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Determination of hydrolyzing and ethanolic potential of cellulolytic bacteria isolated from fruit waste

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ORIGINAL ARTICLE

Abstract

Fruit wastes containing cellulose can be made valuable by cellulolytic bacteria in biofuel production. This study aimed to assess the potential of isolated cellulolytic bacteria to produce bioethanol and its fermentation efficiency. Seven out of 16 bacterial isolates were selected on the basis of their cellulose-degrading potential by providing cellulose as the only carbon source. Their potential to degrade cellulose was determined by different biochemical tests. All strains produced bubbles as indicators of carbon dioxide production in Durham tubes. The maximum hallow on Congo red staining was shown by CA2, CG2, as 54, 40 mm with cellulolytic index 16.3 and 19. Cellulose degradation was evaluated as light pink to maroon color in triphenyl tetrazolium in all strains except CA4 and CB1. Cellulose (2%) added medium was provided to the isolated strains for the period of 10 days to allow fermentation. CG2 and CA2 yielded maximum ethanol as 0.42±0.005 and 0.43±0.011 (g of ethanol/ g of reducing sugar consumed), respectively. Their percent fermentation efficiency was observed as 79.71±0.059% and 75.58±0.011% correspondingly. All strains showed cellulose activity, and the maximum was seen in CA2 and CG2 as 0.265±0.05, 0.27±0.011 µmol/min/L. Molecular characterization suggested that the CA2 and CG2 strains were *Bacillus manliponesis* CA2 and *Bacillus* sp. CG2 (Accession Nos. ON324120 and OM974175). This study elaborates on the capability of bacteria to produce bioethanol by degrading cellulose.

Keywords: Cellulolytic bacteria; ethanol yield; CMCase activity; Lignocellulosic Biomass; Reducing Sugar Content

Introduction

Energy is a significant part of an advanced social economy (Aziz *et al.*, 2023; Chu, 2023). Constant exhaustion of insufficient natural resources is a worldwide problem (Shao *et al.*, 2023). In Pakistan, the power sectors and transport mainly consume 78.5% of petroleum products. However, in the year 2022–2023, the demand of diesel and motor spirit has been reduced by 21.9% because of inflation. About 5.7 billion dollars have been invested on its imports. Whereas, to overcome this problem, consumption of natural gas has been increased up to 3258 MMCFD. In this year, total electricity consumption was 84,023 GWh (Pakistan Economic Survey, 2022–2023).

Some major reasons for the energy crisis are over consumption of oil and petroleum, poor infrastructure, and no focus on renewable energy sources (Chien *et al*., 2021). Today, global warming is an alarming issue of the world (Yakovlev and Belyaev, 2023). Energy produced from fossil fuels has a central place in other environmental problems. Burning of fossil fuels produces CO_2 which is a prime reason (77%) for the greenhouse effect (Wang *et al.*, 2021; Ghezelbash *et al*., 2023).

Ethanol is mostly used as fuel and as a renewable energy source to compensate for the rising prices of oil and petroleum. Ethanol is an ecofriendly biofuel as it has fewer greenhouse gas emissions that eventually help to reduce atmospheric pollution (Bilgili, 2023; Dubey *et al*., 2023). It contains 35% oxygen, which helps in the complete combustion of fuel and it emits a low amount of CO_2. In this way, it will decrease unhealthy tailpipe emissions (Keshavarzi *et al*., 2013). Therefore, its demand and production are increasing rapidly. Now, ethanol is widely used as a liquid biofuel for motor vehicles (Lakra and Bano, 2023).

Arguably, lignocellulosic biomass is present most abundantly and is renewable for energy production. Energy production is in the form of bioethanol; liquid carriers of energy also act as biofuel. Plant biomass consists of polysaccharides in high amount. These polysaccharides mainly include hemicellulose and cellulose with some lignin concentration that forms a matrix where polymer fibers of sugar are embedded (Ben *et al*., 2023). The process of bioethanol production from plant mass requires carbohydrate degradation by enzymes for the release of fermentable sugars. This process is known as saccharification, which is predominantly accomplished by enzymatic or biochemical pathways. It does not produce any byproducts that are inhibitory and thus known as environmentally benign (Gnanasekaran *et al*., 2023). Lignocellulose is the heterogeneous complex of hemicellulose, lignin and cellulose and is a forming factor of plant cell wall (Dang *et al*., 2023). Cellulose is a tough fibrous polysaccharide which is made up of repeating D-glucose having β1, 4-glycosidic linkages (Yao *et al*., 2023). Cellulose is considered to be one of the major organic compounds on earth which can be renewed (Tai *et al*., 2023).

