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Cellulolytic and Ethanologenic Evaluation of *Heterotermes indicola*'s Gut-Associated Bacterial Isolates

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ABSTRACT: Cellulose is the basic component of lignocellulosic biomass (LCB) making it a suitable substrate for bioethanol fermentation. Cellulolytic and ethanologenic bacteria possess cellulases that convert cellulose to glucose, which in turn yields ethanol subsequently. *Heterotermes indicola* is a subterranean termite that causes destructive damage by consuming wooden structures of infrastructure, LCB products, etc. Prospectively, the study envisioned the screening of cellulolytic and ethanologenic bacteria from the termite gut. Twenty six bacterial strains (H1−H26) based on varied colonial morphologies were isolated. Bacterial cellulolytic activity was tested biochemically. Marked gas production in the form of bubbles (0.1−4 cm) in Durham tubes was observed in H3, H7, H13, H15, H17, H21, and H22. Sugar degradation of all isolates was indicated by pink to maroon color development with the tetrazolium salt. Hallow zones (0.42−11 mm) by Congo red staining was exhibited by all strains except H2, H7, H8, and H19. Among the 26 bacterial isolates, 12 strains were identified as efficient cellulolytic bacteria. CMCase activity and ethanol titer of all isolates varied from 1.30 ± 0.03 (H13) to 1.83 ± 0.01 (H21) umol/mL/min and 2.36 ± 0.01 (H25) to 7.00 ± 0.01 (H21) g/L, respectively. Likewise, isolate H21 exhibited an ethanol yield of 0.40 ± 0.10 g/g with 78.38 ± 2.05% fermentation efficiency. Molecular characterization of four strains, *Staphylococcus sp.* H13, *Acinetobacter baumanni* H17, *Acinetobacter sp.* H21, and *Acinetobacter nosocomialis* H22, were based on the maximum cellulolytic index and the ethanol yield. *H. indicola* harbor promising and novel bacteria with a natural cellulolytic tendency for efficient bioconversion of LCB to value-added products. Hence, the selected cellulolytic bacteria can become an excellent addition for use in enzyme purification, composting, and production of biofuel at large.
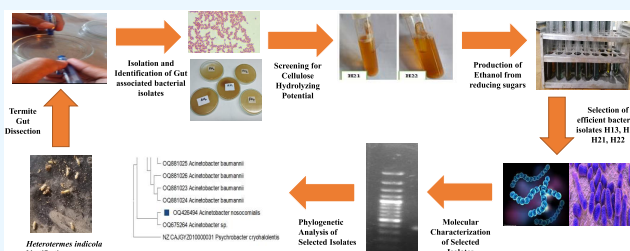
1. INTRODUCTION

Lignocellulosic biomass (LCB) is considered a renewable and inexpensive resource in the world with energy content ($3−4/GJ).[1−3] Pakistan, being an agricultural country, is one of those countries producing abundant lignocellulosic waste. In Pakistan, agricultural waste, especially fruit and vegetable waste, wheat husks, rice husks, cotton sticks, and sugar cane residues, is abundant.[4] Annual massive accumulation of lignocellulosic waste in the form of fruit, vegetable, and agro crops has led to the spoilage of the valuable biomass, which can be processed in a wide range of value-added products, and deterioration of the environment and hence necessitates the requirement to look for new avenues for applicable utilization. The current estimates for lignocellulosic waste, wood, and wood-based residues are 20,494, 25,271, and 1,121 million tons, respectively.[5] According to The Pakistan Business Council,[6] Pakistan's overall estimated biomass potential is 50,000 GW h/year or up to 36% of the country's entire energy scenario.

Naturally, the cellulosic substrate is decomposed by the action of a mixture of hydrolytic enzymes (known as cellulases). The cellulase is a complex enzyme comprising endogenous (endoglucanase) and exogenous (cellobiohydrolase) that work synergistically in cellulose-degrading microorganisms.[7,8] Cellulases of cellulolytic bacteria are involved in the hydrolysis of cellulose, producing sugar derivatives by breaking β-1,4-glycosidic linkage.[9] Globally, cellulases are used due to their remarkable industrial applications, viz, biorefinery, paper, textile, feed, and agriculture industries, over decades. The worldwide demand for industrial cellulases reaches up to eight percent.[10,11] A wide range of microbes, bacteria, and fungi have the ability to degrade the cellulose and cell wall

components of plants. Among them, fungi is the main producer of cellulase.[12,13]

Cellulolytic enzyme systems are present naturally in many organisms and plants to degrade living as well as rotten plant materials. These organisms may serve as impending candidates for the production of biofuel from lignocellulosin substrate.[7,14,15] Currently, attempts are made to screen substantial diversity of the environment and organisms to possess enzymes with amended characteristics of cellulolysis, a process of reliable determination and assessment of cellulase activity. A variety of aerobic, anaerobic, and facultative anaerobic microbes have the potential to breakdown cellulosic biomass.[16,17] The metabolic, physiological, and efficient enzyme machinery of the microbe contributes for considerable carbon flow in the biosphere.[7,15,18] As for animals, LCB is a poor nutritional food source and remains undigested due to lack of cellulolytic machinery and metabolic pathway. Termites play a havoc, being highly destructive to wooden structures and other cellulosic material, causing an estimated amount of $3 billion loss annually worldwide.[19,20] They possess an ability to digest cellulose efficiently.[21−23] Due to their lignocellulosic biomass recycling potential, they play a vital role in tropical, subtropical, and Mediterranean ecosystems.[24,25] In the detritus food chain, they are the main decomposers due to their own cellulolytic system that is enhanced by the presence of cellulose-degrading microbes in their gut.[26−28]

Heterotermes indicola Wasmann, a subterranean termite, belongs to Rhinotermitidae, order Isoptera,[29] and is termed as the lower termite that feeds only on wood. It is found in tropical to subtropical and warm temperate habitats in Pakistan, Sri Lanka, and India.[30] Globally, it is known as the destructive lower termite that cause massive timber damage in agriculture and urban areas.[31,32] *H. indicola* has a highly organized nesting system with diffused colonies and earthen tunnels/galleries to hit timber. Moreover, it has the ability to spread up to 100 m or more by constructing satellite nests underground in woody material and rafters.[33] This insect can also build hanging satellite tubes in search of moisture and food.[34] The highly wood-damaging behavior of termites pointed to the gut's powerful innate cellulolytic system. It may contribute to the fact that the gut of the lower termite has synergistic bacterial, fungal, and protozoan species, whereas higher termites possess only few bacterial species with no protozoans.[35] The hind gut of *H. indicola* is larger than the midgut and contains a diverse range of bacteria and protozoans (symbiont). The microbial communities present in the hind gut of termite are linked to the digestion of wood (nutrient-deficient food source). Symbionts supplement this nutrient-deficient diet by synthesizing other necessary nutrients and stimulate reactions involved in the breakdown of all three major components of wood (cellulose, hemicellulose, and lignin phenolics).[36,37] Symbiotic cellulase systems produce complex cellulolytic enzymes. Thus, it exhibits strong hydrolytic activity (40−88%) against the carboxymethyl cellulose (CMC) substrate in comparison with endogenous cellulase (40−85%) of total gut activity.[38]

It is evident that the termite gut is a complex microhabitat having distinct biotic and abiotic features that offer ecological niches to a diverse range of microbiota.[39] The termite gut is the unique microhabitat for the micobiota and cellulolytic flagellates.[40−42] As a result, scientists from all over the world are now particularly interested in the diversity and role of the microbial community in the termite gut.[42−44] The microbiota

present in the gut of lower termites belong to archaea, eukarya, and bacteria. Methanogen represented the intestinal group of archaea, whereas the protozoans, yeast, and fungi belong to eukarya. The gut bacteria may be Gram-positive, viz, bacteroides, firmicutes, acinetobacter, and spirochaetes. Gut bacteria along with symbiotic protists degrade aromatic compounds, hemicellulose, cellulose, and fixed nitrogen,[45] while higher termites broke down only cellulose by relying on their own enzymes.[46] The cellulolytic bacterial groups of the gut isolated from *Coptotermes curvignathus* were *Bacillus cereus, Chryseobacterium kwangyangense, Acinetobacter sp., Enterobacter cloacae,* and *E. aerogenes.*[47−49] Ali et al.[37] isolated five symbiotic bacterial strains *Paenibacillus lactis, Lysinibacillus macrolides, Stenotrophomonas maltophilia, Lysinibacillus fusiformis,* and *Bacillus cereus* from the gut of lower termite *Psammotermes hypostoma.* A cellulolytic *Bacillus licheniformis* HI-08 with 400 U/mL cellulase activity was reported from the wood-feeding lower termite *Heterotermes indicola.*[50] The CMCase activity of 3.36 and 0.75 U/mL was recorded by *Bacillus subtilis* G4 and *B. subtilis* AS3 isolated from the termite gut.[51,52] Javaheri-Kermani and Asoodeh[53] reported a novel bifunctional (endo and exo glucanases) β-1,4-glucanase producing *Bacillus sp.* CF96 from lower termite *Anacanthotermes sp.* The purified β-1,4-glucanase has the ability to hydrolyze both CMC and avicel.[54]

The literature suggested that the cellulolytic bacterial isolates of termite gut possess great potential for breaking down LCB. There is a great chance to enhance the lignocellulose pretreatment by employing these effective bacterial cultures. Especially, in the canvas of biomass-to-bioenergy valorization, the search for efficient cellulases from the microbial systems is always in high demand. In this prospect, there is a need for exploring the cellylolytic potential of these enzymes for LCB hydrolysis and subsequent solvent production (e.g., acetone, butanol, ethanol, etc.).[27,28,55,56] Hence, termites may be a desirable source of novel cellulolytic microorganisms and cellulases to be applied for the industrial conversion of biomass to biofuel. Termites are efficient wood decomposers[57] that harbor diverse range of symbiotic cellulolytic microbiota. Termites opt different mechanisms for decomposition of wood such as direct ingestion and digestion, substrate modification (such as fragmentation and tunneling), and interactions with bacteria, fungi, and other saproxylic community members.[58,59] Furthermore, these insects may have an impact on nitrogen dynamics in decaying woods by fixing nitrogen and releasing nutrients.[60]

