The global optimum solution was determined by the use of a composite desirability function calculated using the equation below [47]:

$$D = (d_1 \times d_2 \times d_3 \times \ldots\ldots \times d_n)^{1/n} = \left(\prod_{i=1}^{n} d_i\right)^{1/n} \quad (3)$$

where D is the composite desirability; n is the number of responses; and d_1, d_2, d_3 and d_n are the desirability of individual responses.

Validation of optimal operational conditions for lignocellulosic waste bioconversion

Triplicate experiments were performed to validate the predicted levels of the explanatory variables by the bacterium over a 120-h fermentation period in real time. Differences of more than 5% were considered unacceptable; otherwise, the predicted levels were upheld for further studies.

Lignocellulosic waste bioconversion kinetics

A time-series study was conducted to determine when peak saccharification of waste substrates occurred and the best time to harvest reducing sugars for bio-ethanologenesis. A standard curve for pentose sugars was prepared with D-xylose, and the assay was conducted according to the methods of Deschatelets and Yu [48]. The method is based on the formation of furfural from, first, a reaction between pentoses and acetic acid in the presence of the antioxidant thiourea at 70 °C and, second, the subsequent reaction of furfural with *p*-bromoaniline acetate to form a pink-colored product. The absorbance of the test and blank samples was read at 520 nm in a UV–Vis's spectrophotometer (DR6000, HACH, Loveland, CO). The amount of pentose was determined from the linear regression equation obtained from the xylose calibration curve. The study was set up in triplicate 500 mL Erlenmeyer flasks containing 200 g of reaction mixture. The amount of pentose sugar, total reducing sugar, and cellulolytic and xylanolytic activities were determined every 8 h during the 120-h saccharification process. The data were analyzed by one-way ANOVA, and the significance of the means was tested at the 95% confidence level.

Fermentation of lignocellulosic waste hydrolysate into bioethanol

The utilization of reducing sugars in the hydrolysate obtained from lignocellulosic waste bioconversion into ethanol was initiated. Fermentation was mediated by *Saccharomyces cerevisiae* strain UCCM 00054, *Kluyveromyces marxianus* strain NCYC 2303 and a cocktail of both at a 50:50 ratio (% v/v, 10^8 cells/mL).

The stock cultures of the *S. cerevisiae* strain UCCM 00054 and *K. marxianus* strain NCYC 2303 were separately reactivated in yeast extract peptone dextrose (YPD) agar media supplemented with (g/L) yeast extract 10, peptone 20, and glucose 50 (Sigma–Aldrich, MA, USA). The plates were incubated at 30 °C for 48 h. A loopful of each culture was subsequently transferred aseptically to 50 mL of YPD broth medium in a 250 mL Erlenmeyer flask and incubated for 24 h on an orbital shaker (150 rpm, 30 °C). The cells were harvested by centrifugation at 3,075 × *g* for 10 min and washed twice with deionized water. Cell densities of 3×10^8 cfu/mL were prepared for both strains by dilution and plating onto YPD agar. Next, a 2-fold dilution (50:50 mixture) of each culture was prepared using each yeast suspension as a diluent, and a final yeast concentration of 1.5×10^8 cells/mL was obtained.

The various yeast suspensions were used to inoculate a 32-h-old lignocellulosic waste hydrolysate fortified with 1% (v/v) yeast fermentation broth [49]. The yeast fermentation broth contained (g/L) yeast extract 5, KH_2PO_4

20, $MgSO_4.7H_2O$ 10, $(NH_4)_2SO_4$ 20, and $MnSO_4$. H_2O 1 was dissolved in 1000 mL of deionized water. The hydrolysate was first filter-sterilized (Millipore; 0.22 µm) to remove interfering bacteria, and the pH was adjusted to 4.5 using 2 N HCl to prevent further unwanted bacterial conversions of the reducing sugars. The hydrolysate (200 mL) was subsequently inoculated with a yeast cocktail prepared in yeast fermentation broth at 1% (v/v) in 1 L Erlenmeyer flasks. Individual yeast inoculations were also performed for comparison. All flasks were covered with aluminum foil to prevent aerobiosis and incubated for 72 h at 30 °C. The destructive sampling technique was adopted to prevent contamination, wherein a set of three flasks was withdrawn from the experimental set up at 8 h intervals. The flask contents were centrifuged at 3,075 × *g* for 15 min, and the supernatant was used to determine the residual sugar and ethanol concentrations. The ethanol concentration was determined by high-performance liquid chromatography (HPLC Infinity II LC system; Agilent, Santa Clara, CA, USA), and the compounds were separated on an ion exchange column at 60 °C using 0.005 M H_2SO_4 as the mobile phase at a flow rate of 1 mL/min [14]. All the analyses were performed in triplicate, and the data were analyzed via one-way analysis of variance in GraphPad Prism 8. Significant means were separated by Duncan post hoc multiple comparisons test at the 95% confidence level. The ethanol yield (%) was calculated relative to the estimated theoretical amount, which assumes complete conversion of reducing sugars in the lignocellulose hydrolysate into ethanol according to the expressions below [14]:

$$Yield\,of\,ethanol = \frac{Concentration\,of\,ethanol\ (g/l)\ in\,fermentation\,broth \times 1}{Sugar\,consumed\ (g/l)} \quad (4)$$

$$Fermentation\ efficiency\,(\%) = \frac{Yield\,of\,ethanol \times\ 100}{Theoretical\,ethanol\,yield} \quad (5)$$

where;

Theoretical ethanol yield calculations from the reducing sugars of lignocellulosic biomass were performed as described in Zhao et al. [50].

$$Volumetric\,ethanol\ productivity\ (g/l/h) = \frac{Ethanol\ concentration\ (g/l)\ in\,fermentation\,broth}{Fermentation\,time\ (h)} \quad (6)$$

Results and discussion

Selection of efficient lignocellulosic waste hydrolyzing bacterium

Two hundred and fifty-six morphologically distinct bacteria were isolated in pure culture from the five samples studied (Table S1, supplementary material). Only 34 (13.28%) of the bacteria were cellulolytic on solid (agar) media supplemented with carboxy-methyl cellulose as a carbon source, suggesting that a low proportion of cellulose-degrading bacteria occur in natural environments. Secondary screening of the 34 bacteria in liquid media revealed that only 9 isolates could release the enzyme in sufficient amounts to demonstrate activity in dilute solutions (Table S2). Strain CGS28 had the highest zone diameter (4.2 cm) on agar plates and the highest cellulolytic activity (13.5±2.87 FPU/mL) in liquid media (Table S2). Only a very few bacteria possess the level of natural cellulolytic activity reported here. Demissie et al. [51] reported a cellulolytic index of 3.1 mm for *Bacillus* sp. CD1 when CMC was used as a carbon source.

