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Highlights

- The Optical density of *Streptomyces* strain COB increased from 0.9 to 1.41 when sugar cane bagasse was used as the sole carbon source.
- The GC-FID showed that 43.08 g/L ethanol was generated from 5.0g sugarcane bagasse using *Streptomyces coelicolor* strain COB as inoculum under aerobic batch fermentation.
- Ten carboxylic acids including formic acid, glycolic acid, tartaric acid, acetic acid, citric acid, oxalic acid, malic acid, lactic acid, n-valeric acid, and 3-hydroxybutyric acid were identified as biochemical organic acids by-products.

Sustainable generation of bioethanol from sugarcane wastes by *Streptomyces coelicolor* strain COB KF977550 isolated from a tropical estuary

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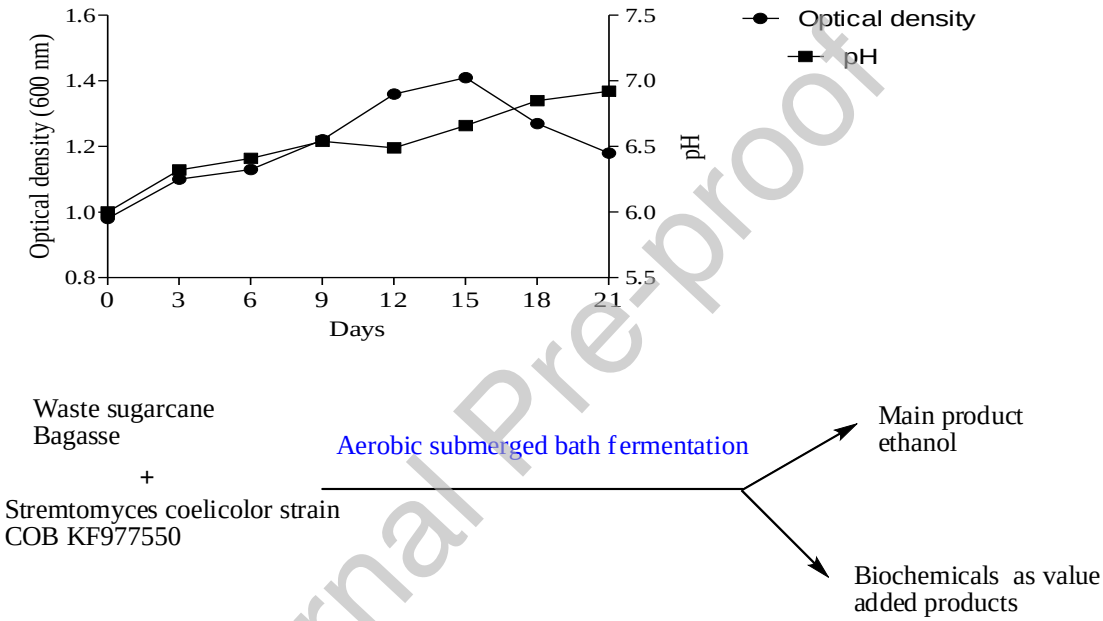
Graphical abstract

Sugarcane bagasse was exploited as a renewable substrate for obtaining bioethanol using *Streptomyces* strain COB under aerobic submerged batch fermentation. Detection of the metabolic products was determined using a combination of FTIR, GC-FID and GC-MS. The Optical density of *Streptomyces* strain COB increased from 0.9 to 1.41 at optimum pH of 6.66. The GC-FID analysis showed that 43.08 g/L ethanol was generated. Interestingly, the results showed the presence of diverse biochemicals released into the medium in addition to the main product ethanol.



Abstract

The damaging effect and challenges associated with the use of fossil fuel is enormous and very costly. Biofuels could be obtained from plant biomass wastes which are known to be sources of environmental pollution and breeding grounds for vectors of diseases. Sugarcane bagasse was exploited as a renewable substrate for obtaining bioethanol using *Streptomyces* strain COB KF977550 as inoculum. Submerged aerobic batch fermentation was performed in flasks containing mineral salts medium supplemented with 5.0 g (w/v) sugarcane bagasse. Incubation was done in a shaker (150 rpm) at 30 ^{o}C for 21 days. Microbial growth was assessed by measurement of the optical density (O.D $_{600nm}$) at 3-day intervals. Fractional distillation was carried out in batch mode using a simple fractional distillation setup. Metabolic products were determined using GC-FID. Further analyses were performed using FTIR and GC-MS. The optical density of *S.coelicolor* strain COB KF977550 increased from 0.9 to 1.41. The GC-FID showed that 43.08 g/L ethanol was generated. Interestingly, the results showed the presence of diverse biochemicals released into the medium in addition to the main product ethanol. Ten carboxylic acids including formic acid, glycolic acid, tartaric acid, acetic acid, citric acid, oxalic acid, malic acid, lactic acid, n-valeric acid, and 3-hydroxybutyric acid were identified as biochemical organic acids by-products.