The objectives of the current study are to screen and characterize molecularly the bacterial isolates from subterranean termite *Heterotermes indicola* for evaluation of their cellulolytic and ethanologenic potential. Microorganisms are exploited for bioconversion of natural renewable biomass to meet the demands of the future for energy and chemical precursors. However, termites possess excellent intestinal polysaccharide-degrading symbiosis (wide variety of bacteria and protozoans) over approximately 150 million years. Within the termite gut, ecosystem lignocellulose (cellulose and hemicellulose) is efficiently degraded while lignin contents are weakly attacked. The development of cellulose hydrolysis and biomass bioconversion processes/techniques, as explored in this study, may benefit from understanding of the principles of cellulose degradation in the naturally occurring polymer-degrading ecosystem from termite. The gut of termite may be a probable source to screen novel microbiota to be used for a
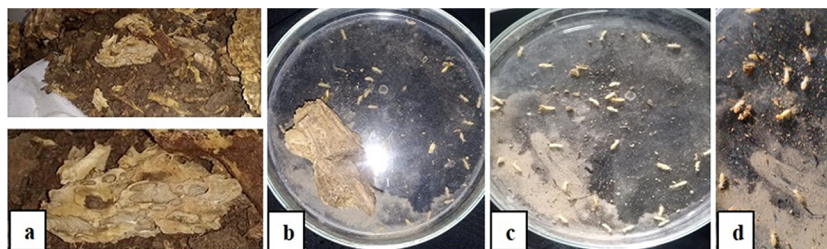
Figure 1. *Heterotermes indicola*. Termite collection (a), isolation of the termite (b, c), and identification of the soldier insect (d) (The photographs are original, unpublished and the effort of the first author).

Table 1. Colonial Characteristics of Bacterial Isolates Following Streaking on Cellulose-Supplemented Agar Medium

sr. no	isolated bacteria	size	color of colony	margin	surface texture	elevation	optical feature	pigmentation	consistency
1	H1	0.5 mm	creamy white (pinkish)	entire	smooth	flat	opaque	no	brittle
2	H2	2 mm	off-white	undulate	smooth	flat	opaque	yes (light pink)	butyrous
3	H3	3 mm	creamy white	entire	granules	convex	opaque	no	butyrous
4	H4	1 mm	white	undulate	granules	flat	transparent	no	butyrous
5	H5	2 mm	off-white	undulate	smooth	concave	opaque	yes	viscous
6	H6	2 mm	creamy white	entire	smooth	dome	opaque	no	butyrous
7	H7	1.5 mm	creamy white	entire	smooth	dome	opaque	Yes (brownish)	viscous
8	H8	pinpoint	off-white	undulate	granules	raised	opaque	no	butyrous
9	H9	3 mm	white	entire	smooth	flat	opaque	no	butyrous
10	H10	pinpoint	White	undulate	granule	flat	transparent	no	butyrous
11	H11	pinpoint	off-white	spreading	granule	irregular	opaque	no	butyrous
12	H12	2 mm	creamy white	entire	smooth	dome	opaque	no	butyrous
13	H13	2 mm	off-white	undulate	granule	concave	opaque	no	butyrous
14	H14	1 mm	white	entire	smooth	flat	opaque	no	butyrous
15	H15	3 mm	white	spreading	granule	flat	opaque	no	butyrous
16	H16	1 mm	white	undulate	granule	flat	transparent	no	butyrous
17	H17	3 mm	white	spreading	granule	flat	opaque	no	viscous
18	H18	3 mm	creamy white	undulate	smooth	flat	opaque	no	butyrous
19	H19	2 mm	white	entire	granule	flat	transparent	no	butyrous
20	H20	1 mm	white	spreading	granule	dome	opaque	no	butyrous
21	H21	pinpoint	off-white	entire	smooth	flat	opaque	no	butyrous
22	H22	1 mm	off-white	undulate	granule	dome	opaque	no	butyrous
23	H23	pinpoint	white	entire	smooth	flat	opaque	yes	viscous
24	H24	pinpoint	white	undulate	granule	dome	transparent	no	butyrous
25	H25	2 mm	white	spreading	granule	irregular	opaque	no	viscous
26	H26	2 mm	off-white	entire	smooth	dome	opaque	no	butyrous

wide range of industrial applications, viz, biofuel production and food industry.[61]

2. RESULTS

2.1. Termite Collection. The termites collected were identified as *Heterotermes indicola* on the basis of large longer than broad yellowish brown rectangular heads and well developed slender mandibles that were slightly curved near the tip and were crossed while closing both. Labrum is tongue shaped with a needle-like tip (Figure 1).

2.2. Sampling of Termite for Bacterial Screening. Twenty six bacterial strains were identified from the termite's gut and given codes H1−H26 based on the colonial morphology. The prefix H stands for *Heterotermes indicola*. All isolated bacteria were cultured on 2% cellulose-supplemented medium to get pure cultures and were preserved in the form of glycerol stock for further study.

2.3. Morphological Characterization of Isolated Bacteria. Colonial characteristics including colony size, color, elevation, margin, texture, optical features, pigmentation,

and consistency of bacterial isolates on the cellulose-enriched medium were recorded in Table 1.

The colonies vary from pinpoint to 2.5 cm as H8, H10, H11, H21, H23, and H24 were pinpoint, while H3, H9, H15, H17, and H18 showed 2.5 cm colony diameter. The color of the colonies were creamy white (H1, H3, H6, H7, H12, and H18), off-white (H2, H5, H8, H11, H13, H21, H22, and H26), and white for the remaining isolates. The margins of all colonies were entire, except H2, H4, H8, H10, H13, H16, H18, H22, and H24 (undulate) and H11, H15, H17, H20, and H25 (spreading). The textures of H1, H2, H5, H6, H7, H9, H12, H14, H18, H21, H23, and H26 were smooth, whereas other isolates were granular. The elevations observed were convex (H3), concave (H5 and H13), raised (H8), irregular (H11 and H25), dome-like (H6, H7, H12, H20, H22, H24, and H26), and flat for rest of the bacterial isolates. All isolates were opaque, except H4, H10, H16, H19, and H24 that were transparent. All bacterial isolates produced nonpigmented colonies except pigmented colonies of H2, H5, H7, and H23. Consistency of all isolates were butyreous except H5,H7, H17, H23, and H25 that were viscous.

2.4. Microscopic Examination of Bacterial Isolates. Microscopic cell characteristics of bacterial strains were studied by culturing on a cellulose-enriched medium. Cellular characteristics include cell shape, cell size, and cell type as shown in Table 2 and Figure 2. All bacterial strains were rod-

Table 2. Cellular Characteristics of Bacterial Isolates on Cellulose-Supplemented Medium

sr. no.	isolated bacteria	gram stain reaction	length (μm)	diameter (μm)	shape
1	H1	gram-negative	4.2	2	diplococci
2	H2	gram-positive	5	2 0.7	rods with pointed ends
3	H3	gram-negative	2	1.5	short rod, chain
4	H4	gram-positive	5	2.5	long rod, chain
5	H5	gram-positive	2	1	diplococci
6	H6	gram-negative	2.3	1.5	single rod
7	H7	gram-negative	3	1.5	spiral rod
8	H8	gram-positive	2	1	single rod
9	H9	gram-positive	3	2	coccus
10	H10	gram-negative	2	1.5	single rod
11	H11	gram-positive	4	2.3	cluster coccus
12	H12	gram-positive	2	0.5	diplococci
13	H13	gram-negative	2.2	0.56	coccus
14	H14	gram-positive	3	1.49	cluster coccus
15	H15	gram-positive	4	2	rods with oval ends
16	H16	gram-positive	2	1	spiral rods
17	H17	gram-negative	5	2	long rods, chain
18	H18	gram-positive	5	2.9	rods with pointed ends
19	H19	gram-positive	5	1.89	long rods
20	H20	gram-positive	3	1	spiral rods
21	H21	gram-negative	2	0.58	slightly curved rod
22	H22	gram-negative	2.5	0.5	single rod
23	H23	gram-negative	3	2	coccus
24	H24	gram-negative	5.6	3	long rods, chain
25	H25	gram-positive	4	2	short rods, chain
26	H26	gram-positive	3	1	coccus

shaped including long, short rods (with pointed or oval ends) except H1, H5, and H12 (diplococci), H9, H13, H23, and H26 (coccus), and H7, H16, and H20 (spirila). The size ranged from 2×0.5 to 5.6×3 μm. The observed maximum size, i.e., 5.6×3 μm, was in H24, while a minimum of 2×0.5 μm was in H12. The size of spiral bacteria varied from 3×1

μm with one to two twists. All bacterial strains were Gram-positive, whereas H1, H3, H6, H7, H10, H13, H17, H21, H22, H23, and H24 were Gram-negative bacteria.

2.5. Analysis of Cellulose-Hydrolyzing Potential for Bacterial Isolates. In the present study, qualitative biochemical tests were performed to detect the utilization of cellulose by bacterial strains. These tests include gas production, color development, and clear zone formation via Durham tubes, tetrazolium chloride (TTC) indicator, and Congo red stain. Table 3 and Figure 2 interpreted the degradation of cellulose by different indicators. Bubbles of variable size 0.1−4 cm in Durham tubes were produced by H3, H7, H14, H15, H17, H21, H22, and H24 within 2−8 days. All bacteria produced a maroon color with TTC to depict the speedy degradation of cellulose except H23. Four bacterial isolates showed negative response, while 22 bacteria produced a clear zone as positive response by Congo red staining. The highest as well as lowest cellulolytic indexes were recorded as 5 (H21) and 0.67 (H9, H15).