Identity of the efficient lignocellulose waste hydrolyzing bacterium

The strain CGS28 was identified by biochemical analysis, culture morphology, microscopy (Fig. 2a) and 16 S rRNA partial-gene sequencing as a strain of *Thermobifida fusca*, with 99% sequence identity to *Thermobifida fusca* (GenBank accession number: AB562464.1) (Fig. 2b). The bacterium and its sequence data were deposited at the University of Calabar Collection of Microorganisms (UCCM) under the code name *Thermobifida fusca* strain UCCM 00158.

Approximately 80% of the bacteria that hydrolyze cellulosic biomass in nature have been reported to belong to the firmicutes and actinobacteria, especially members of the genera *Clostridium* [52] and *Streptomyces* [53, 54]. The actinomycete *Thermobifida fusca* strain UPMC 901 was previously reported to demonstrate the ability to hydrolyze lignocellulose under thermophilic conditions [27].

One-factor-at-a-time selection of most appropriate lignocellulose carbon substrate for the bacterium

To identify the most susceptible plant biomass to lignocellulolytic activity by the study bacterium and enhance its potential for improved biotechnological relevance in line with the United Nations' sustainable development goals, a suitable medium was developed around lignocellulosic waste as a carbon source. Figure 3a shows that cellulase activity, measured in filter paper units per milliliter (25.87±2.44 FPU/mL), was significantly ($p<0.05$) greater in the RHP treatment than in the other treatments, followed by the corn cob powder treatment (20.23±2.11 FPU/mL). This difference may be attributed to the higher

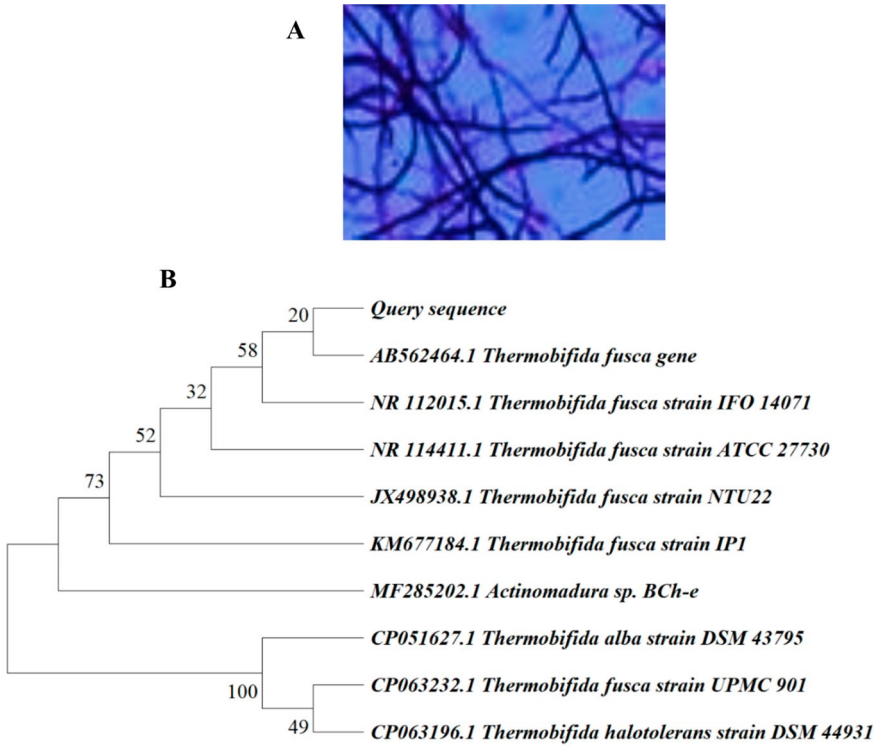

Fig. 2 The lignocellulolytic bacterium (**a**) Microscopy after Gram reaction and (**b**) Phylogenetic relationship between strain CGS28 (query sequence) and NCBI closely related 16 S rRNA partial gene sequences created in MEGA 11 software using neighbor-joining approach

cellulose but lower lignin content of rice husk and corn cob lignocellulosic materials compared to those of other sources [55]. Tukey's multiple comparisons test of the ordinary one-way ANOVA revealed that cellulase activity mediated by CMC was not significantly different ($p>0.05$) from that mediated by SCB, GBM or SDP, suggesting that any of these factors could mediate cellulase production in the *Thermobifida fusca* strain UCCM 00158 in place of CMC.

One-factor-at-a-time selection of nitrogen source for lignocellulose waste degradation

When RHP served as a carbon source during nitrogen screening (Fig. 3b), peptone mediated a significantly greater total cellulase activity (53.30±3.76 FPU/mL) than did all the other nitrogen sources, suggesting that the optimal carbon/nitrogen ratio for efficient degradation of organic carbon in rice husk powder was supplied by this combination. Nitrogen metabolism has been.

reported to require significant input of metabolic energy and carbon skeletons with which it links carbon metabolism through the GS/GOGAT cycle [56]. Ammonium nitrate (27.02±4.98 FPU/mL) and yeast extract (28.84±2.49 FPU/mL) provided the least increase in cellulolytic activity on RHP and may have repressed lignocellulolytic gene expression in the bacterium [57]. Nature and concentration of nitrogen are significant considerations for optimal microbial growth and gene expression during lignocellulose degradation involving cellulases and xylanases [58].

One-factor-at-a-time selection of appropriate inoculum size for lignocellulose degradation by the bacterium

OFAT experiments revealed that an inoculum size of 10^9 cfu/mL was significantly greater ($F=44.68$, $p<0.0001$, $r^2=0.9504$) than other inoculum densities and therefore mediated the highest degree of cellulolysis by the strain with a cellulase activity of 89.17±5.40 FPU/mL (Fig. 3c). Notably, there was a sequential improvement in cellulolytic activity from a baseline of 13.5 FPU/mL to 89.17 FPU/mL, which is a 6.61-fold improvement that underscores the relevance of OFAT statistical experimentation. Many microbial fermentations have been reported to proceed with inoculum sizes between 10^7 and 10^9 cfu/mL [59]. The optimal inoculum size for a fermentation process is a function of nature of the producing organism and product of interest [60].