Key words: Bioethanol; sugarcane bagasse; biomolecules; lignocellulose wastes; *Streptomyces.*

Introduction

The negative impact of fossil fuel on the environment is a global concern, particularly greenhouse gas emissions [1]. The challenges associated with the use of fossil fuel is enormous and costly in some developing oil-producing countries. For example, Nigeria (West Africa) is an oil-producing country but going through a crisis emanating from over-dependence on fossil oil. Sadly, the issue of unrest and continuous pollution of land and water from oil spillage has badly affected the livelihood of the populace. Thus, Nigeria resorted to the importation of fuel with consequent Naira depreciation and high cost of importation.

The uncertainty of fossil fuel as a global dependable economy is further pronounced by the outbreak of COVID-19. The International Energy Agency (IEA) in their review as of April 2020 reported that oil demand was greatly hit, down by nearly 5% in the first quarter, especially by the cut down in mobility and aviation, which accounted for nearly 60% of global oil demand. The worldwide road transport activity was near 50% below the 2019 average and aviation was 60% below as at end of March 2020 [2].

Based on the report by the Renewable Fuel Association of 2018, the world bioethanol production outreached 27,050 million gallons in 2017 from 13,123 million in 2013 which is an indication that the International bioethanol market is at a very dynamic stage. The determination for bio-based “clean technologies” is propelled by environmental pollution issues. In some developed nations including the USA, Brazil, Europe, and China, significant advances have been made towards bioconversion of plant biomass into bio-based products such as bioethanol [3] The USA has been reported to be the largest producer of ethanol with 6.521 and 15,800 million gallons in 2007 and 2017 respectively. Brazil followed behind with 5.019 and 7.060 respectively in 2007

and 2017, The E.U from 570 to 1,415, China 486 to 875 while the rest of the world was reported to produce 315 and 1450 million in 2007 and 2017 respectively [4, 5, 6, 7].

Although Nigeria is endowed with a wealth of renewable resources, however, the contribution of renewable resources to the biobased economy in Nigeria is currently negligible. Lignocellulose and agricultural wastes are generated in large quantities in Nigeria because of a lack of appropriate storage and preservation systems. This often gives rise to environmental hazards because they are indiscriminately disposed of by burning (leading to respiratory disorders in humans) or by dumping into water bodies. Refuse in water bodies generates anoxic condition resulting in the death of aquatic animals, serves as a breeding ground for mosquitoes and serves as nutrients for pathogenic microorganisms [8, 9, 10]. Moreover, this act is a waste of resources that could be harnessed for biotechnological purposes. The hydra-headed challenges imposed by fossil fuel could be addressed through sustainable bioconversion of wastes into bio-based consumables such as bioethanol and organic acids mostly used as polymers, additives, and preservatives in the food, medical, pharmaceutical cosmetic, and automotive industries [3, 11].

The utilization of sugarcane bagasse for producing ethanol has gained more attention but the recalcitrance nature in this substrate demands a pretreatment step to unlock the lignin and hemicellulose structures that serve as a hindrance to access the cellulose potion for bioethanol production. The conventional pretreatment process removes lignin and enhances the penetration of hydrolysis agents [12, 13]. However, most of the chemical methods for the pre-treatment of lignocellulose wastes are expensive and may add to the cost of production and ultimately lead to environmental degradation. The biological pre-treatment method which involves the use of whole microorganisms such as fungi and bacteria or their enzymes in the pre-treatment of lignocellulose wastes appears to be a better choice. The use of microorganisms for the pre-