Numerous microorganisms can grow themselves on carbohydrate-related polymers like cellulose. But many of them can also grow on substrates different from cellulose. These microorganisms include *Xanthomonas, Pseudomonas, Bacillus, Cytophaga, Vibrio,* and *Cellulomonas* genera of bacteria*, Verticillium, Trichoderma, Pestalotiopsis, Alternaria, Cirrenalia, Aspergillus, Neurospora, Fusarium, Stachybotrys* and *Calathella* from fungi. Whereas anaerobic bacteria like *Ruminococcus* sp., *Clostridium* and *Fibrobacter* are mainly restricted to only cellulosic substrates and oligomers that are derived from cellulose (Behera *et al*., 2017). Nearly 80% of the current cellulosic ethanol capacity installed in industries is enzymatically produced from *Trichoderma reesei* (Bischof *et al*., 2016). Some other microbes are also employed to generate ethanol from cellulose, and they are also from different genera of fungi, like *Aspergillus* and *Penicillium*. Some rare exceptions are there (Payne *et al*., 2015).

One of the suitable sources of sustainable energy is second-generation ethanol that might be formed from various renewable products. Newspaper, a feedstock containing cellulose, is an emerging option for bioethanol production. This is due to its higher potential to displace fossil fuels, low cost of feedstock, reduced GHG (greenhouse gas) emissions (Byadgi and Kalburgi, 2016). Now the challenge is to produce maximum ethanol by degenerating biomass. It can be done by the synergism of enzymes. These enzymes include cellobiose dehydrogenases, beta-glucosidases, monooxygenases, and endoglucanases. Furthermore, detailed knowledge about the interaction of lignocellulosic biomass and enzymes, enzyme bioengineering and enzyme mixture optimization is needed to advance second-generation bioethanol (Barbosa *et al.*, 2020). Due to the ability of cellulases to hydrolyze biomass containing cellulose and its transformation into bioethanol of high demand, they are the center of attraction all over the world. Though cellulases have been used for decades at an industrial level, there is constant hard work to obtain higher yield of ethanol. Knowing that microbes are the original sources of these enzymes, isolation of new microbes as potential sources of enzymes is important (Illić *et al*., 2023). Rotten fruits might be a source of cellulolytic bacteria. This study aims to evaluate enzymatic activity as well as the potential to produce ethanol from bacteria isolated from rotten fruits.

Materials and Methods

Bacterial isolation

Rotten fruit juice was used to isolate bacteria. These fruits include *Malus domestica* (apple), *Musa balbisiana* (banana), *Psidium guajava* (guava) and *Vitis vinifera* (grapes) that were purchased from Township fruit Market, Lahore.

Serial dilutions of fruit juice were made. Bacteria were isolated by spreading these dilutions on cellulose containing medium (cellulose 20 g, peptone 1.5 g, NH_4SO_4 0.1 g, KH_2PO_4 0.1 g, $MgSO_4$ 0.05 g, yeast extract 1 g, agar 2 g, $CaCl_2$ 0.1 g by dissolving in 1000 mL of distilled water) following the protocol of Zhang *et al*., (2011).

Selection of these colonies was made by the difference in their morphology, colony size and color. After selection, these strains were streaked on agar plates and assigned some code like CA2, CA3, CA4, CG1, CG2, CG3, CB4, CGr2 and CGr4.