Plackett-Burman design selection of significant medium parameters for lignocellulose degradation by the bacterium

The Pareto chart (Figure S1) of the PBD suggested that four out of the eleven variables screened, namely, rice husk powder, corn cob powder, inoculum volume and

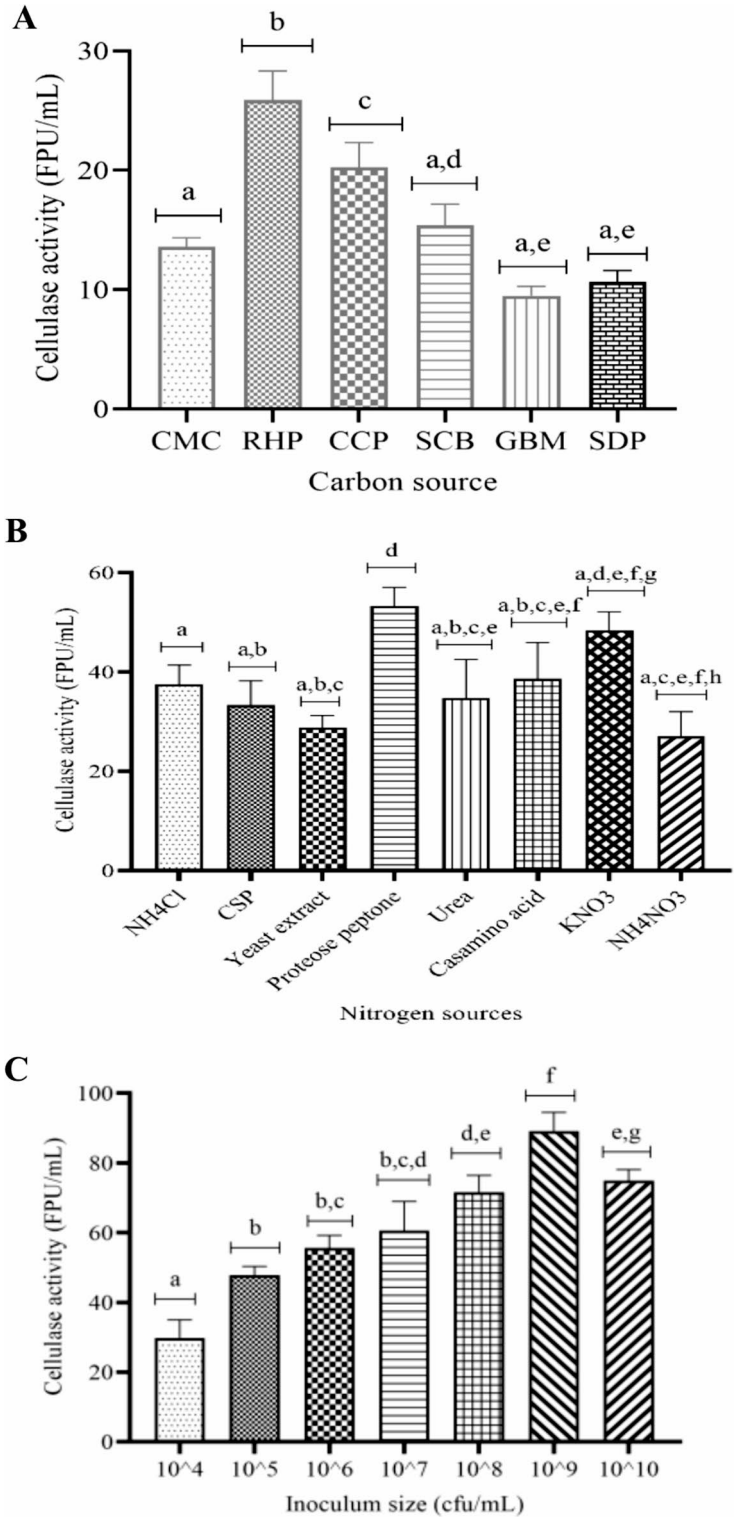

Fig. 3 One-factor-at-a-time screening of **A** – carbon source; **B** – Nitrogen source; **C** – Inoculum size for efficient bioconversion of rice husk lignocellulosic waste biomass by *Thermobifida fusca* strain UCCM 00158. Similar alphabets indicate no significant difference between or among means while dissimilar alphabets indicate significant difference between or among mean values. Bar values are means of triplicate determinations and error bars are standard deviations from the means at 5% significance level

peptone, significantly enhanced cellulase activity in response to the bacterium to 197.83 FPU/mL (Table S3 a and b, supplementary material), which occurred in the experimental run 10. Sensitivity analysis indicating the relative contributions of each of the significant variables to the bioprocess revealed that the bioprocess was most sensitive to RHP, which contributed 36.36% to the model. This was followed by peptone (10.40%), inoculum volume (10.26%) and CCP (6.09%). The adjusted r^2 of 0.7744 indicated that the model was accurate and could explain 77.44% of the variability in the response due only to the significant factors, with a predicted r^2 of 0.6293. Because the predicted and adjusted r^2 values did not differ by more than 0.2, they were considered to be in reasonable agreement regarding PBD model adequacy. Additionally, the adequate precision of the model (13.602) was greater than 4.0, indicating an adequate signal and suggesting that the model could be used to navigate the design space. Analysis of variance (ANOVA) of the PBD (Table S4a) showing only significant variables revealed that the first-order factorial model for cellulolytic activity was significant at $F=13.87$, $p=0.0003$, suggesting that there was only a 0.03% chance that the large F value variability could occur due to noise. The first-order model built by the coefficients of the significant main factors is given by Eq. 7 below:

$$Y = 161.35 + 17.40\,RHP + 7.12\,CCP + 9.24\,INV + 9.30\,Peptone \quad (7)$$

where Y is cellulolytic activity.

Because a curvature was detected in the model (Table S4b), the factorial (first-order) model was considered inappropriate for explaining the variability of the data, suggesting that a higher model would have to be developed. Additionally, examination of the curvature term revealed that it was significant ($F=21.45$, $p=0.0007<0.05$), suggesting that the study could use straightforward response surface methodology (RSM), where a higher-order model would be developed without recourse to the path of steepest ascent experimentation [61]. This indicated that the variable levels used in the PBD experiments were already close to the optimum region and would need very little adjustment.

Response surface modeling of significant variables for maximum lignocellulose bioconversion by *Thermobifida fusca* strain UCCM 00158

The matrix of the Box–Behnken design (BBD) in coded units and the experimental and predicted values of cellulase and xylanase activities determined by RSM modeling of the four significant variables from the PBD are presented in Table 1. The table reveals that the highest cellulase activity (214.05 FPU/mL) and xylanase activity (325.52) were obtained in run 1 of the experiment under conditions set at high levels of RHP and peptone (+1, +1), while CCP and INV were held at the center points (0,0), the levels of which were obtained from the PBD experiment. Analysis of variance of the two models (Tables 2 & 3) revealed that both models were significant (cellulase activity: $F=53.63$, $p<0.00001$; xylanase activity: $F=75.73$, $p<0.0001$), suggesting that there is a less than 0.01% chance that the variability in either model would occur due to noise. The 'fit statistics summary' revealed that the adjusted goodness-of-fit, $r^{2,}$ of the cellulase activity model Y_1 was 0.9634, indicating a reasonable fit of the predicted data with the experimental data and explaining 96.34% of the variation in the experimental region (Table 2). With the predicted r^2 (0.9253) in reasonable agreement with the adjusted r^2 and an adequate precision of 20.9872 affirming an adequate signal, the model was found to be reliable.