treatment of wastes largely depends on the ability of the microorganisms to produce the required enzymes. Many fungal and bacterial species are able to breakdown the cellulose components of plant biomass [14]; however, their ability to synthesize the required enzymes necessary to break down the lignin protecting the cellulose and hemicellulose components is a challenge. The removal of the lignin component is essential for the release of fermentable sugars from plant biomass wastes for the production of biochemicals such as bioethanol. Marine/estuarine microbes are yet to be fully exploited, unlike their soil counterparts. Because they are well adapted to the harsh environmental conditions of their habitat; they are likely candidates that can withstand the harsh environmental conditions of biotechnological processes. Previously *S. coelicolor* strain COB KF977550 isolated from a tropical estuary have utilized Kraft lignin (65%) and released into its growth media laccase and peroxidase; two major enzymes required for lignin breakdown [9]. The organism degraded the lignin and carbohydrate components of wood residue (sawdust) by 50 and 48% respectively without chemical pre-treatment [15]. Based on the capabilities displayed by these strains, it could be a good candidate for the sustainable production of bioethanol from plant biomass wastes if properly harnessed. Therefore, this project aims to focus on the utilization of sugarcane bagasse wastes as renewable raw material for the production of bioethanol using *S. coelicolor* strain COB KF977550 because of its environmental friendliness, reduced cost, and creation of new technologies. In addition, we intend to by-pass the expensive saccharification and other chemical pretreatment steps to reduce cost and avoid degradation of the environment. Though *Streptomyces* spp is known to be capable of breaking down lignocellulose materials, we hope *S. coelicolor* strain COB KF977550 will be able to break down sugarcane bagasse without chemical pretreatment and simultaneously or subsequently generate bioethanol.

Materials and Methods

Description of microorganism used in this study

S. coelicolor strain COB KF977550 was isolated from a tropical estuarine in Lagos, Nigeria. The organism was identified as previously described [15]. In previous laboratory studies, this strain has demonstrated substantial capabilities to utilize wood residues, grasses, papers, and sugarcane bagasse for growth [16]. It also showed varied abilities to utilize kraft-lignin and lignin- related aromatic compounds[9, 17]. Before use, the strain was resuscitated to check for purity using Luria Bertani Agar.

Physical and mechanical pre-treatment of bagasse waste samples

Sugarcane bagasse (1kg) was collected into a sterile Ziploc bag from a local farm located at Ikorodu, Lagos State, Nigeria. It was rinsed with sterile distilled water and dried in the oven at 80 ºC until the weight is constant after which the samples were grounded into small particles using a hammer mill. The samples were stored in labeled sterile plastics with a lid until used.

Submerged fermentation

Growth of *S. coelicolor* strain COB KF977550 was performed under aerobic submerged batch fermentation using Erlenmeyer flasks (2000 mL). Each flask contains 1000 mL of mineral salts medium (pH 7.2). Sugarcane bagasse (5.0 g) was used as the carbon source. There were two control flasks. Control (A) contained the sugarcane bagasse and medium without the microbial strain while the control (B) contained only the *S. coelicolor* strain COB KF977550 without sugarcane bagasse, this is to monitor contamination. The flasks were incubated at 30 ºC on a rotary shaker (150 rpm) for 21 days. The experiment was carried out in triplicate. Microbial

growth was evaluated by measurement of the optical density (O.D 600nm) at an interval of 3 days. Preliminary detection of the metabolic products was determined using GC-FID (Hewlett Packard (HP) 5890 series II, California, USA) with an OV-3 glass column pack. The column temperature was 200 ºC while the injector and detector temperatures were 200 oC and 270 ^{o}C respectively with an N_2 carrier gas and H_2 at a flow rate of 22 mL/min and temperature/ramping rate of 5^{o}C/min.

Fractional distillation to obtain bioethanol.

Fractional distillation was carried out in batch mode using a simple fractional distillation setup. Since ethanol boils at approximately 78 ^{o}C, the distillation of the bioethanol was achieved below 80 ^{o}C using a heating mantle (Labline distiller LSC model, 230V, 300W, 5A). Removal of water from the sample after distillation was done by adding 5 g of anhydrous sodium sulfate to 25 mL of the presumed bioethanol. It was then filtered using filtered paper (Whatman Cat.No. 1001 125 mm) and stored in a vial at 4 ^{o}C in a refrigerator until needed for IR and GCMS analyses.