On the basis of qualitative analysis (color development by TTC indicator, gas production in Durham tubes, and hallow formation by Congo Red dye), 12 bacterial isolates (highlighted in Table 3) were selected for further study, i.e., cellulolytic and ethanologenic titer. Cellulolytic activity as depicted by conversion of cellulose to glucose is recorded in Table 4. The selected 12 bacterial isolates showed a tendency to release extracellular enzymes for cellulose hydrolysis. Bacterial isolates depicted the activity (μmol/min/mL) in the range of 1.30 ± 0.03 (H13) to 1.83 ± 0.01 (H21). Bacterial isolates H17 and H21 showed the maximum potential. The strains H17, H21, and H22 were selected for identification on the maximum cellulolytic potential.

2.6. Production of Ethanol from Cellulose as Substrate. Data related to ethanol titer by selected isolates are recorded in Table 5. Maximum ethanol was produced on different days by different isolates. Highest ethanol (g/L), i.e., 7.21 ± 0.01, 6.54 ± 0.01, 7.00 ± 0.01, and 5.64 ± 0.01, was generated by H13, H17, H21, and H22, respectively, on the eighth day of incubation followed by a decrease in content. In other strains, the maximum values were observed between days 7 and 8, and that was the indication of ethanol tolerance by bacterial isolates. Table 6 presents the calculated ethanol yield and fermentation efficiency (FE) in 2% CMC-supplemented fermentation medium. H21 showed a remarkable 0.40 g/g yield with 78.38% FE. Bacterial isolates H13, H17, and H22 exhibited 0.37−0.38 g/g yield with 71−73% FE. On the basis of the excellent ethanol yield, four isolates H13, H17, H21, and H22 were selected for molecular identification.

In the fermentation medium, carbon source was supplied in the form of CMC (2%) and yeast extract (0.65%). Figure 4 displays the correlation of reducing sugars in the fermentation medium and ethanol contents. It is hypothesized that ethanologenesis in the fermentation medium tends to cause a decrease in the remaining reducing sugars. The ethanol contents and the remaining reducing sugars in the medium are inversely proportional to each other. More or less inclination in reducing sugars with varied increase in ethanol contents was exhibited by different bacterial isolates. The increasing trend of ethanol contents from day 2 in H6, H13, H21, and H22 and less reducing sugars in the medium were detected, whereas, more reducing sugars with a slow increase in contents in the medium by H14, H23, H25, and H26 were recorded.
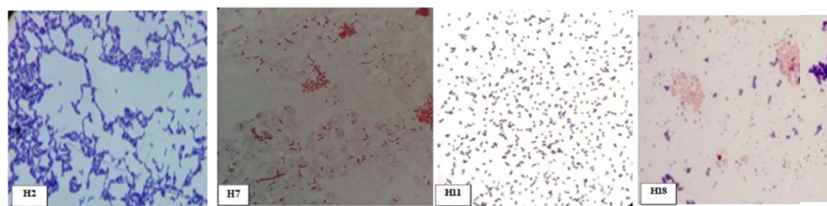
Figure 2. Gram reaction presented by different bacterial isolates as Gram-positive rods (H2, H18), Gram-negative rods (H7), and Gram-positive cocci (H11)(Photographs are original, unpublished and taken by the first author).

Table 3. Biochemical Evaluation of Cellulolysis by Various Bacterial Isolates[a,b,c,d,e]

sr. no.	bacterial isolates	bubble formation in Durham tubes	color development with TTC	clear zones diameter by Congo red staining (mm)	diameter of bacterial colonies	cellulolytic index
1	H1	−	+++ (maroon)	1.5	0.5	2.0
2	H2	−	+++ (maroon)		2.0	0
3	H3	++ (1 cm, day 3)	+++ (maroon)	7.0	3.0	1.33
4	H4	−	+++ (maroon)	4.0	1.0	3.0
5	H5	−	+++ (maroon)	4.0	2.0	1.0
6	H6	+ (0.1 cm, day 8)	+++ (maroon)	4.0	2.0	1.0
7	H7	−	+++ (maroon)		1.5	0.0
8	H8	−	+++ (maroon)		0.1	0.0
9	H9	−	+++ (maroon)	5.0	3.0	0.67
10	H10	−	+++ (maroon)	0.5	0.2	1.5
11	H11	−	+++ (maroon)	0.42	0.2	1.1
12	H12	−	+++ (maroon)	4.0	2.0	1.0
13	H13	+++ (1.5 cm, day 3)	+++ (maroon)	4.0	2.0	1.0
14	H14	+++ (1.6 cm, day 2)	+++ (maroon)	3.0	1.0	2.0
15	H15	+ (0.1 cm, day 5)	+++ (maroon)	5.0	3.0	0.67
16	H16	−	+++ (maroon)	3.0	1.0	2.0
17	H17	+ (0.2 cm, day 6)	+++ (maroon)	11.0	3.0	2.67
18	H18	−	+++ (maroon)	7.0	3.0	1.33
19	H19	−	+++ (maroon)		2.0	0.0
20	H20	−	+++ (maroon)	5.0	1.0	4.0
21	H21	+++ (4 cm, day 2)	+++ (maroon)	1.2	0.2	5.0
22	H22	+++ (3 cm, day 2)	+++ (maroon)	5.0	1.0	4.0
23	H23	+ (0.1 cm, day 2)	light pink, + +	0.62	0.2	2.1
24	H24	++ (1 cm, day 3)	+++ (maroon)	0.82	0.2	3.1
25	H25	+ (0.2 cm, day 4)	+++ (maroon)	4.0	2.0	1.0
26	H26	+ (0.1 cm, day 5)	+++ (maroon)	6.0	2.0	2.0

[a]Cellulolytic index = (clear zone diameter−bacterial colonies diameters)/ bacterial colonies diameter. [b]+++ Positive, strong response. [c]++ Positive, intermediate response. [d]+ Positive, weak response [e]− Negative response.

Figure 5a reveals that consumption of reducing sugars tends to be increased as the experiment continued for 10 days. It is assumed that consumed reducing sugars may be used for ethanologenesis and bacterial biomass production. Growth tendencies are presented in Figure 5b. All bacterial isolates have a long lag phase up to day 3 except H6, H13, H15, H25, and H26 and a short decline phase, i.e., days 9 and 10. The log phase varied between days 3 and 8. Bacterial growth measurement exposed the fact that the highest ethanol contents were observed in the log phase with actively dividing cells by all bacterial isolates with a good end product of ethyl alcohol.

2.7. Molecular Characterization of Selected Bacterial Isolates. A phylogenetic study on isolates H13, H17, H21, and H22 based on 16S rRNA gene sequences specified that the similarity and the closest relative strains to selected isolates were *Staphylococcus sp.*H13(99.28%), *Acinetobacter baumanni* H17 (99.17%), *Acinetobacter sp.* H21 (98.01%), and *Acinetobacter nosocomialis* H22 (97.32%) with 98% similarity correspondingly. From phylogenetic trees, Figure 6 depicts

that *Staphylococcus sp.,* H13 differed from its closest clades. However, *Staphylococcus sp.* Strain H13 showed the highest similarity/identity with Staphylococcus sp. strain L11 with 28 bootstraps. *Acinetobacter baumanni* H17 showed the closest resemblance with *Acinetocabter baumanni* OIFC 189 clone with 57 bootstraps (Figure 7). Figures 8 and 9 indicated that the selected species *Acinetocabter Sp.* H21 and *Acinetocabter nosocomialis* H22 showed a close relation with *Acinetocabter baumanni* and *Acinetocabter baumanni* AbCTX5, respectively, with boot straps 41 and 33.

3. DISCUSSION

The current study deals with the screening, identification, and characterization of cellulolytic and ethanologenic bacteria from termites. The termite collected from Jinnah Hospital and Botanical garden, Punjab University, Lahore was identified as *Heterotermes indicola*. The identification was made on the basis of behavior to make large number of tunnels as well as traveling long distances in tunnels and morphological features, viz, size and shape of the head, mandible, labrum, position of
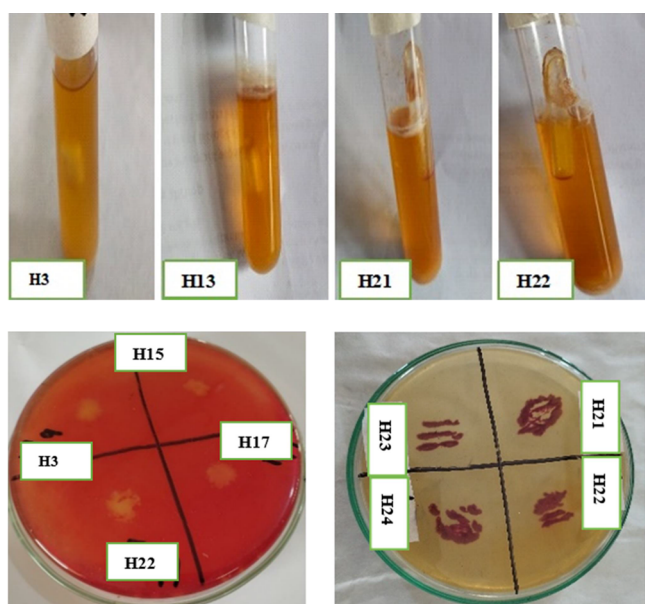
Figure 3. Bubble formation as observed in Durham tubes (upper row), clear zones by Congo red staining, and maroon color development with TTC (lower row) by different cellulolytic bacterial isolates (The photographs are original, unpublished and taken by the first author).