Isolation of efficient cellulose degrading bacteria

Degradation of cellulose in Durham tubes

The liquid medium was prepared by mixing (%) 1.5 peptone, 1 yeast extract, 0.1 NH_4SO_4, 0.1 KH_2PO_4, 0.05 $MgSO_4$ and 2 cellulose. Durham tubes containing liquid media were inverted into the test tubes having 10 mL fermenting media. They were then incubated at 37°C over the period of 15 days (Meyer and Yarrow, 1998). Carbon dioxide gas production was recorded as a positive response.

Evaluation of cellulose degradation with Congo red staining

Bacterial ability to catalyze cellulose like polymers of carbohydrates into simpler sugars by Congo red staining followed by the process guided by Zhang *et al.*, 2006. The medium containing cellulose 20 g, peptone 1.5 g, NH_4SO_4 0.1 g, KH_2PO_4 0.1 g, $MgSO_4$ 0.05 g, yeast extract 1 g, Agar 2 g, $CaCl_2$ 0.1 g was dissolved in 100 mL of distilled water (Zhang *et al.*, 2011). Petri plates with sterilized cellulose-supplemented medium were spread with bacteria and incubated for 16 hours at 37°C. Solution of Congo red dye (1% in distilled water) was prepared by stirring continuously for fifteen minutes. It is then poured into the petri dishes containing bacterial colonies and kept at 37°C for 30 min. Post 30 minute's incubation, extra stain was drained out and NaCl (1%) solution was used to de-stain it. For de-staining, petri plates with NaCl solution were incubated for 30 min at 37°C. The same procedure was repeated for three to four times to get clear hallows. Cellulose degradation was depicted by clear zones surrounding bacterial isolates.

Analysis of sugar degradation using TTC (triphenyl tetrazolium salt)

The media composition (%) involved cellulose 2, peptone 1.5, NH_4SO_4 0.1, KH_2PO_4 0.1, $MgSO_4$ 0.05, yeast extract 1, agar 2, and $CaCl_2$ 0.1. The medium was sterilized at standard conditions (121°C, 15 psi, 15 minutes). TTC solution was prepared by adding 0.05 g TTC in 10 mL autoclaved distilled water. TTC solution and medium was mixed when the temperature of medium is about 50°C and were dispensed into petri dishes. Each isolated bacteria was streaked on these petri dishes and incubated at 37°C for 1–3 days. The appearance of colony color from pink to maroon indicated sugar degradation.

Morphological characterization

To evaluate colonial as well as cellular characteristics of the bacteria, 2% cellulose supplemented medium was used. Colonial size, margin, color, consistency, elevation, optical density and texture were studied (Bergey, 1994). Colonies were Gram stained for the examination of microscopic characteristics. For the measurement of colonial size, an ocular meter was used.

Study of fermentation kinetics

Preparation of cellulose-supplemented synthetic culture media

The synthetic culture media was made by adding 20 g of cellulose, 6.5 g of yeast extract, 2.6 g of $(NH_4)_2SO_4$, 2.72 g of KH_2PO_4, 0.8 g of $MgSO_4$ $7H_2O$, 0.3 g of $CaCl_2$, 0.00042 g of $ZnCl_2$, 1.5 g of citric acid and 6 g of sodium citrate in 1 liter of distilled water. This mixture was then autoclaved.

Preparation of inoculum

To initiate bacterial growth, inoculum was made. It included 20 g cellulose, 0.7 g peptone, 0.05 g ammonium sulphate, 0.05 g potassium dihydrogen phosphate, 0.025 g magnesium sulphate, 0.05 g calcium chloride, and 0.5 g yeast extract in 50 mL of distilled water. Selected strains of bacteria were inoculated into this sterilized medium and incubated at 37°C overnight.

Fermentation experiment

Bacterial culture (1%) was inoculated in cellulose supplemented synthetic medium. It was then placed in an incubator for 10 consecutive days at its standard temperature (37°C). Daily samples were taken to evaluate the contents of reducing sugar and ethanol production at the wavelength of 540 nm and 590 nm, respectively. Conversion factor for cellulose to sugars is 1.11 (Soni *et al.*, 2010). Growth of bacteria in fermentation medium was checked by spectrophotometer at the 600 nm wavelength.