Only two of the 12 predictor terms of the cellulase activity model failed to contribute significantly ($p>0.05$) to the model. These included the two-way interaction terms X_1X_3 and X_2X_4, corresponding to the rice husk powder/inoculum volume and corn cob powder/peptone, respectively. The suggested quadratic model for $Y_{1,}$ which relates the means and standard deviations of cellulase activity, Y_1 to the independent variables, is presented in Eq. 8 below.

$$\begin{aligned} Y_1 = {} & 195.43 + 16.68X_1 - 11.41X_2 + 18.51X_3 \\ & + 23.32X_4 - 51.64X_1X_2 - 6.25X_1X_3 \\ & + 24.20X_1X_4 - 12.85X_2X_3 - 3.28X_2X_4 \\ & + 58.18X_3X_4 - 29.96{X_1}^2 - 78.63{X_2}^2 \\ & - 63.34{X_3}^2 - 28.50{X_4}^2 \end{aligned} \quad (8)$$

RHP-Rice husk powder; CCP-Corn cob powder; INV-Inoculum volume; PEP-Peptone; r^2 – coefficient of determination (goodness-of-fit), df-degrees of freedom.

Sensitivity analysis of cellulolytic activity model

The sensitivity analysis of a model refers to the relative contributions of the variables to the model in linear form, such as first-order sensitivity; Sa; or interaction form, such as second-order sensitivity (Sb). The coefficients of the predictor terms in combination with their F-statistic and p values are useful metrics for discussing the relative contributions of the variables to the model [62]. The cellulase activity model showed that the bioconversion process was most sensitive to the level of peptone ($F=49.28$, $p<0.0001$) for first-degree sensitivity, Sa. This effect may be clearer from Eq. 8, which reveals that the highest linear effect on cellulase activity (+23.32) was elicited by peptone. Protein is a nitrogen source and fulfills a major nitrogen requirement for cellular metabolism. Nitrogen,

Table 1 Box-Behnken designed matrix of a surface methodology showing significant variables in coded levels, and the experimental and predicted values of responses

Run	A: RHP	B: CCP	C: INV	D: PEP	eCact	pCact	eXact	pXact
1	1	0	0	1	214.05	201.17	325.52	322.22
2	0	-1	-1	0	39.06	33.52	243.85	245.06
3	0	0	-1	-1	111.28	119.95	235.95	236.12
4	0	-1	1	0	96.63	96.24	239.84	239.77
5	0	1	0	1	100.07	96.92	248.12	248.91
6	0	0	1	1	200.29	203.6	272.83	276.27
7	-1	0	0	-1	111.84	121.16	251.19	252.73
8	1	0	0	-1	107.93	106.14	277.84	277.79
9	0	0	0	0	188.65	195.43	268.39	263.57
10	0	0	0	0	179.09	195.43	256.15	263.57
11	-1	0	-1	0	70.35	60.69	220.64	217.8
12	0	1	1	0	45.73	47.71	255.74	252.77
13	0	0	-1	1	41.38	50.23	218.67	222.1
14	1	0	-1	0	105.72	106.57	257.42	257.15
15	1	1	0	0	30.97	40.47	284.73	285.78
16	0	0	0	0	208.54	195.43	257.05	263.57
17	0	-1	0	1	120.67	126.31	290.73	288.09
18	0	1	-1	0	39.56	36.39	203.47	201.77
19	0	0	1	-1	37.47	40.61	227.48	227.65
20	1	0	1	0	129.84	131.08	291.85	292.85
21	0	0	0	0	210.59	195.43	269.63	263.57
22	-1	0	0	1	121.18	119.41	244.61	242.9
23	-1	1	0	0	101.48	110.38	233.47	235.51
24	0	-1	0	-1	78.39	73.11	249.38	246.75
25	1	-1	0	0	163.49	166.57	301.28	302.85
26	-1	-1	0	0	27.45	29.93	246.18	248.73
27	-1	0	1	0	119.48	110.21	229.38	227.81
28	0	1	0	-1	70.92	56.85	254.85	255.65
29	0	0	0	0	190.29	195.43	266.63	263.57

RHP-rice husk powder; CCP-corn cob powder; INV-inoculum volume; PEP-peptone; *e*Cact-experimental cellulolytic activity; *p*Cact-predicted cellulolytic activity; *e*Xact-experimental xylanolytic activity; *p*Xact-predicted xylanolytic activity

which is frequently a limiting nutrient in organic material biodegradation, is required for the synthesis of nitrogenous compounds, including amino acids, for polymerization into requisite enzymes involved in the catabolic pathways of lignocellulosic materials. The element is required in a proportionate ratio to carbon for the synthesis of proteins, nucleic acids, reducing power, and energy currency for bacterial growth, reproduction and functioning [58]. The effect of nitrogen was followed by that of inoculum volume (+18.51) and rice husk powder (+16.68), with the level of corn cob powder (-11.41) making the least contribution to the cellulase activity model. This finding suggested significant ($F=11.81$, $p=0.004<0.05$) inhibition of cellulase activity and indicated that the efficiency of the combined substrate should be monitored very carefully.

The second-order sensitivities are represented as 2D (contours) and 3D (surface plots) in Fig. 4. The interaction between RHP and CCP (X_1X_2) inhibited cellulase activity the most, with a coefficient of -51.64, at which only 150 FPU/mL of total cellulase activity was observed. This finding suggested that bioprocessing may benefit more from using one lignocellulosic material at.

a time. The interaction between RHP (X_1) and peptone (X_4) contributed to the second-order sensitivity of the bioprocess, with a coefficient of 24.20 and a maximum cellulase activity of 180 FPU/mL. However, the interaction between INV and peptone (X_3X_4) made the greatest contribution to the cellulase activity model, with a contribution to cellulase activity of 200 FPU/mL. Cellulase activity was therefore most sensitive to these two variables, and optimized control of their levels alone led to 91% total cellulase activity (221.93 FPU/mL) in this study.