Extraction, IR and Gas Chromatography-Mass Spectrophotometry analyses

The extraction of other components alongside the bio-ethanol was carried out using dichloromethane (DCM). The solvent (500 mL) was added to every 100 mL of the sample solution. This was shaking vigorously over a magnetic stirrer for 1 h after which filtration was carried out. The filtrate was thereafter dried using 10 g of anhydrous sodium sulfate to every 100 mL. This is to ensure that every trace of the water molecule is completely removed. The dried filtrate was concentrated under pressure using a rotary evaporator (Stuart digital water bath (RE

300 DB model) connected to an SHB III Water aspirator vacuum pump). The semi-solid concentrate obtained was kept in amber glass vials and refrigerated at 10 ^{o}C for further analysis.

Fourier Transform Infrared Spectroscopy (FTIR) measurements of the crude fermentation extract and distillate were done using a Bruker-Alpha FTIR Spectrophotometer (Bruker Optics GmbH Ettlinger, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector. The Alpha – p The Alpha – p ATR attachment is furnished with a solitary-reflection diamond ATR bisection and a spring stacked machine-driving press for compressing solid specimens at the ATR waveguide surface with consistent duplicable pressure. Details were documented in the MIR spectral range (400 – 4000 cm^{-1}) at a spectral resolving power of 2 cm-1. 24 scans standards for backgrounds and specimen spectral. This was performed at an atmospheric condition (Temperature = 24 ± 1°C).

Gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC–MS) analyses were performed using GC Agilent Technologies 7890B GC-System coupled with MS 5975C GC/MSD. The mobile phase was helium and with a stationary phase (0.25 μm HP-5 5% cross-linked with phenylmethyl polysiloxane) with an internal diameter of 0.320nm and length 30m The volume injected was 1μl per run.

Results

Growth studies

The results of the growth experiment showed that the optical density of *Streptomyces* strain COB KF977550 increased from 0.9 to 1.41. Strain COB recorded the highest growth when the pH value of the medium was 6.66 (Fig.1)

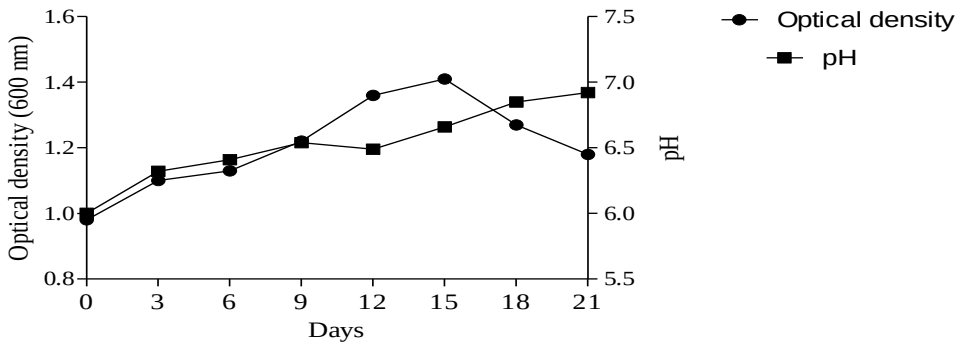


Fig 1: Growth profiles of pure cultures of *Streptomyces coelicolor* strain COB KF977550 in sugarcane bagasse as the carbon source.

Preliminary detection and quantification of metabolic products using GC-FID

The preliminary results showed that *S. coelicolor* strain COB KF977550 utilized sugarcane bagasse for the production of bioethanol alongside other biomolecules after 21 days' fermentation. Based on the GC-FID results, the highest ethanol yield of *S. coelicolor* strain COB KF977550 by day 21 was 43.08 gL^{-1} as summarized in Table1**.** The table shows the progressive increase in the quantity of ethanol produced over three weeks with approximately 43% optimum yield.

Table 1: Generation of ethanol with time by strain COB medium containing sugarcane bagasse as carbon source

Day	Quantity of ethanol (g/L)	Control (No isolate)
0	0	ND
3	0.08	ND
6	0.15	ND
9	10.86	ND
12	15.02	ND
15	21.72	ND
18	38.62	ND
21	43.08	ND

ND-Not detected by GC-FID in the growth medium

IR Analysis

In the FTIR analysis of bioethanol obtained from the medium containing strain COB, showed the presence of peaks characteristics of monomeric alcohol at 3365 - 3350 cm^{-1} absorption (OH stretching with strong and broad appearance). This broad peak was not noticeable when compared to the spectra obtained from the control sample. Although the spectra of the control indicate the absence of bio-ethanol but show an evident strong sharp peak at 2922-2852 cm-1 (hydrocarbon sp^3-C-H), C=O stretching at 1710 - 1740 cm^{-1} absorption and C-H bending at 1458 - 1514 cm^{-1} absorption. This confirmed that the hydrocarbons of the sugarcane bagasse have not been converted to ethanol and other biochemicals found in the medium containing strain COB. The presence of a range of carboxylic acids in the medium containing strain COB, such as oxalic

acid, etc. are suspected and also indicated in the GC-FID chromatogram of analysis of composition and structure of fermentation products in hydrolysate (Table 2).