For computation of ethanol yield and fermentation efficiency, the following formulae were used:

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

$$\text{Fermentation efficiency} = \frac{\text{Practical ethanol yield}}{\text{Theoretical ethanol yield}} \times 100$$

Evaluation of enzymatic activity

Processing for crude enzyme extraction

For the production of bacterial cellulase enzymes, basal media of 7.0 pH was made by adding magnesium sulphate

0.01%, Na_2HPO_4 0.7%, $Na_3C_6H_5O_7$ 0.05%, KH_2PO_4 0.2% and CMC (carboxy methyl cellulose) 2% as a carbon source. Test tubes containing basal media were inoculated with 1% culture of cellulolytic bacteria separately and placed in incubator at 37°C and kept on shaking at 200 rpm for about 3 days. For 15 min, this incubated bacterial culture was centrifuged at 1000 rpm. Filtrate was termed as crude extract of enzyme. Its activity was determined by evaluating reducing sugar in substrate buffer. For substrate buffer (2%) preparation, 2 g CMC was added into 0.2 M acetate buffer (100 mL). Acetate buffer (0.2 M) was made by adding $C_2H_3NaO_2$ (54.43 g) and CH_3COOH (12 mL) into distilled water (1988 mL) and mixed until homogenized. NaOH (10 N) was used to adjust pH at 5.

Enzyme assay

DNS protocol was used to assess reducing sugars in substrate buffer (Miller *et al*., 1960). Substrate buffer (1 mL) was added to 0.5 mL of enzyme extract. It was then placed in water bath for 30 minutes at 50°C followed by the addition of DNS (dinitro salicylic acid) 3 mL and five minutes boiling. When cooled, absorbance of the mixture was determined at the wavelength of 640 nm via spectrophotometer.

Enzyme activity (µmol/min/mL) was determined by the expression given below:

$$\text{Enzyme activity} = \frac{\text{O.D of Sample} \times \text{S.F.} \times 1000 \times \text{R.V.}(\text{mL})}{\text{M.W.}(\text{CMC}) \times \text{I.T}(\text{min}) \times \text{T.C.E}(\text{mL})}$$

Where
Standard factor (S.F) = 10.69 µmol/min/mL
Reaction volume (RV) = 1.5 mL [enzyme extract (0.5 mL); CMC buffer (1 mL)]
Molecular weight (M.W) of CMC = 162.1406 gram/mol
Incubation time (I.T) = 30 min
Total crude enzyme (T.C.E) = 0.5 mL

Molecular characterization of efficient cellulolytic isolates

Extraction of genomic DNA of bacterial isolates CG2 and CA2 was done. Samples were sent to Genomed Laboratories, Pakistan for bacterial gene sequencing. The similarity of their sequences was reviewed using BLAST (http://www.ncbi.nlm.nih.gov/blast) and their accession numbers were obtained. Phylogenetic trees were constructed using software MEGA X version 10 based on the phylogenetic relationships of nucleotide sequences.

Statistical analyzing tests

Presented values for all tests were taken in triplicates and analyzed on Minitab (ver 16.1.1.). Duncan test was performed followed by one way analysis of variance.

Results

Bacterial isolation

Fruit waste (guava, grapes, banana, and apple) was collected from the fruit market Township, Lahore. Table 1 showed the location, date of sample collected and number of bacterial strains isolated on cellulose supplemented medium. Out of 16 bacteria, seven were screened on the basis of their cellulolytic ability.

Morphological characteristics

Solid cellulose-containing medium (2%) was used to grow bacteria. Characteristics of bacterial colonies like color, size, texture, consistency, elevation and margin were analyzed (Table 2).

A total of 7 bacterial isolates were selected on cellulose-supplemented medium. The surface texture of all bacterial colonies was smooth. Margins of bacterial isolates were circular except for CGr1 and CA2 which were undulate. The color of all colonies was pale yellow except CG2 that was creamy white while CG1 was light green. All colonies were convex except for CGr1 and CA2 which were flat and CGr2 which was raised. Size of CG1, CG2 and CA4 was 2 mm, CGr1 and CA2 were 3 mm, CGr2 was 1.5 mm and CB1 was pinpoint. All colonies were opaque except CGr1 and CA2 as these were translucent. Consistency of all the colonies was butyreous except CG1

Table 1. Isolation of different cellulose degrading bacteria from fruit waste on selected medium.