Table 4. Estimation of Cellulolytic Potential of Different Bacterial Isolates to Hydrolyze Cellulose[a]

bacterial Isolates	enzyme activity (μmol/min/mL)
H3	1.65 ± 0.01[B]
H6	1.41 ± 0.01[D]
H13	1.30 ± 0.03[E]
H14	1.52 ± 0.01[C]
H15	1.67 ± 0.02[B]
H17	1.79 ± 0.02[4]
H21	1.83 ± 0.01[A]
H22	1.62 ± 0.02[C]
H23	1.44 ± 0.01[D]
H24	1.56 ± 0.01[C]
H25	1.54 ± 0.02[C]
H26	1.55 ± 0.01[C]

[a]Data embodied means ± SEM. Significance is recorded by different letters at $p \leq 0.05$ by single-factor ANOVA.

antennal segments, and tooth of workers and soldiers by following the web based keys.[62−65] *Heteroterme indicola* (Wasmann), *Odontotermes obesus* (Rambur), *Microtermes obesi* (Holmgren), and *Coptotermes heimi* (Wasmann) are reported as the most common and damaging species for wood and wooden infrastructures in Pakistan. *Heterotermes indicola* in Pakistani regions has been found to cause massive damage to wooden structures in houses. On the basis of massive wooden damage, it is ranked as the most destructive domestic pest in Lahore.[66,67] The purpose of study is the isolation, characterization, and exploitation of termite harboring bacteria for cellulolysis and ethanologenesis. The gut of termites possesses large diversity of cellulolytic bacteria involved in digestion and degradation of lignolellulosic substrate and sugars.[23,25,68−70] Hence, the study supported the idea of the presence of cellulose-degrading bacteria in the digestive tract of termites. Twenty six bacterial isolates were selected on CMC-

supplemented medium from the samples of both sites. Few bacterial isolates were cultured in the laboratory. The gut of termite possesses diversified microbiota. The reason for less species of cultured bacteria may be the absence of real gut environment and natural nutrition.[25,71,72] As the study involves the isolation of cellulolytic bacteria from the gut of termite, using microbiological pure culturing, it is not necessary to maintain/provide the gut environment to the isolate, though food in the form of cellulose was provided. The study was successfully conducted, aiming to generate knowledge and creativity based on the potential benefits from the natural gut microbiota from the termite gut. In the natural gut environment, these microbes have a symbiotic relationship. However, further research is needed to optimize the cellulolytic potential of the isolated bacteria and to be used as a consortium to improve the enzymatic hydrolysis of LCB at a commercial scale. Among bacteria isolated from *H. indicola*, the 17 isolates belong to bacilli and the remaining were cocci. Ali et al.[37] isolated bacilli in subterranean termite *Psammotermes hypostoma*. In *Macrotermes michaelseni*, large frequencies of bacilli[49] and cocci[18] were observed. The diversity of bacteria in termites may differ due to variation in the food type, soil composition, and geographical regions.[73]

Bacterial isolates were isolated on CMC as the sole carbon source because of the only soluble form of cellulose.[74−76] The current study deals with the estimation of the ethanologenic and cellulolytic potentials of bacteria. Hence, different biochemical tests were performed to assess the fermentative and cellulolytic abilities of carboxy methyl cellulose. Cellulolysis is the breakdown or digestion of cellulose microfibril into oligosaccharides and monosaccharids.[77] Bacterial cellulases supported the cellulolysis of a complex substrate to digest them into monomers.[78] Carbon dioxide production by fermentation of monomeric sugars in Durham tubes was assessed in the form of bubbles. Durham tube testing is not in common practice because slow fermenting bacteria cannot be detected efficiently.[79] The sugar hydrolysis was also detected by two indicators, viz., TTC and Congo red stain. All isolates showed positive color development with TTC. Sugar hydrolysis by bacteria can be detected employing TTC which appeared as bright colored (light pink to dark maroon) formazones.[80−82] In living cells, TTC is converted from colorless to colored triphenyl formazon because it is a good electron acceptor.[83,84]

Congo red staining appeared as a sensitive and rapid test for screening of cellulose-degrading bacteria.[85] Except for four, all bacterial isolates formed varied zones of clearance around their colonies. The enzymes diffused into the medium by aging/lysis of bacterial cells to interact with the dye which in turn reduced the color of the dye.[86] Congo red stain is a sulfonatedazo dye, and bacterial plasma membrane is impermeable to the dye. The high cellulolytic activity resulted in high zones of clearance and low retention of the dye.[87−89] Hallow formation surrounding the bacterial colonies by Congo red is helpful to assess the cellulolytic potential and, in turn, cellulolytic index. For the computation of index, the diameter of hallows was divided by the bacterial colony diameter. Bacterial isolates showed varied cellulolytic index with the highest value 5.0 (H21). The hallow around the colonies after Congo red staining indicates production of extracellular cellulases by them.[90] Cellulolytic bacteria produce cellulase enzymes that will hydrolyze the cellulose present in the medium. Cellulose hydrolysis is evident by hallow formation because of the

Table 5. Ethanol Titer (g/L) from Cellulose by Different Bacterial Isolates[a]

days	H3	H6	H13	H14	H15	H17	H21	H22	H23	H24	H25	H26
1	1.11 ± 0.02[C]	2.04 ± 0.1[H]	1.57 ± 0.02[I]	1.20 ± 0.01[H]	1.67 ± 0.01[H]	1.97 ± 0.01[H]	2.64 ± 0.01[H]	1.73 ± 0.01[J]	0.67 ± 0.03[I]	0.71 ± 0.03[J]	0.89 ± 002[J]	2.18 ± 0.01[I]
2	1.51 ± 0.03[BC]	2.09 ± 0.01[H]	2.33 ± 0.01[H]	1.22 ± 0.01[H]	1.70 ± 0.01[H]	3.16 ± 0.01[F]	3.18 ± 0.02[G]	3.03 ± 0.01[I]	1.70 ± 0.04[F]	0.95 ± 0.01[I]	1.48 ± 0.03[H]	2.24 ± 0.01[H]
3	2.86 ± 0.05[AB]	2.54 ± 0.01[G]	2.45 ± 0.02[G]	1.99 ± 0.01[F]	2.44 ± 0.02[G]	3.42 ± 0.02[E]	3.24 ± 0.01[G]	3.40 ± 0.01[H]	1.72 ± 0.01[E]	1.04 ± 0.02[H]	1.68 ± 0.03[G]	2.63 ± 0.02[F]
4	2.10 ± 0.02[ABC]	4.64 ± 0.01[D]	4.62 ± 0.03[E]	3.30 ± 0.03[D]	3.53 ± 0.01[D]	4.11 ± 0.01[C]	3.67 ± 0.02[F]	3.91 ± 0.01[F]	1.96 ± 0.03[D]	3.12 ± 0.02[E]	2.22 ± 0.02[E]	2.67 ± 0.03[F]
5	2.32 ± 0.02[ABC]	5.08 ± 0.02[C]	5.23 ± 0.02[D]	4.00 ± 0.03[C]	3.57 ± 0.04[D]	4.17 ± 0.01[C]	5.30 ± 0.02[C]	4.39 ± 0.01[D]	1.97 ± 0.03[D]	3.23 ± 0.05[D]	2.63 ± 0.03[C]	3.18 ± 0.02[D]
6	2.34 ± 0.04[ABC]	5.20 ± 0.03[B]	5.78 ± 0.01[C]	4.02 ± 0.02[C]	3.90 ± 0.03[C]	4.29 ± 0.03[B]	5.34 ± 0.02[C]	5.00 ± 0.01[C]	3.12 ± 0.02[C]	3.37 ± 0.03[C]	2.87 ± 0.02[B]	3.52 ± 0.01[C]
7	2.55 ± 0.01[ABC]	5.54 ± 0.02[A]	6.22 ± 0.03[B]	4.84 ± 0.03[A]	4.22 ± 0.01[B]	4.36 ± 0.01[B]	5.87 ± 0.07[B]	5.42 ± 0.01[B]	4.22 ± 0.02[B]	3.68 ± 0.02[B]	3.36 ± 0.03[A]	5.63 ± 0.02[A]
8	3.18 ± 0.01[A]	5.52 ± 0.02[A]	5.91 ± 0.03[A]	4.59 ± 0.01[B]	4.44 ± 0.01[A]	6.54 ± 0.01[A]	7.00 ± 0.01[A]	5.64 ± 0.02[A]	4.92 ± 0.02[A]	4.57 ± 0.02[A]	2.36 ± 0.01[D]	3.62 ± 0.03[B]
9	1.71 ± 0.03[ABC]	3.64 ± 0.01[F]	3.77 ± 0.01[F]	2.67 ± 0.02[E]	3.30 ± 0.03[E]	3.58 ± 0.01[D]	4.77 ± 0.02[D]	4.09 ± 0.01[E]	1.55 ± 0.04[G]	1.73 ± 0.02[F]	2.03 ± 0.01[F]	2.98 ± 0.02[E]
10	1.70 ± 0.04[ABC]	4.36 ± 0.02[E]	2.44 ± 0.01[G]	1.76 ± 0.01[G]	2.60 ± 0.04[F]	2.17 ± 0.01[G]	4.06 ± 0.03[E]	3.59 ± 0.01[G]	1.28 ± 0.02[H]	1.07 ± 0.01[G]	1.26 ± 0.02[I]	2.51 ± 0.02[G]

[a]Values represent means ± SEM. Significance is recorded by different letters at $p \leq 0.05$ by single-factor ANOVA.

reaction between Congo red and 1,4-glycosidic present in the cellulose polymer.[91] The principle of Congo red staining is that the dye diffuses into the agar medium. The higher solubility of the enzymes will result in the form of larger hallow/clear zones (Jo et al.).[92] . The variation in the cellulolytic index value can be attributed to the isolates' capacity to hydrolyze the cellulose present in the medium through the release of endo-β-1,4-glucanase (CMCase). CMCase is an enzyme generated by the cellulolytic bacteria that breaks down the β-1,4 glycoside bond in the CMC medium.[93,94] The isolates possessing cellulolytic index higher than 1.5 or 2.0 may be considered as an efficient cellulase producer.[77] These findings corroborated with the cellulolytic index reported by Kakkar et al.[69] in gut bacteria from *Odontotermes parvidens*, Raheli et al.[25] in *Macrotermes michaelseni*, and in different termites.[77]