According to the xylanase activity model ($F=75.73$, $p<0.0001$; adjusted $r^2=0.9739$), Y_2 explained 97.39% of the variability, as indicated by the changes in the levels of the predictor variables (Table 3), with sufficient prediction capability (predicted $r^2=0.9599$), which was in reasonable agreement with the adjusted r^2. The highly adequate precision of 38.799 signal-to-noise ratio

Table 2 Analysis of variance table for cellulase activity response of BBD-RSM

Source	Sum of Squares	df	Mean Square	F-value	*p*-value
Model	99392.86	14	7099.49	53.63	< 0.0001
X_1-RHP	3340.67	1	3340.67	25.23	0.0002
X_2-CCP	1563.17	1	1563.17	11.81	0.004
X_3-INV	4110.33	1	4110.33	31.05	< 0.0001
X_4-PEP	6524.47	1	6524.47	49.28	< 0.0001
X_1X_2	10665.73	1	10665.73	80.56	< 0.0001
X_1X_3	156.38	1	156.38	1.18	0.2955
X_1X_4	2341.59	1	2341.59	17.69	0.0009
X_2X_3	660.49	1	660.49	4.99	0.0423
X_2X_4	43.1	1	43.1	0.3255	0.5773
X_3X_4	13539.65	1	13539.65	102.27	< 0.0001
X_1^2	5822.83	1	5822.83	43.98	< 0.0001
X_2^2	40105.3	1	40105.3	302.93	< 0.0001
X_3^2	26019.52	1	26019.52	196.54	< 0.0001
X_4^2	5268.71	1	5268.71	39.8	< 0.0001
Residual	1853.46	14	132.39		
Lack of Fit	1112.38	10	111.24	0.6004	0.7665
Pure Error	741.08	4	185.27		
Cor Total	1.01E + 05	28			
Fit statistics					
r^2	0.9817		Standard deviation	11.51	
Adjusted r^2	0.9634		Mean	112.5	
Predicted r^2	0.9253		Coefficient of variation (%)	10.23	
Adequate precision	20.9872				

RHP - Rice husk powder; CCP - Corn cob powder; INV - Inoculum volume; PEP - Peptone; r^2 – coefficient of determination (goodness-of-fit), df-degrees of freedom

Table 3 Analysis of variance table for xylanase activity response of BBD-RSM

Source	Sum of Squares	df	Mean Square	F-value	*p*-value
Model	19755.14	14	1411.08	75.73	< 0.0001
X_1-RHP	8172.95	1	8172.95	438.65	< 0.0001
X_2-CCP	688.26	1	688.26	36.94	< 0.0001
X_3-INV	1566.82	1	1566.82	84.09	< 0.0001
X_4-PEP	897.7	1	897.7	48.18	< 0.0001
X_1X_2	3.69	1	3.69	0.1979	0.6633
X_1X_3	164.99	1	164.99	8.86	0.01
X_1X_4	736.04	1	736.04	39.5	< 0.0001
X_2X_3	791.86	1	791.86	42.5	< 0.0001
X_2X_4	577.92	1	577.92	31.02	< 0.0001
X_3X_4	980.63	1	980.63	52.63	< 0.0001
X_1^2	567.42	1	567.42	30.45	< 0.0001
X_2^2	143.64	1	143.64	7.71	0.0148
X_3^2	3742.71	1	3742.71	200.88	< 0.0001
X_4^2	6.3	1	6.3	0.3381	0.5702
Residual	260.85	14	18.63		
Lack of Fit	93.96	10	9.4	0.2252	0.9745
Pure Error	166.89	4	41.72		
Cor Total	20015.99	28			
Fit statistics					
r^2	0.987		Standard deviation	4.32	
Adjusted r^2	0.9739		Mean	255.96	
Predicted r^2	0.9599		Coefficient of variation (%)	1.69	
Adequate precision	38.799				

RHP - Rice husk powder; CCP - Corn cob powder; INV - Inoculum volume; PEP - Peptone; r^2 – coefficient of determination (goodness-of-fit), df-degrees of freedom

(> 4.0) indicates an adequate signal, suggesting that the model could be used to navigate the design space. The lack of fit test *F*-statistic of 0.2252 was not significant ($p = 0.9745 < 0.05$), implying that the model was adequate for explaining data variations about the region of optimum. The interaction between RHP and CCP (X_1X_2) and the quadratic term peptone (X_4^2) did not contribute significantly ($p > 0.05$) to the xylanase activity model. A comparison between the predicted r^2 and adjusted r^2 values revealed that the difference was not up to 2.0, suggesting reasonable fit agreement between the two metrics. The xylanase activity model, Y_2, is presented in Eq. 9 below:

$$\begin{aligned} Y_2 = {} & 263.57 + 26.10X_1 - 7.57X_2 + 11.43X_3 \\ & + 8.65X_4 - 0.96X_1X_2 + 6.42X_1X_3 \\ & + 13.56X_1X_4 + 14.07X_2X_3 - 12.02X_2X_4 \\ & + 15.66X_3X_4 + 9.35{X_1}^2 - 4.71{X_2}^2 \\ & - 24.02{X_3}^2 + 0.99{X_4}^2 \end{aligned} \quad (9)$$

Sensitivity analysis of xylanolytic activity model

The model revealed that the process was most sensitive to the concentration of RHP (X_1: +26.10), followed by the volume of inoculum (X_3: +11.43), suggesting that these two parameters contributed the most to the linear forms of the bioprocess. The second-order sensitivity, Sb, showed that the inoculum volume/peptone interaction made significant contribution to the bioprocess (+15.66), followed by the corn cob powder/inoculum volume interaction (X_2X_3), with a maximum xylanase activity of 301 U/mL. The interaction between corn cob powder and peptone (X_2X_4) significantly inhibited the bioprocess and led to a final xylanase activity of only 280 U/mL. The interaction between RHP and peptone (X_1X_4) led to the highest xylanase activity (321 U/mL), suggesting that these two parameters contributed more than 96.27% of the total xylanase activity (333.44 U/mL). The selection of peptone as nitrogen source for efficient degradation of lignocellulose substrates by xylanase is supported by earlier researches [63, 64].