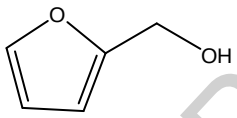
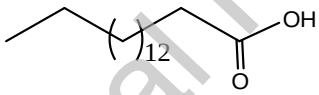
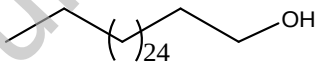
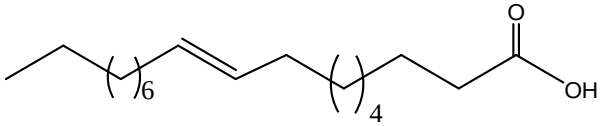
Table 2: GC–FID analysis of composition and structure of fermentation products in hydrolysate of COB.

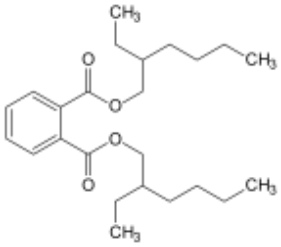
Degradation Product	**RT (min)**	**Area**	**Height**	**MW g/mol**	**Boiling Point (^{O}C)**
Formic acid	3.300	1517,8650	43.018	46.03	100.8
Glycolic acid	4.033	747,6520	34.742	76.05	112
Ethanol	5.016	8045,8830	546.478	46.07	78.37
Tartaric acid	6.450	5107750	19.515	150.087	275
Acetic acid	7.150	11392510	129776	60.05	117.92
Citric acid	7.533	4041.0130	221.694	192.124	310
Oxalic acid	7.816	4293.5395	315.423	90.03	190
Malic acid	8.600	4854.7350	423,663	116.07	150
Lactic acid	9.200	18308,8960	1793563	90.08	122
n-Valeric acid	11.483	7472820	62,680	102.13	186
3-Hydroxybutyric	14.566	166.3655	15.596	104.1	269

GC-MS Analyses

GC-MS is the primary tool employed for the identification of the microbial fermentation products in this study. The chromatograms and mass spectra obtained were analyzed using the library of the National Bureau of Standards for common compounds. The GC-MS spectra and main compounds identified in GCMS quantitative analysis (Sample COB) are shown in Table 3.

Table 3: GC-MS analysis of composition and structure of fermentation products in hydrolysate of strain COB

Product	**Structure**	**RT (min)**
2-Furanmethanol ($C_5H_6O_2$)		8.57
n-Hexadecanoic acid ($C_{16}H_{32}O_2$)		12.12
Octacosanol ($C_{28}H_{58}O$)		12.33
Octadec-9-enoic acid ($C_{18}H_{34}O_2$)		13.98

Bis(2-ethylhexyl) phthalate ($C_{24}H_{38}O_4$)		18.84

Discussion

Among the actinomycetes, *Streptomyces* species have been credited with various abilities to produce different arrays of lignocellulolytic enzymes [18 - 20] using different lignocellulose substrates as contained in our previous report [16]. This endowment undoubtedly enabled *S. coelicolor* strain COB KF977550 to utilize sugarcane bagasse as a growth substrate as indicated by the increase in the optical density of the medium from 0.9 to 1.41 in this study. It is well established that *Streptomyces* species are a group of microorganisms that performs optimally under aerobic conditions [21]. Aeration probably contributed to the ability of *S. coelicolor* strain COB KF977550 to release the requisite enzymes, attack the sugarcane bagasse, and subsequently utilize it for growth and release of metabolites. In addition, the mechanical pretreatment reduced the substrate size, creating large surface areas for easy access by the strain.