The bacterial strains isolated for the current study had efficient cellulase producers to convert cellulosic microfibrils into oligosaccharides and monosaccharides. The isolates H21 and H17 showed the highest activity as 1.83 ± 0.01, 1.79 ± 0.024 μmol/min/mL respectively. The results were corroborated with findings of Ali et al.[37] who recorded the CMCase activity from 0.22 to 2.28 U/mL by *Paenibacillus lactis*, *Lysinibacillus macrolides*, *Stenotrophomonas maltophilia*, *Lysinibacillus fusiformis*, and *Bacillus cereus* isolated from lower termite *Psammotermes hypostoma*. On the contrary, Bacillus species B1, B2 and *Brevibacillus sp.* Br3 isolated from higher termite *Bulbitermes sp.* showed high 138.77 U/g endoglucanase, 32.16 U/g exoglcanases and 104.96 U/g xylanase activities under solid state fermentation, respectively.[95,96] Similarly cellulolytic activity was observed as 0.9 μmol/mL/min CMCase by *Paenibacillus sp.*[97] 2.40 IU/mL CMCase as well as 1.43 FPU/mL by *Phanerochaete chrysosporium*,[98] 1.07 FPU/mL in alkaline rice straw[99] and 1.9 FPU/mL by *T. reesei* in steam-treated wheat straw.[100] The varied findings such as 0.71 FPU/mL was reported in powdered rice straw by *C. thermocellum*,[101] 10.5FPU/mL by *A. cellulolyticus*[102] and 154.58 U/gds with *Trichoderma reesei* in sugar cane bagasse.[103] *Aspergillus niger* released 0.1813 IU/mL cellulases,[104] 25.6 U/mL by *Streptomyces*,[105] and 16.2 IU/g with *Trichoderma reesei*.[106] Iqbal et al.[107] investigated the maximum CMCase potential (480 ± 4.22 μM/mL/min) after seventh day of

incubation at specific condition. All of these findings from the literature differed from the investigations of the present study.

The present study attempted to screen ethanologenic bacterial strains on 2% CMC-supplemented medium. Bacterial isolates H13, H17, H21, and H22 produced maximum ethanol g/L as 7.21 ± 0.03, 6.54 ± 0.01, 7.00 ± 0.01, and 5.64 ± 0.02 on day 8. Similar results, i.e., 10.8 g/L ethanol contents were recorded at a maximum concentration by *Streptomyces sp.* identified from *Microcerotermes sp.* The results of ethanol contents corroborated with the findings of 8.3 g/L employing *B. subtilis* in potato wastes,[108] while the ethanol assay 15.73 ± 0.44, 14.22 ± 0.15, and 17.73 ± 0.25 g/L recorded by Chandel et al.[109] via enzymes prepared from *P. stipitis*, *A. oryzae* MTCC 1846, and *S. cerevisiae* VS3 correspondingly differed from the findings of the current study. Ethanol yields (g/g) 0.37, 0.38, 0.40, and 0.37 with the percent FE of 71.70, 73.53, 78.38, 72.56 were calculated for H13, H17, H21, and H22 respectively. The findings of Rudolf et al.[110] and Abedinifar et al.[111] agreed with the results who reported an ethanol yield of 0.30 g/g from *S. cerevisiae* on sugar cane, 0.36−0.43 g/g by *Mucor indicus*, as well as 0.37−0.45 g/g via *S. cerevisae* in enzymatically treated rice straw. *Zymomonas mobiliz* generated 60.5 g/L ethanol content with 0.30 g/g yield using solid carob pods and wheat bran mixture under submerged fermentation.[112,113] These findings varied from the current values in terms of ethanol titer. In the simultaneous saccharification and fermentation of potato peels by *S. cerevisiae*, an ethanol yield of 0.32 g/g was obtained by Chohan et al.[114] In another report, *Z. mobiliz* fermented the potato peels for the highest ethanol yield after day 5, which was higher than *S. cerevisiae* after day 7. The selected bacterial isolates produced maximum ethanol on day 7−8 using CMC. The findings are comparable with the data obtained by Mazaheri and Pirouzi.[115] The isolated microbes may tolerate the ethanol contents and ferment the sugars.

The present study endeavored to figure out the consumption of cellulose in the fermentation medium before and after the experiment by the reducing sugar analysis. During the fermentation study, the reducing sugars were utilized to produce ethanol contents. Both factors were inversely proportional to each other. Furthermore, an increasing trend in sugar consumption was noticed up to the termination of the

Table 6. Calculated Ethanol Yield (g/g) and FE (%) from CMC by Different Bacterial Isolates[a,b,c]

	H3		H6		H13		H14		H15		H17	
days	Yi	FE	Yi	FE	Yi	FE	Yi	FE	Yi	FE	Yi	FE
1	0.21 ± 0.12	40.83 ± 0.21	0.16 ± 0.03	30.75 ± 0.19	0.15 ± 0.11	28.56 ± 0.01	0.17 ± 0.21	34.10 ± 0.19	0.21 ± 0.02	40.98 ± 2.90	0.25 ± 0.01	49.65 ± 1.45
2	0.25 ± 0.02	48.94 ± 0.17	0.15 ± 0.02	29.50 ± 0.14	0.21 ± 0.04	41.42 ± 0.01	0.16 ± 0.12	31.81 ± 0.20	0.19 ± 0.03	37.84 ± 2.02	0.29 ± 0.04	57.16 ± 1.25
3	0.21 ± 0.08	40.70 ± 0.23	0.17 ± 0.07	33.43 ± 0.19	0.16 ± 0.03	32.24 ± 0.07	0.23 ± 0.07	45.80 ± 1.27	0.23 ± 0.06	44.22 ± 1.05	0.30 ± 0.02	58.06 ± 3.04
4	0.24 ± 0.02	46.12 ± 0.02	0.29 ± 0.40	56.90 ± 0.21	0.31 ± 0.30	60.03 ± 0.06	0.30 ± 0.40	58.40 ± 1.55	0.27 ± 0.02	53.28 ± 3.87	0.34 ± 0.30	67.27 ± 1.77
5	0.27 ± 0.40	53.45 ± 0.08	0.31 ± 0.14	60.15 ± 0.05	0.31 ± 0.02	60.72 ± 0.05	0.33 ± 0.22	65.47 ± 3.92	0.27 ± 0.06	52.91 ± 2.44	0.33 ± 0.12	65.46 ± 1.71
6	0.28 ± 0.01	54.23 ± 0.10	0.31 ± 0.01	60.33 ± 0.18	0.34 ± 0.01	66.32 ± 0.02	0.33 ± 0.01	64.03 ± 1.10	0.28 ± 0.20	55.37 ± 2.85	0.32 ± 0.02	63.25 ± 3.15
7	0.27 ± 0.21	52.30 ± 0.15	0.32 ± 0.02	63.52 ± 0.41	0.37 ± 0.14	71.70 ± 0.05	0.33 ± 0.21	65.18 ± 1.62	0.29 ± 0.19	57.22 ± 1.78	0.30 ± 0.01	59.70 ± 1.50
8	0.29 ± 0.21	57.63 ± 0.26	0.31 ± 0.23	61.25 ± 0.11	0.33 ± 0.31	64.74 ± 0.32	0.32 ± 0.11	61.98 ± 1.58	0.29 ± 0.11	57.69 ± 1.88	0.38 ± 0.32	73.53 ± 0.15
9	0.13 ± 0.03	26.22 ± 0.05	0.20 ± 0.02	39.87 ± 0.17	0.21 ± 0.32	41.30 ± 0.59	0.17 ± 0.01	33.78 ± 1.34	0.21 ± 0.01	40.80 ± 2.10	0.21 ± 0.21	40.62 ± 1.13
10	0.12 ± 0.41	24.08 ± 0.11	0.24 ± 0.31	47.79 ± 0.19	0.14 ± 0.19	26.91 ± 0.12	0.11 ± 0.31	21.58 ± 1.34	0.16 ± 0.21	32.35 ± 2.29	0.13 ± 0.15	24.51 ± 0.62

	H21		H22		H23		H24		H25		H26	
days	Yi	FE	Yi	FE	Yi	FE	Yi	FE	Yi	FE	Yi	FE
1	0.24 ± 0.01	46.22 ± 1.31	0.16 ± 0.01	31.58 ± 1.51	0.46 ± 0.13	89.37 ± 0.04	0.12 ± 0.01	23.60 ± 2.45	0.20 ± 0.02	39.57 ± 3.31	0.28 ± 0.01	54.80 ± 1.58
2	0.25 ± 0.07	48.52 ± 1.43	0.27 ± 0.09	53.52 ± 0.89	0.42 ± 0.15	82.30 ± 0.03	0.14 ± 0.01	26.96 ± 0.04	0.29 ± 0.01	56.68 ± 2.17	0.27 ± 0.01	52.16 ± 1.95
3	0.25 ± 0.02	48.91 ± 1.14	0.28 ± 0.05	55.14 ± 2.77	0.31 ± 0.05	60.33 ± 0.18	0.14 ± 0.02	26.55 ± 0.12	0.31 ± 0.07	61.69 ± 0.21	0.29 ± 0.06	56.54 ± 0.90
4	0.27 ± 0.01	53.66 ± 1.37	0.30 ± 0.10	59.57 ± 2.40	0.23 ± 0.20	44.69 ± 0.05	0.39 ± 0.02	75.62 ± 4.28	0.32 ± 0.09	63.09 ± 0.57	0.27 ± 0.7	52.88 ± 1.45
5	0.38 ± 0.32	74.28 ± 1.80	0.32 ± 0.11	63.43 ± 2.24	0.16 ± 0.23	31.40 ± 0.52	0.36 ± 0.09	70.45 ± 0.03	0.33 ± 0.14	65.28 ± 2.46	0.29 ± 0.11	57.20 ± 1.44
6	0.37 ± 0.10	72.06 ± 2.60	0.36 ± 0.31	70.08 ± 2.30	0.24 ± 0.11	47.02 ± 1.05	0.37 ± 0.22	73.26 ± 2.68	0.33 ± 0.02	63.95 ± 3.08	0.30 ± 0.21	58.24 ± 0.12
7	0.39 ± 0.11	77.30 ± 1.62	0.37 ± 0.12	71.86 ± 3.10	0.30 ± 0.21	59.53 ± .95	0.38 ± 0.25	75.01 ± 0.18	0.35 ± 0.4	68.48 ± 0.09	0.36 ± 0.21	70.72 ± 2.53
8	0.40 ± 0.10	78.38 ± 2.05	0.37 ± 0.21	72.56 ± 1.70	0.35 ± 0.51	68.32 ± 1.18	0.39 ± 0.22	76.98 ± 0.21	0.23 ± 0.33	45.46 ± 0.20	0.26 ± 0.32	50.66 ± 0.61
9	0.31 ± 0.31	61.49 ± 1.74	0.27 ± 0.31	52.73 ± 2.13	0.10 ± 0.22	20.40 ± 0.33	0.14 ± 0.22	27.12 ± 0.17	0.19 ± 0.01	36.52 ± 2.36	0.20 ± 0.11	39.22 ± 1.31
10	0.26 ± 0.32	50.07 ± 0.06	0.24 ± 0.32	46.90 ± 1.73	0.08 ± 0.13	16.60 ± 0.30	0.08 ± 0.09	15.87 ± 0.21	0.12 ± 0.2	22.69 ± 2.89	0.19±0.22	37.83 ± 0.78