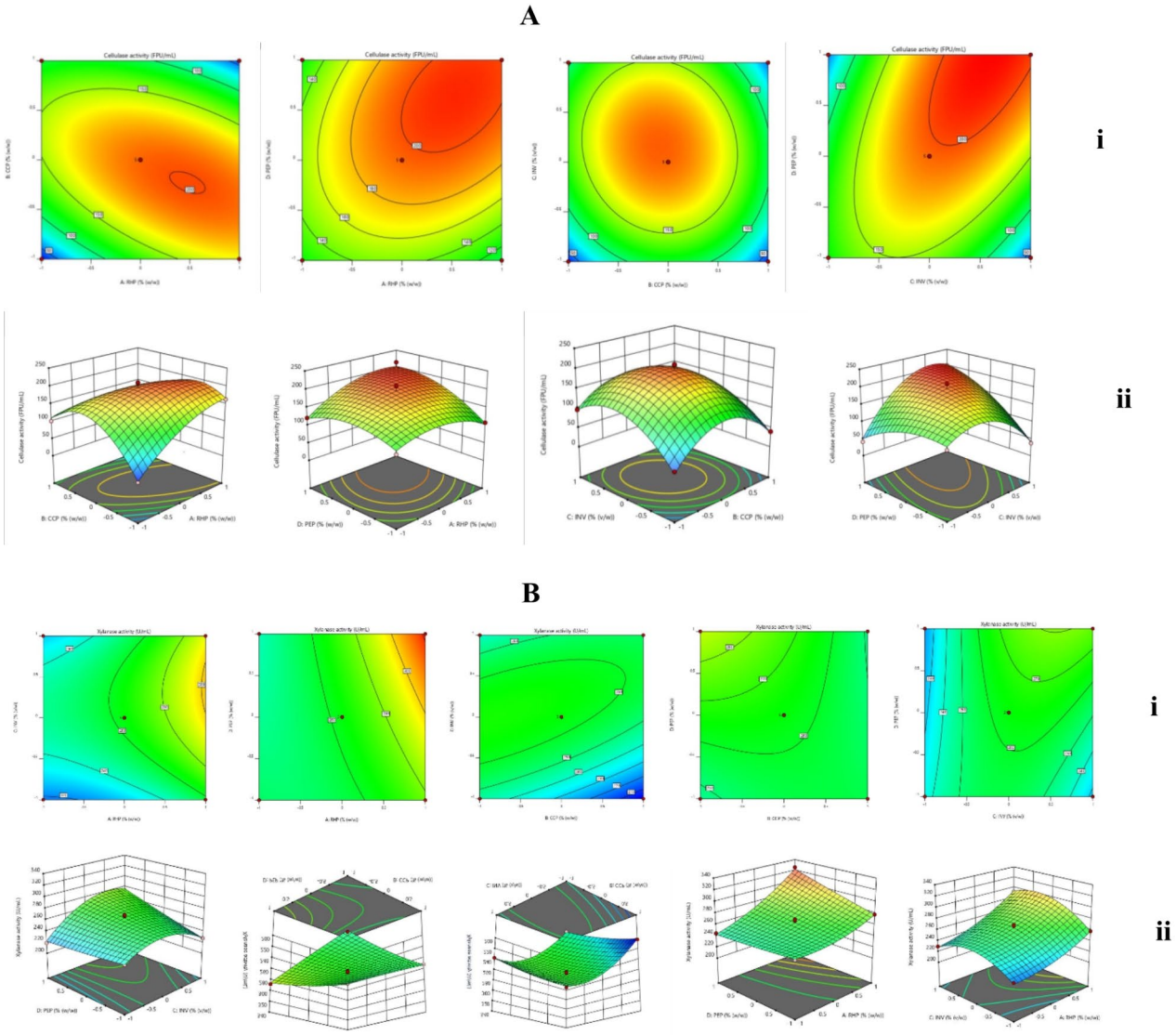

Fig. 4 Contour (i) and surface (ii) plots of significant two-way variable interactions in (**A**) celluolytic activity and (**B**) xylanolytic activity BBD-RSM models for lignocellulosic waste saccharification by *Thermobifida fusca* strain UCCM 00158; - the blue and red colours indicate respectively, lowest and highest values of interacting variables within the range of which the optimum value is supposed to lie. The closer the colour is to red, the better; the green and yellow colours are intermediates and frequently indicate where the region of optimum lies

Optimization of BBD-RSM models

The multi-objective optimization of the bioprocess revealed that maximum cellulase and xylanase activities of 221.93 FPU/mL and 333.44 U/mL, respectively, could be obtained in the solid-state fermentation of binary lignocellulosic waste under conditions set as (X_1, X_2, X_3, X_4) = (0.992, -0.018, 0.625, 0.999). These conditions corresponded to 59.92% RHP, 24.82% CCP, 41.25% INV and 5.998% peptone calculating from the actual levels in BBD-RSM (Table S5). This is the first report on the optimization of lignocellulosic biomass bioconversion under solid-state fermentation nutrient conditions. Gao et al. [65] recently optimized the environmental conditions for lignocellulose bioconversion by the endophytic fungus *Chaetomium globosum* and obtained interesting results. The large difference between their results and those of the present study may arise from the nature of the organism used and the duration of the saccharification process. Additionally, these authors used a recovered enzyme cocktail for saccharification, which suggested that many of the saccharification products, viz. simple sugars, in the present study may have been consumed by the actinomycete after 20 h. Real-time validation of these conditions revealed a cellulase activity of 221 FPU/mL and xylanase activity of 335.06 U/mL, suggesting that these conditions could be adopted for SSF of the binary lignocellulosic waste substrates of rice husk and corn cob.

Kinetics of saccharification

Since the major objective of the study was to circularize the bioconversion process by fermenting the lignocellulosic waste hydrolysate into bioethanol in accordance with renewable energy requirements, it was necessary to monitor reducing sugar accumulation during the optimized bioprocess. This is because lignocellulolytic actinomycetes are physiologically interested in using the obtained reducing sugars, namely, glucose (from cellulose) and xylose (from xylan or hemicellulose), as the sole sources of carbon and/or energy, not to produce ethanol. Prolonged contact between the *Thermobifida fusca* strain UCCM 00158 and the hydrolysate could therefore lead to reduced amounts of simple sugars available for fermentation into ethanol. The results of the time-course study are presented in Fig. 5. Peak cellulolytic activity (441.04-466.12 FPU/mL) occurred between 16 and 32 h, and another peak (463.31-470.36 FPU/mL) occurred between 56 and 64 h. These activities were significantly greater than the final activity recorded at 120 h in the preceding section, confirming the fear of concomitant consumption of simple sugars and possible enzyme degradation. The reported peak cellulolytic activities coincided with the peaks of total reducing sugar (120.89–124.09 g/L) and another 122.0 g/L which occurred 32 h later. Since the amount of pentoses during these periods ranged from 23.98 to 49.48 g/L, it is safe to conclude that.

the reducing sugars that accumulated during these periods were mostly hexoses, particularly glucose, which is a monomeric unit of cellulose. These findings also suggest that the cellulose component of lignocellulose was the first of the three major lignocellulose components to be attacked by lignocellulolytic enzymes during the saccharification process.

Xylanolytic activity reached its peak of 748.02 at 40 h, which remained fairly constant until 80 h. This coincided with a peak pentose sugar accumulation of 54.37–59.10 g/L, suggesting efficient bioconversion of the hemicellulose component of lignocellulose during this period. The lower xylanolytic activity observed at the end of 120 h (330 U/mL) compared to 80 h (770 U/mL) suggests the presence of little hemicellulose substrate for catalysis by xylanase. This result is comparable to that of Gao et al. [65], who reported *Chaetomium globosum* xylanolytic activity of up to 987.33 U/g. It would therefore be biotechnologically sensible to harvest reducing sugars for fermentation into bioethanol during peak cellulolytic and xylanolytic activities at 64 h.