Serial No.	Rotten Fruits	Location of Sampling	Date of Sampling	Isolated Bacteria
1	Guava	Township, LHR	6th Nov., 2017	CG1, CG2, CG3, CG4
2	Grapes	Township, LHR	6th Nov., 2017	CGr1, CGr2, CGr3, CGr4
3	Banana	Township, LHR	6th Nov., 2017	CB1, CB2, CB3, CB4
4	Apple	Township, LHR	6th Nov., 2017	CA1, CA2, CA3, CA4

Table 2. Colonial characteristics of bacterial isolates after 24 hours incubation at 37°C following streaking on cellulose containing medium

Serial No	Isolated Bacteria	Size	Color of Colony	Margin/ Edges	Surface Texture	Elevation	Optical Features	Consistency	Pigmentation
1	CA2	3 mm	Pale yellow	Undulate	Smooth	flat	Translucent	Butyreous	No pigment
2	CA4	2 mm	Pale yellow	Circular	Smooth	Convex	Opaque	Viscous	No pigment
3	CB1	Pin-point	Pale yellow	Circular	Smooth	Convex	Opaque	Butyreous	No pigment
4	CG1	2 mm	Light Green	Circular	Smooth	Convex	Opaque	Viscous	Light Green
5	CG2	2 mm	Creamy white	Circular	Smooth	Convex	Opaque	Butyreous	No pigment
6	CGr1	3 mm	Pale yellow	Undulate	Smooth	Flat	Translucent	Butyreous	No pigment
7	CGr2	1.5 mm	Pale yellow	Circular	Smooth	Raised	Opaque	Butyreous	No pigment

Table 3. Microscopic characteristics of bacterial isolates after 24 hours incubation at 37°C following streaking on cellulose containing medium (Bergey, 1994).

Serial No.	Isolated Bacteria	Gram Staining	Size		Shape
			Length (µm)	Diameter (µm)	
1	CA2	G+ve	4	2	Rods
2	CA4	G+ve	2	1	Diplococci (attach with each other)
3	CB1	G+ve	2	1	Diplococci (attach with each other)
4	CG1	G+ve	2	1	Diplococci (attach with each other)
5	CG2	G+ve	2	1	Diplococci (attach with each other)
6	CGr1	G+ve	5	2	Rods
7	CGr2	G+ve	4	1	Rods

and CA4 that were viscous. All the colonies produced no pigment except for CG1 which produced light green color in colony as well as on medium.

Microscopic characteristics of bacterial isolates

Bacterial microscopic characteristics were observed that included cell size, type and shape (Table 3**)**. All seven bacteria appeared to be diplococci except CGr1, CGr2 and CA2 that were rod shaped. All bacterial strains were Gram +ve bacteria. These bacteria ranged in size from 2 µm × 1 µm – 5 µm × 2 µm. The maximum size *i.e.,* 5 µm × 2 µm was observed in rod-shaped bacterium (CGr1) and the minimum size *i.e.,* 2 µm × 1 µm was observed in diplococci bacterium (CG1, CG2, CB1 and CA4) as shown in Figure 1.

Potential of bacterial isolates to ferment cellulose by different biochemical tests

The ability of bacteria to utilize cellulose was assessed by staining by Congo red, development of color on TTC and the production of gas inside Durham tubes (Table 4). All strains produced bubbles in Durham tubes at the 2nd, 3rd and 7th fermentation days (Figure 2). Clear zones were produced by all the bacteria by staining through Congo red. Variation in zone size was seen from 1.4 cm to 2.9 cm (CA4). On TTC, most bacterial strains depicted the highest cellulose degradation by showing the maroon color whereas light pink color was shown by CA2 and CA4 and CB1 did not show any color (Figure 3).