[a]Values represent means ± SEM. Significance is recorded by different letters at $p \leq 0.05$ by single-factor ANOVA. [b]Yi, ethanol yield. [c]FE, fermentation efficiency.

experiment even though the ethanol contents decreased after day 8. Moreover, the maximum ethanol contents were detected in the exponential growth phase in all bacterial isolates. Bacterial cells are the competent source for production of cellulases in a liquid medium. The cellulolytic product, i.e., glucose, serves as the sole carbon source for bacterial biomass and fermentation, which in turn reduces the sugar yield. Similarly, the ethanol production rate is going to be decreased by the lowering sugar level.[116] Contrary to this, high glucose concentrations may inhibit enzyme activity and growth of bacteria.[117] The more effectual uptake and consumption of sugars by microbes are the main factors for efficient ethanol production.[118] *Saccharomyces cerevisiae* uses the Embden-Meyerhof-Parnas (EMP) glycolytic pathway, whereas *Zymomonas mobiliz* utilizes the Entner-Doudoroff (ED) pathway for ethanol yield.[119] *Zymomonas mobiliz* produces biomass by employing the ED pathway than the EMP pathway, utilizing *Escherichia coli* and *S. cerevisiae*. Subsequently, more carbon source will be available for fermentation with 2.5-fold greater

ethanol productivity in *Z. mobiliz* than *S. cerevisiae*.[120] Likewise, Clostridia harbors lignocellulosic enzymes that are secreted naturally to hydrolyze polymeric sugars to fermentable monomeric sugars, both hexoses and pentoses.[121,122]

Four selected competent bacterial strains were characterized molecularly as *Staphylococcus sp.* H13, *Acinetobacter baumanni* H17, *Acinetobacter sp.* H21, and *Acinetobacter nosocomialis* H22 with 98% similarity. Several researchers had reported different bacterial species from gut of termite capable of lignocellulose degradation such as *Alcaligenes faecalis* HI-1,[123] Cellulomonas,[124,125] Acinetobacter, *Bacillus cereus*, and *Enterobacter aerogenes*.[48]

From the past decade, several studies for screening of cellulolytic bacteria from termites indicated that bacteria have competence to hydrolyze crystalline cellulose completely.[126,127] The bacteria grow rapidly than yeast and fungi and hence can be used widely for cellulase production and can be optimized efficiently for certain cultural conditions.[128] The study was a stepping stone in establishing the large-scale
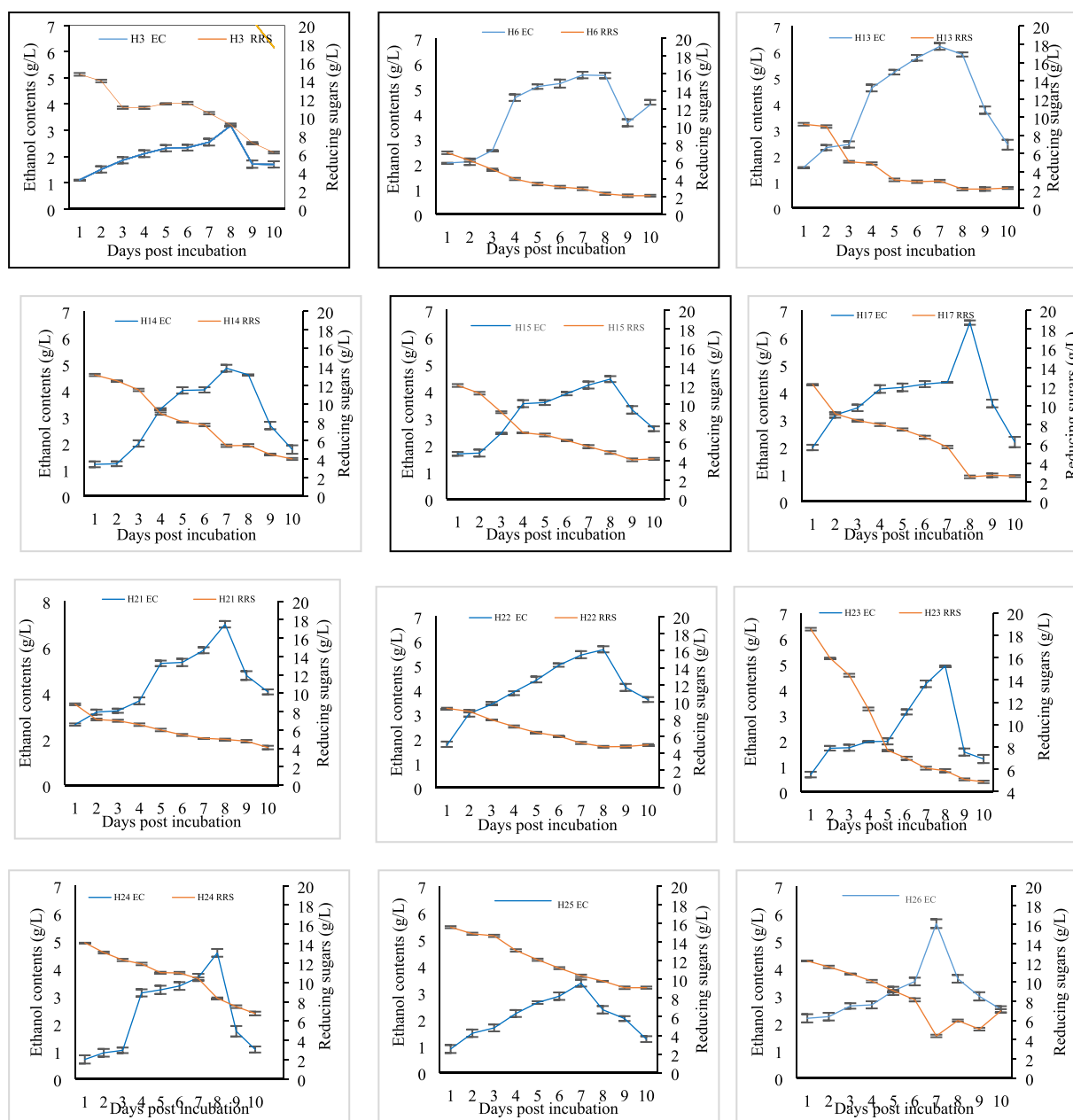
Figure 4. Ethanol and remaining reducing sugar contents in the fermentation medium supplemented with 2% cellulose by bacterial isolates. Bars denote average ± standard error mean.

process development of bioenergy production. As with every passing day, energy sources of the world are diminishing at alarmingly high speed, there is a great need to explore more efficient, sustainable, and renewable alternates.[129,130] Cellulose and hemicellulose present in the LCB, in the prospect, can contribute as the prime source substrates for bioenergy generation. The systematic conversion of cellulose (via the employment of cellulolytic bacteria) into fermentable sugar is considered as the most critical step in the biofuel production.[131] Once this step is optimized in as a cost-competitive way, it may lead to the establishment of eco-viable process development.[132] Hence, these selected bacterial isolates may be proved as efficient candidates for cellulosic biomass transformation into bioethanol commercially and can be employed in plant waste degradation to yield fermentable sugars.

The cellulases obtained from microbes can be used in large scale for bioremediation of cellulosic wastes (Dixit et al.),[133] for large-scale cellulosic substrate conversions into value-added products, e.g., biofuels (Ahmad et al.),[129] biofertilizers (Yu et al.),[134] animal feed (Azizi-Shotorkhoft et al.),[135] pulp and paper (Karthika et al.),[136] textile (Bussler et al.).[137] As the study deals with the employment of native cellulolytic bacterial isolates, metabolic engineering can further enhance the cellulolytic and ethanologenic abilities of the microbes. In this regard, the detailed study on understanding of key metabolic pathways leads to desirable outcomes.