Fermentation of lignocellulosic waste hydrolysate into bioethanol

The lignocellulosic waste hydrolysate harvested after 64 h contained 124.60 g of total reducing sugar/L of hydrolysate. Trends of five parameters of lignocellulose hydrolysate fermentation by the axenic and mixed cultures of *Saccharomyces cerevisiae* UCCM 00054 and *Kluyveromyces marxianus* NCYC 2303 strains are presented in Fig. 6a-e. Figure 6a compared the amount of total reducing sugar consumed by the three fermenting systems and revealed that the order of total reducing

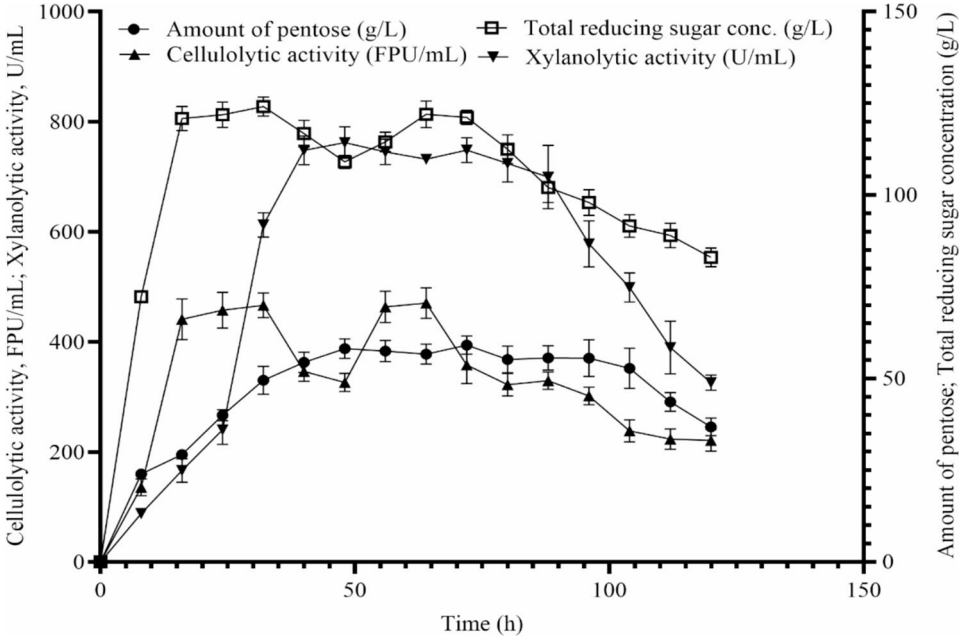

Fig. 5 Kinetics of lignocellulose binary waste saccharification by cellulolytic and xylanolytic activities of *Thermobifida fusca* strain CGS28. Total reducing sugar concentration refers to the amount of reducing pentoses and hexoses obtained from lignocellulose waste hydrolysis by the study actinomycete; Data are means of triplicate determinations. Error bars are standard deviations from the means at 5% significance level

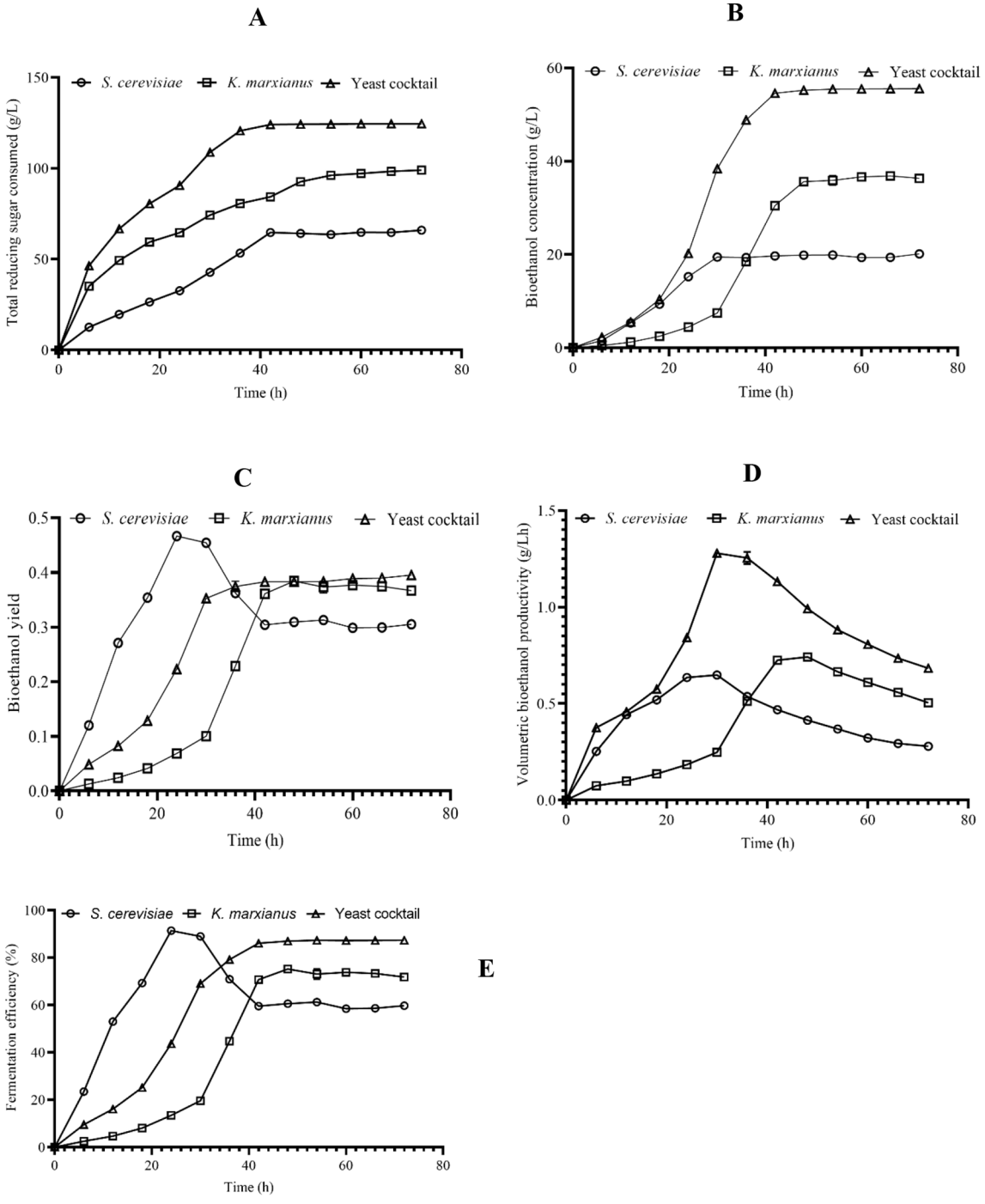

Fig. 6 Comparison of bioethanol fermentation parameters (**A**) total reducing sugar consumed (**B**) bioethanol concentration (**C**) Bioethanol yield (**D**) volumetric bioethanol productivity and (**E**) fermentation efficiency of the three fermenting systems including *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and a 50:50 cocktail of both. Data are means of triplicate determinations and error bars are standard deviations from the means at 5% significance level

sugar consumption among the fermenting systems was *S. cerevisiae*<*K. marxianus*<yeast cocktail with final consumed sugar concentrations of 65.94 g/L, 99.04 g/L and 124.53 g/L, respectively. This indicates that about 100% of the total reducing sugar in the hydrolysate was consumed by the yeast cocktail while the axenic cultures of *S. cerevisiae* and *K. marxianus* only.