Although Streptomycetes are known to prefer neutral to alkaline environmental pH, they commonly occur at extraordinary fluctuating pH and nutritional conditions. From the GC-FID result, the *Streptomyces* strain COB KF977550 used in this study produced the highest bioethanol quantity when the pH was 6.6 at a room temperature of 25 oC. The dependence of 10 species of *Streptomyces* spp on pH of nutrient tolerance was determined by Kontro et al. [22]. The authors reported that the growth pH range of *Streptomyces* spp. are dependent on media composition and nutrient constituents. They submitted a minimum pH of 4.0 to 7.0 and the maximum pH of between 8·5 and 11·5 for the growth of *Streptomyces*. Similarly, in their study on the isolation of *Streptomyces indoligenes* sp. nov. from rhizosphere soil of Populus euphratica, Luo et al. [23] reported a pH range of 5-12 and temperature range of 10 - 45 oC.

Although yeasts especially *Saccharomyces* species are acidophilic in nature and are adjudged to be the best producers of ethanol. However, they are mostly adapted to do so under anaerobic conditions. *Streptomyces coelicor* spp are well respected as secondary metabolite producers and for their ability to adapt to adverse conditions. Even though *S. coelicolor* may not grow in the absolute absence of oxygen, Keulen et al. [21] provided evidence that the organism is able to grow micro aerobically and sustain viability for several weeks under strictly anaerobic conditions. They also opined that resting and germinated spores are capable of surviving rapid exposure to anaerobiosis.

In this study, the *S. coelicolor* strain COB KF977550 was able to produce a significant amount of ethanol under aerobic conditions. In most alcohol-producing industries, yeast is rinsed with sulphuric acid at intervals before the start of a new fermentation to remove bacterial contaminants. Sometimes the removal of the bacterial contaminant is done by the use of antibiotics and biocidal chemicals which may add to the cost of production and uncontrolled release of the spent medium may lead to antibiotic resistance in the environment. Moreover, bacteriological contamination during yeast fermentation may cause flocculation of yeast thereby inhibiting fermentation [24]. *Streptomyces* spp are natural producers of bioactive compounds such as antibiotics [25], which enables them to rival other microorganisms that come in contact with them, hence the issue of contamination is minimal or eradicated during fermentation.

Another thumb up for the *S. coelicolor* strain COB KF977550 used in this study is its ability to access the cellulose portion without the usual rigorous and expensive pretreatment steps. When Zhanga et al. [13] enhanced enzymatic hydrolysis of sugarcane bagasse using ferric chloride catalyzed organosolv pretreatment and Tween 80, the pretreatment removed most of the impurities that could serve as inhibitors to the production of ethanol. In this study, there was no chemical pretreatment, hence varied organic acid with higher or equal peak areas with ethanol (as shown by the GC-FID) could have inhibited the further release of ethanol. In addition, some quantities of ethanol may have evaporated over time especially with the use of shakers for aeration. Despite all the obvious obstacles, *S. ceolicolor* strain COB KF977550 produced a substantial amount of ethanol (48 g/L) from sugarcane waste in this study.

FTIR and GC-FID analyses were used to confirm the identity of the aerobic fermentation products. Interestingly, the results showed the presence of diverse biochemicals released into the medium in addition to the main product ethanol. Ten carboxylic acids including formic acid, glycolic acid, tartaric acid, acetic acid, citric acid, oxalic acid, malic acid, lactic acid, n-valeric acid, and 3-hydroxybutyric acid were identified as biochemical organic acids by-products. Additional value-added chemicals identified on further analyses by GC-MS method include 2-furan methanol, n-hexadecanoic acid, octacosanol, 9-octadecenoic acid, and bis(2-Ethylhexyl) phthalate. It is interesting to note that some of these value-added chemicals have been reported as products in the depolymerization of lignin [26, 27].

Conclusions

Biochemical generation of bioethanol from lignocellulosic wastes holds tremendous potential in terms of meeting energy needs, reduced cost, and providing environmental benefits if carefully harnessed. Furthermore, estuarine bacteria are vast, amenable, capable of metabolic activities under wide growth conditions, and are known to possess robust enzymatic capabilities that could be fully exploited for possible biotechnological applications. Lignocellulose wastes which are generated in large quantities through agricultural practices that would have otherwise cause environmental pollution problems appear to be good raw materials. Microbial conversion offers a cheap and safe method of not only disposing of the residues but also has the potential to convert lignocellulose and agricultural residues into value-added biomolecules.

Acknowledgments

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Declaration of Competing Interest

We declare no conflict of interest.

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