4. CONCLUSIONS

The present study supported the novel approach to search and screen competent *Heterotermes indicola*'s gut-associated bacteria possessing novel and efficient cellulolytic enzymes. The
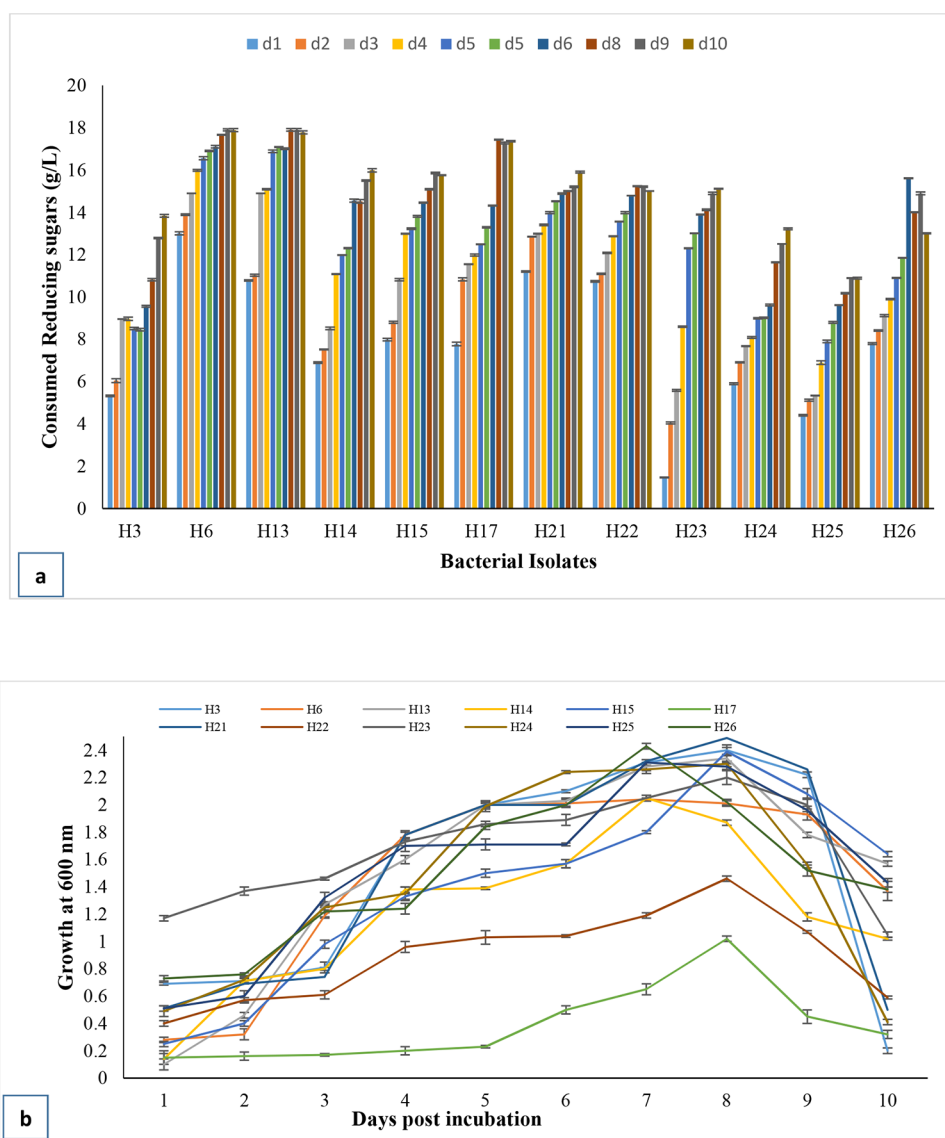
Figure 5. Consumed reducing sugar contents (a) and growth (b) of selected bacterial isolates in a fermentation medium.

data revealed the competency of *Acinetobacter sp.* H21 screened from *H. indicola*' gut with a cellulolytic index of 5.0 and CMCase activity as 1.83 ± 0.01 umol/mL/min. Ethanol titer with *Acinetobacter sp.* H21 is observed as 7.00 ± 0.01 g/L having an ethanol yield of 0.40 g/g and 78.38% FE.

The gut of higher termites proved as a natural and potential source for screening of novel cellulolytic bacteria and enzymes. The screened cellulolytic bacteria possess the ability for biotransformation of cellulose into glucose and can be employed for biofuel production, enzyme purification, and composting.

5. MATERIALS AND METHODS

5.1. Sampling of Termite for Bacterial Isolation. In the current study, *H. indicola* workers were collected from the highly infested, standing trees of *Poplus euramericana* using forceps and a chisel from two sampling areas, i.e., Botanical garden, University of Punjab (31° 520′ N, 74° 358′ E), and Jinnah hospital, (31° 495′ N, 74° 286′ E) Lahore, Pakistan on 13th July, 2021. At both locations, termites were found in wood, forming tunnels in soil leading to wood (Figure 1).

Random samples of *Heterotermes indicola* termites were taken from the decomposing logs present in both the study areas. A clean plastic container containing a minimum of several dozen termites, along with a sample of wood and soil, was brought back to the laboratory and kept at 26 °C and total darkness. The termite samples were identified morphologically based on the size and shape of heads and mandibles. The termites feed on the bark and soft parts around the base and the stems of trees. Twenty five termites were isolated and cleaned by disinfecting surfaces with 70% ethyl alcohol and stored at 4 °C by keeping in sterilized plastic bags. Within 48 h, termites were dissected for sampling of gut to be proceeded for bacterial isolation.

The gut of 24 termites were dissected using sterile dissecting tools (with 70% alcohol for 30 s) in a sterile air flow safety cabinet (laminar flow). The gut of the dissected termites was separated. The suspension was made in triplicates by crushing the 8 guts with a sterile glass rod in 2 mL of sterilized phosphate buffer saline (0.9% PBS) in a sterile glass vial. Phosphate buffer saline was prepared by mixing (g/L) Na_2HPO_4, 1.44, KCl, 0.2, NaCl, 8, KH_2PO_4, 0.24 by adjusting
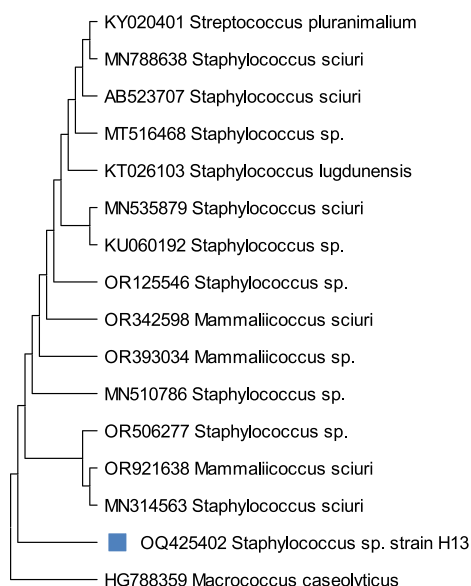
Figure 6. Phylogenetic tree of *Staphylococcus sp.* H13 constructed by the neighbor-joining method with the bootstrap test showing replicate percentage and associated taxa. The tree with the highest log likelihood (-3137.86) is shown. Maximum Composite Likelihood tool computed evolutionary distances with 31 nucleotide sequence analysis by deletion of ambiguous pair wise positions. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories ($+G$, parameter = 0.1031)]. Final data set has 1535 positions.

Figure 8. Phylogenetic tree of *Acinetobacter sp.* H21 by the neighbor-joining method with the bootstrap test showing replicate percentage and associated taxa. The tree with the highest log likelihood (-4277.18) is shown. Maximum Composite Likelihood tool computed evolutionary distances with 31 nucleotide sequence analysis by deletion of ambiguous pair wise positions. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories ($+G$, parameter = 0.0916)]. The rate variation model allowed for some sites to be evolutionarily invariable ([$+I$], 24.25% sites). Final data set has 1501 positions.

pH at 7.4. The suspension (0.1 mL) was plated on CMC-enriched media at 37 ± 1 °C for 1 day. Spread plate method was adopted for bacterial strain isolation from the gut contents. The composition (%) of CMC-supplemented medium was cellulose 2 g, peptone 1.5 g, yeast extract 1 g, MgSO$_4$ 0.05 g, (NH$_4$)$_2$SO$_4$ 0.1 g, KH$_2$PO$_4$ 0.1 g, and CaCl$_2$ 0.1 g with agar 2

g.[138] It could be assumed that the medium having CMC, yeast extract, and peptone served as the main carbon source for efficient ethanologenic fermentation. Bacterial isolates were selected based on the morphological characteristics of colonies.
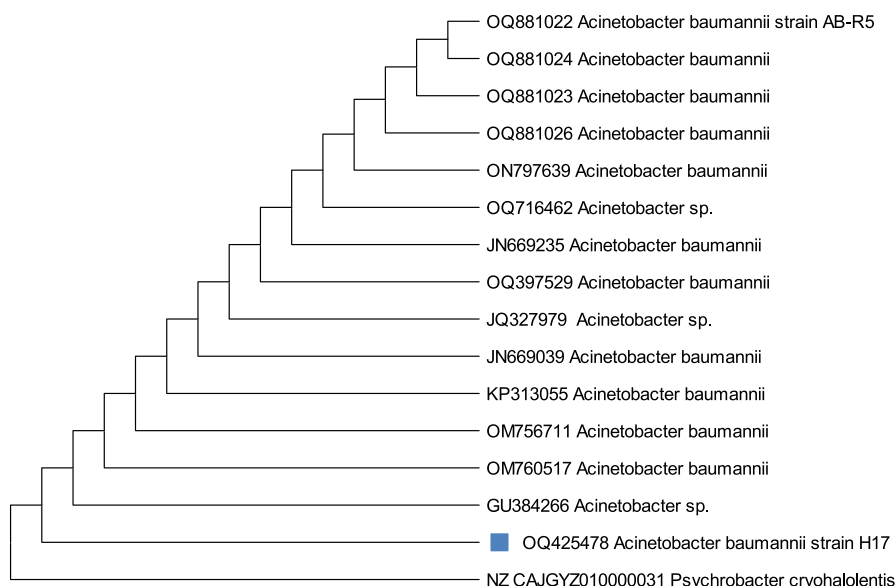