consumed 52.9% and 79.49%, respectively at the end of 72 h. The low final proportion of reducing sugar consumed from the lignocellulosic waste hydrolysate by *Saccharomyces cerevisiae* may be attributed to its poor

tolerance to a number of stress factors encountered in the hydrolysate [66]. Some of these stress factors include pentose sugars which the yeast naturally cannot metabolize [18] and lignin by-products like acetic and formic acids, furfural and phenolic compounds which are inhibitory to cellular metabolism in the yeast [67]. Comparatively, the higher reducing sugar consumption by *K. marxianus* may be attributed to its superior tolerance to inhibitory compounds, and its ability to utilize a range of pentose and hexose sugars in the hydrolysate [68]. The greater consumption of reducing sugars by the yeast cocktail indicates synergistic relationship between the yeast strains which led to improved tolerance to inhibitory substances and broadened range of utilizable sugars in the hydrolysate. A commonly used technique in recent times to broaden the range of utilizable sugars in lignocellulose hydrolysate is to express pentose fermenting genes in *Saccharomyces cerevisiae*. However, since *Kluyveromyces marxianus* appears more tolerant to inhibitory compounds in lignocellulose hydrolysate, it has become a major chassis for second-generation bioethanol production hence the preferred expression host [69].

In theory, the amount of reducing sugar consumed determines the amount of ethanol produced. However, the cellular requirement of carbon atoms from the sugar consumed may not allow for the realization of the theoretical yield of ethanol. Time-course of ethanol concentration in this study is depicted in Fig. 6b and indicates that overall ethanol concentration increased with increase in duration of fermentation. This suggests that in the initial stages of fermentation, the amount of sugar consumed may have been channeled mostly into biomass accumulation rather than ethanol production [70]. Overall, ethanol concentration after 72 h of fermentation ranged from 20.12 g/L in *S. cerevisiae* to 55.57 g/L in the yeast cocktail. Ethanol concentration between 11 g/L and 28 g/L was earlier reported by Cunha et al. [71] when an engineered strain of *Saccharomyces cerevisiae* was employed to ferment lignocellulose hydrolysate. Tavarez et al. [72] reported an ethanol concentration of 34.13 g/L in batch fermentation with *K. marxianus* strain which does not differ significantly from the 35.6 g/L ethanol in the present study. Contrariwise, a very low ethanol concentration of 3.739 g/L was reported when *S. cerevisiae* fermented the hydrolysate of corn stalk lignocellulose for 72 h. There is therefore great variation in ethanol concentration generated by fermentation of lignocellulose hydrolysate, the variation of which may not be unconnected with fermenting strain, lignocellulose source and initial pretreatment.

Figure 6c depicts bioethanol yield which is a measure of the amount of ethanol produced from a corresponding amount of sugar consumed. The figure shows that *S. cerevisiae* had better ethanol yields than both *K. marxianus* and the yeast cocktail, especially in the initial stages (early 30 h) of fermentation when its yield was 0.467. During alcoholic fermentation, *S. cerevisiae* is reported to give an ethanol yield very close to the theoretical maximum of the amount of glucose consumed. The yeast's ethanol yield reduced significantly to 0.3 by 72 h in this study suggesting that the best time to harvest ethanol from lignocellulosic waste fermentation with the yeast is between 24 and 30 h. Barring inhibition from toxic compounds in lignocellulose hydrolysates, *Saccharomyces cerevisiae* is reported to have an ethanol yield of 0.90 to 0.93 from the amount of sugar consumed because of the EMP pathway it adopts for the process [70].

The volumetric bioethanol productivity was also compared among the three fermenting systems (Fig. 6d). The results show that the yeast cocktail was the most productive in terms of ethanol by volume per unit time with a maximum volumetric bioethanol productivity of 1.2 g/L/h between 30 and 36 h. At no time during the course of fermentation was volumetric ethanol productivity in the axenic cultures better than those mediated by the yeast cocktail. By 72 h, productivity by the yeast cocktail dropped to 0.68 g/L/h which was comparatively higher than observed for the axenic cultures. However, volumetric ethanol productivity by *K. marxianus* was reported to reach 1.99 g/L/h by 12 h [72] in glucose medium which was not the case in this study where it was just 0.1 g/L/h.

Figure 6e depicts efficiencies of the three fermenting systems and indicates that *S. cerevisiae* was the most efficient within the first 24–30 h with a fermentation efficiency of 88.9–91.3%. The corresponding efficiencies of the other systems at this point were 69% and 19% for the yeast cocktail and *K. marxianus* respectively. As fermentation progressed into the 42nd hour, fermentation efficiencies of the yeast cocktail and *K. marxianus* shot up to 86% and 70.7% respectively while that of *S. cerevisiae* took a nose-dive to 59.5%. The ability of *K. marxianus* strains to cope with lignocellulose hydrolysate inhibitors have been reported as an important mechanism for higher efficiency in bioethanol fermentation of lignocellulosic hydrolysate than most yeast strains [68]. The yeast is reported to exhibit potential to turn on molecular and physiological mechanisms that facilitate detoxification of inhibitory compounds before embarking on bioethanol fermentation [73]. From 42 h onwards, fermentation efficiencies of all three test systems remained significantly unchanged until the end of fermentation. L'ainez et al. [74] reported higher fermentation efficiency of 92.88% by *K. marxianus* compared to 87.63% by *S. cerevisiae* while fermenting lignocellulose hydrolysate. This difference may be strain-specific, and may also depend on hexose/

pentose sugar ratio in the lignocellulose hydrolysates fermented, among other factors.

In conclusion, a promising cockroach-gut symbiotic lignocellulosic waste-degrading bacterium, *Thermobifida fusca* strain UCCM 00158, was isolated in this study. Sequential statistics facilitated the development of a model for solid-state mixed rice husk-corn cob lignocellulosic waste saccharification, on which future lignocellulose-to-bioethanol bioconversion studies may rely. The model guided the development of an efficient pentose-hexose sugar fermentation cocktail (50:50% v/v) of naturally occurring yeast species for promising circular bioeconomic fermentation of lignocellulosic waste hydrolysate into bioethanol with significant ethanol concentration and commendable volumetric productivity. The technique employed has sufficient potential for environmentally-safe and sustainable second-generation bioethanol production with a significant reduction potential of environmental lignocellulosic agro-industrial waste.

Supplementary Information

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Supplementary Material 1

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Author contributions
ME and CE conceived the idea; CE, UE, PE, DU and DI acquired software and conducted the investigations; RA, AA and ME supervised investigations, analyzed data, prepared figures, interpreted results and wrote the original draft; ME and SA reviewed and edited the original draft into a final draft of the manuscript. All authors reviewed and approved the final version of the manuscript for submission.

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