Evaluation of fermentation kinetics

Reducing sugar consumption

A supplementary amount of cellulose (2%) was added into the fermentation medium. Besides this, the trivial quantity of yeast extract was also added. All cellulolytic bacteria consumed cellulose as alone source of carbon. On the first day of fermentation, the reducing sugar estimated differed from 1.729±0.004 gram per liter in CB1 to 6.971±0.089 gram per liter in CA2. On tenth day of fermentation, the least reducing sugar was seen in CGr2 as 2.18±0.043. As the days of incubation passed, a decreasing trend in remaining reducing sugars was seen as recorded in Figure 5. The bacteria used reducing sugars to form bioethanol as well as the bacterial biomass.

Ethanol production

Seven cellulolytic bacteria were allowed to ferment cellulose for about 10 days. Ethanol produced during these days is shown in Figure 4. Ethanol titer showed

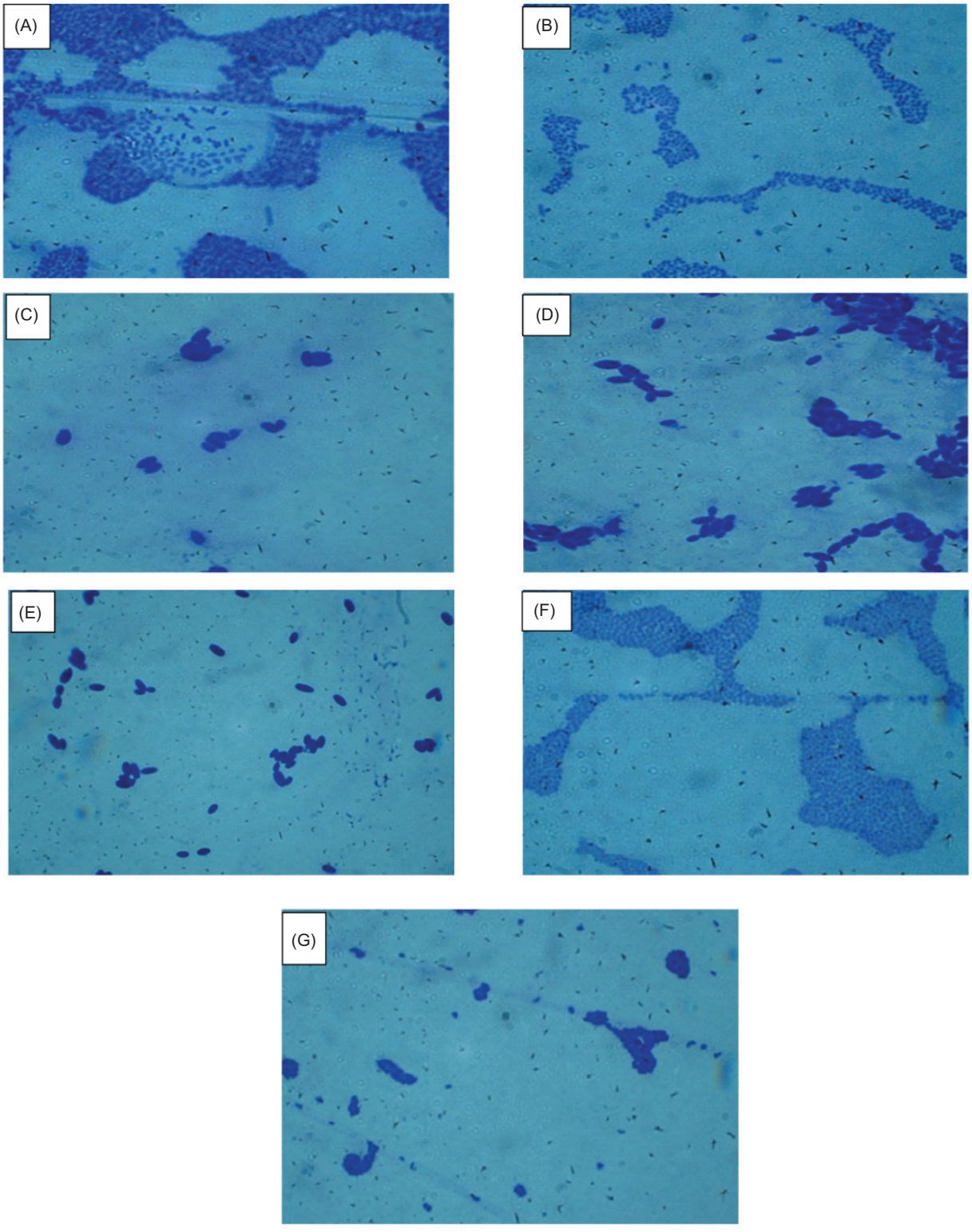

Figure 1. Cellular characteristics showing gram-positive reaction (A–G) and shapes as Diplococci CG1, CG2, CA4, CB1 (A, B, F, G), rods CGr1, CGr2, CA2 (C–F).