Figure 7. Phylogenetic tree of *Acinetobacter baumanni* H17 by the neighbor-joining method with the bootstrap test showing replicate percentage and associated taxa. The tree with the highest log likelihood (-3696.54) is shown. Maximum Composite Likelihood tool computed evolutionary distances with 31 nucleotide sequence analysis by deletion of ambiguous pair wise positions. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories ($+G$, parameter = 0.2439)]. The rate variation model allowed for some sites to be evolutionarily invariable ([$+I$], 41.21% sites). Final data set has 1560 positions.
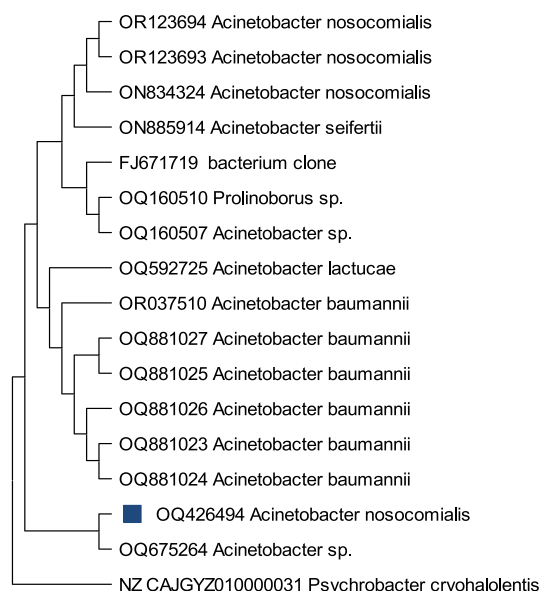
Figure 9. Phylogenetic tree of *Acinetobacter nosocomialis* H22 by the neighbor-joining method with the bootstrap test showing replicate percentage and associated taxa. The tree with the highest log likelihood (−3730.65) is shown. Maximum Composite Likelihood tool computed evolutionary distances with 31 nucleotide sequence analysis by deletion of ambiguous pair wise positions. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+*G*, parameter = 0.5376)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 37.37% sites). Final data set has 1504 positions.

Streak plate method was adopted for the pure culturing of isolated strains.

5.2. Biochemical Evaluation for Sugar Degradation.
The FE and sugar degrading ability were assessed by various biochemical testings. The isolated bacteria formed gas in Durham tubes, clear zones after Congo red staining, and maroon color by TTC. The medium for Durham tubes testing composed of (%) yeast extract 1, peptone 1.5, $(NH_4)_2SO_4$ 0.1, KH_2PO_4 0.1, $MgSO_4$ 0.05, and cellulose 2.[138] The medium was dispensed in test tubes (10 mL) and Durham tubes (2 mL). The filled Durham tubes were placed inversely in a test tube containing liquid medium followed by inoculation of bacterial isolates. The fermentation medium dispensed test tubes were incubated at 37 ± 1 °C in static position for 10 days. The gas was detected in the form of bubbles in Durham tubes, and the length of the bubble was recorded.[139]

Congo red staining is established as a qualitative technique for the detection of sugar degradation during fermentation.[18] Cellulose-supplemented medium was prepared by mixing yeast extract 1 g, $MgSO_4$ 0.05 g, cellulose 2 g, peptone 1.5 g, $(NH_4)_2SO_4$ 0.1 g, KH_2PO_4 0.1 g, and $CaCl_2$ 0.1 g with agar agar 2 g following the protocol laid by Zhang et al.[138] All selected bacterial isolates were streaked on the cellulose-supplemented medium with autoclaved tooth picks for incubation for 16 hours at 37 ± 1 °C. One percent aqueous Congo red stain was flooded on streaked Petri plates with bacterial growth followed by reincubation for half an hour for 30 min at 37 ± 1 °C. The destaining of the bacterial culture was done by 1% aqueous NaCl solution. The sodium chloride-flooded bacterial cultures were incubated again for 30 min at 37 ± 1 °C for the removal of extra and unbound stain. For fine results, the destaining step was repeated three to four times.

The sugar polymer degradation by bacterial cultures was evident in the form of hallow Petri plates.

The cellulose and TTC-enriched medium were used to evaluate the growth and color development of the bacterial isolates.[138] Cellulose-supplemented medium (yeast extract 1 g, $MgSO_4$ 0.05 g, cellulose 2 g, peptone 1.5 g, $(NH_4)_2SO_4$ 0.1 g, KH_2PO_4 0.1 g, $CaCl_2$ 0.1 g, and agar 2 g) was mixed with TTC solution (10 mL of 0.5% aqueous TTC) after autoclaving. The inoculated Petri plates were kept for incubation overnight at 37 ± 1 °C. The development of pink to maroon color of colonies was interpreted as the positive test.

5.3. Screening of Bacterial Isolates Based on Morphology.
Different cellulolytic bacteria were screened for identification on the basis of colonial and cellular features on 2% cellulose-supplemented medium.[140] The observed colonial features for bacteria were color, size, elevation, texture, margin, optical feature, pigmentation, and consistency. For cellular characteristics, Gram's reaction was performed. Sizes of stained bacterial cells were measured via micrometry using ocular and stage micrometers.

5.4. Fermentation Experiments.
The composition of synthetic media with little modification was adopted by following the protocols of Chaudhary et al.[130] The selected bacterial isolates were revived for the fermentation experiment in 2% CMC-enriched liquid medium. The bacterial cultures were incubated at 37 ± 1 °C for 24 h and kept on shaking. Synthetic medium contained (g/L) CMC 20, $ZnCl_2$ 0.00042, $(NH_4)_2SO_4$ 2.6, $MgSO_4 \cdot 7H_2O$ 0.8, KH_2PO_4 2.72, yeast extract 6.5, sodium citrate 6, $CaCl_2$ 0.3 and citric acid 1.5. One percent inoculum was used to start the fermentation. Fermentation was carried out in narrow-necked and screw-capped glass bottles. The fermentation of the inoculated synthetic medium continued statically at 37 ± 1 °C for 10 days. Estimation of reducing and ethanol contents were performed by drawing samples daily. Spectrophotometric measurement of bacterial growth was performed at 600 nm. Ethanol yield (Yi) and fermentation FE were computed by the following expressions;

$$Yi(g/g) = \frac{\text{Ethanol produced}\left(\frac{g}{L}\right)}{\text{Reducing sugar consumed}\left(\frac{g}{L}\right)}$$

$$FE = \frac{\text{Practical ethanol yield}}{\text{theoretical ethanol yield}} \times 100$$

5.5. Cellulolytic Activity.
The basal medium for crude enzyme preparation include (g/L) magnesium sulfate 0.01, disodium hydrogen phosphate 0.7, sodium citrate 0.05, potassium dihydrogen phosphate 0.2, yeast extract 0.1, and CMC 2 and pH 7.0 according to protocols established by Abu-Gharbiya et al.[141] The inoculated basal medium was agitated with 200 rpm at 37 ± 1 °C for 72 h. The supernatant after 15 min of centrifugation (1000 rpm) served as the bacterial crude enzyme. For the cellulolytic assay, the substrate buffer was prepared by mixing 2% CMC in acetate buffer (pH 5.0, 0.2 M). The ingredients of 0.2 M acetate buffer were (g/L) sodium acetate trihydrate 54.43, glacial acetic acid 12 mL. For the assay, 1.0 mL of substrate buffer was mixed with 0.5 mL of crude enzyme. The enzyme mixture was warmed for 30 min at 50 °C. Reducing sugars were measured by addition of 3 mL of DNS reagent followed by boiling in a water bath (5 min). The color change was measured spectrophotometrically at 640 nm. The color change in the DNS reagent indicated the conversion

of cellulose into monomeric sugars.[142] Cellulolytic potential was calculated by the following expression.

$$\text{Enzymatic activity (μmol/min/mL)} = [\text{Sample OD} \times \text{Standard factor}$$
$$(10.64) \times 1000 \times \text{Reaction volume (1.5 mL)}]$$
$$/[\text{Moleculer weight (150.13)} \times \text{Total crude enzyme (0.5 mL)}$$
$$\times \text{Incubation time (30 min)}]$$

5.6. Molecular and Phylogenetic Characterization of Selected Bacterial Isolates. Four efficient bacterial isolates H13, H17, H21 and H22 were selected for 16s rRNA based molecular characterization on the basis of maximum CMCase potential, ethanol yield, and FE. The bacterial DNA from the colony was extracted with 5 mM NaOH and heated at 95 °C and 1 M Tris HCl followed by centrifugation. PCR master mixture (Thermo Fischer) and bacterial colony lysate (2 ul) were used for 16 S rRNA amplification. 35 polymerase chain reactions proceeded at 98 °C denaturation (10 s), 53 °C annealing (30 s) and 72 °C (1 min) extension temperatures. LabGenetix, Lahore, Pakistan facilitated the sequencing and BLAST homology querying of the amplified gene from bacterial isolates following the GenBank database (http://www.ncbi.nlm.nih.gov/blast). The sequenced genes were submitted to the NCBI database under GenBank accession numbers of OQ425402H13, OQ425478H17, OQ425493H21, and OQ426494H22. Based on the phylogenetic tree construction, the main branch with a bootstrap value of 100% was formed, namely, the genus branch cluster with the respective isolates.

The evolutionary history was inferred by using the Maximum Likelihood method, the Jukes-Cantor model,[143] and the Kimura 2-parameter model.[144] The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Jukes-Cantor model and Composite Likelihood (MCL) approach and then selecting the topology with the superior log likelihood value. Evolutionary analyses were conducted in MEGA11 software ver, 10 Biodesign Institute, Tempe, USA.[145] The accuracy and authenticity of the phylogenetic tree was examined with higher bootstraps. To check the reliability of the phylogenetic tree branches, bootstrap analysis is considered as best tool which interpreted the best use of model data sets.[146]

5.7. Statistical Evaluation of Data. The experimental data was recorded by means with standard error of means. One-way analysis of variance (Duncan Multiple Range, Minitab Software, LLC, USA,Ver 17.1.1.) was used as a statistical tool for data evaluation.

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Notes

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