Table 4. Gas production and cellulose degradation by fermentation using different bacterial isolates.

Serial No.	Bacteria Isolated	Durham Tubes (Gas production)	Tetrazolium Salt (Color production)	Congo Red (Clear zones mm)	Bacterial Colony Diameter (mm)	Cellullolytic Index
1	CA2	+ Gas	+ Light Pink	52	3	16.3
2	CA4	++ Gas	– No color	29	2	13.5
3	CB1	++ Gas	– No color	4	Pin-point (0.5)	7.0
4	CG1	++ Gas	++++Maroon	23	2	10.5
5	CG2	++ Gas	+++ Maroon	40	2	19.0
6	CGr1	++ Gas	++ Maroon	30	3	9.0
7	CGr2	++ Gas	+ Maroon	20	1.5	12.4

Cellulolytic index = (clear zone diameter−bacterial colonies diameters)/bacterial colonies diameter
+++ Positive, strong response
++ Positive, intermediate response
+ Positive, Weak response
– Negative response

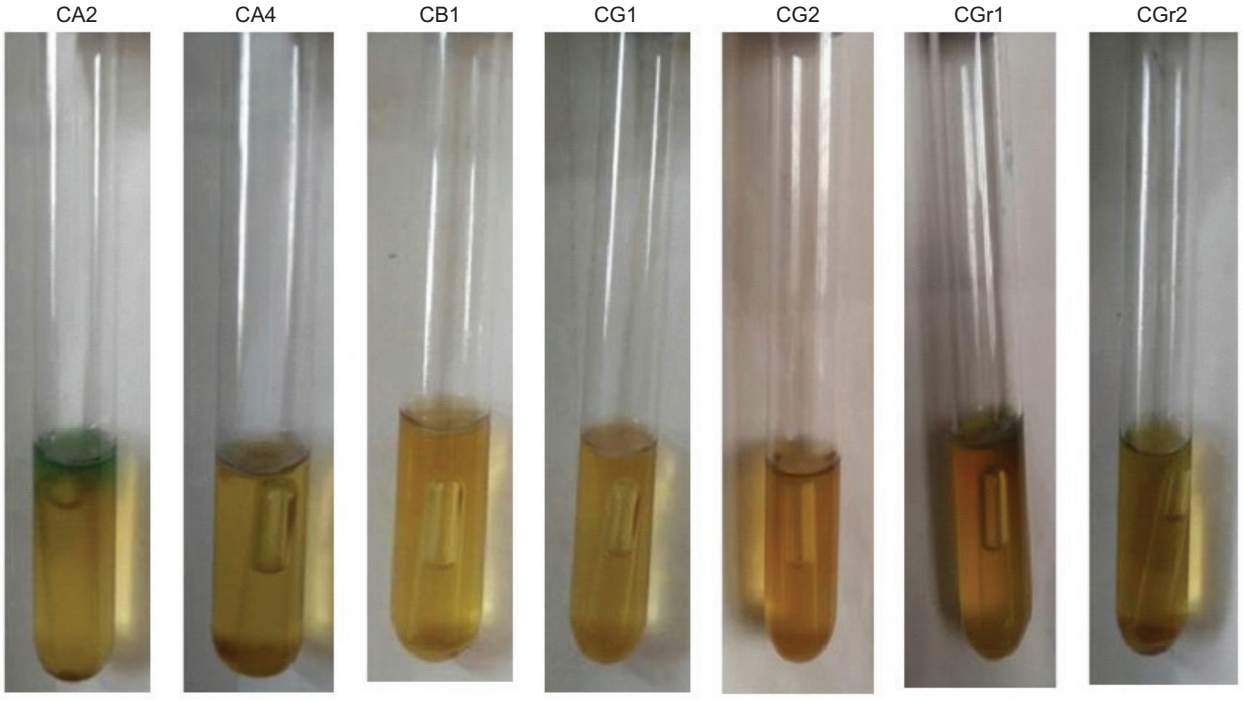

Figure 2. Gas production observed in Durham tubes by CA2, CA4, CB1, CG1, CG2, CGr1 and CGr2.

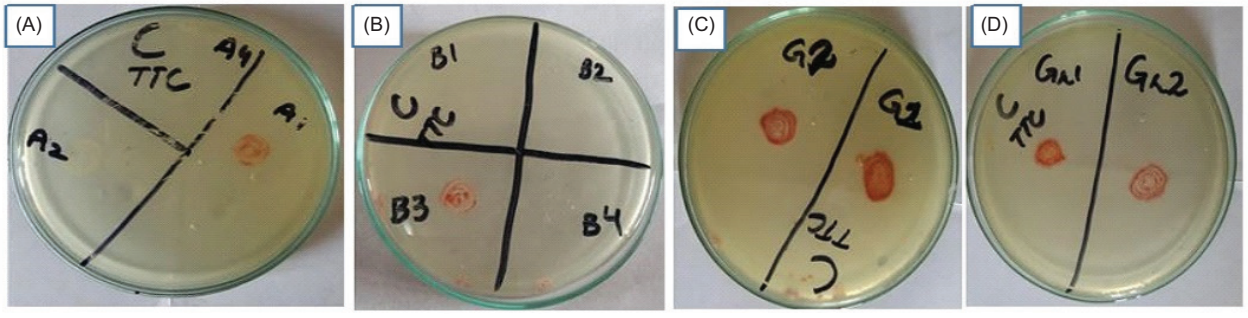

Figure 3. Cellulose degradation evaluated by development of maroon color on TTC by CA2 (A), CB1 (B), CG1, CG2 (C) and CGr1, CGr2 (D).

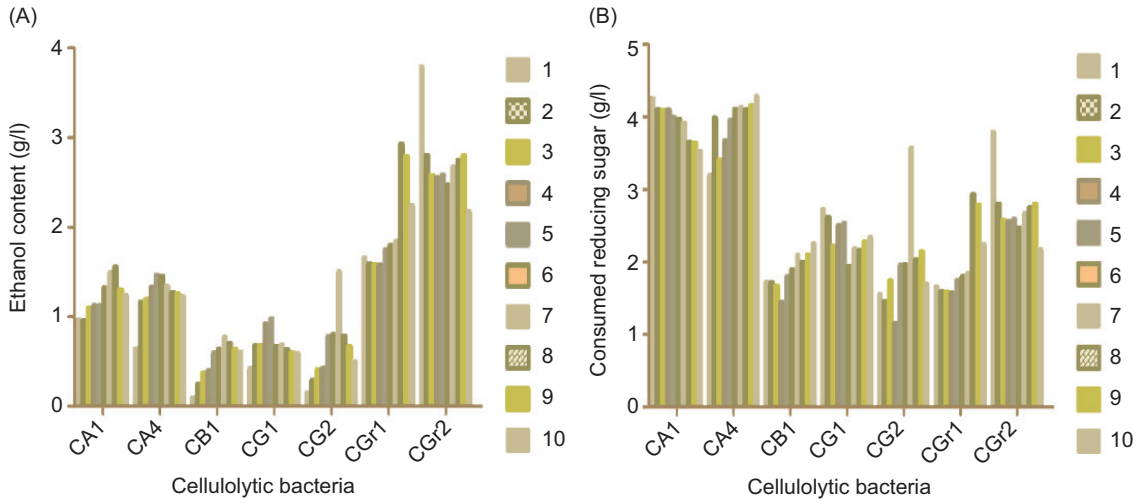

Figure 4. Ethanol (A) and consumed reducing sugar (B) contents in fermentation medium by cellulolytic bacteria within 10 days.

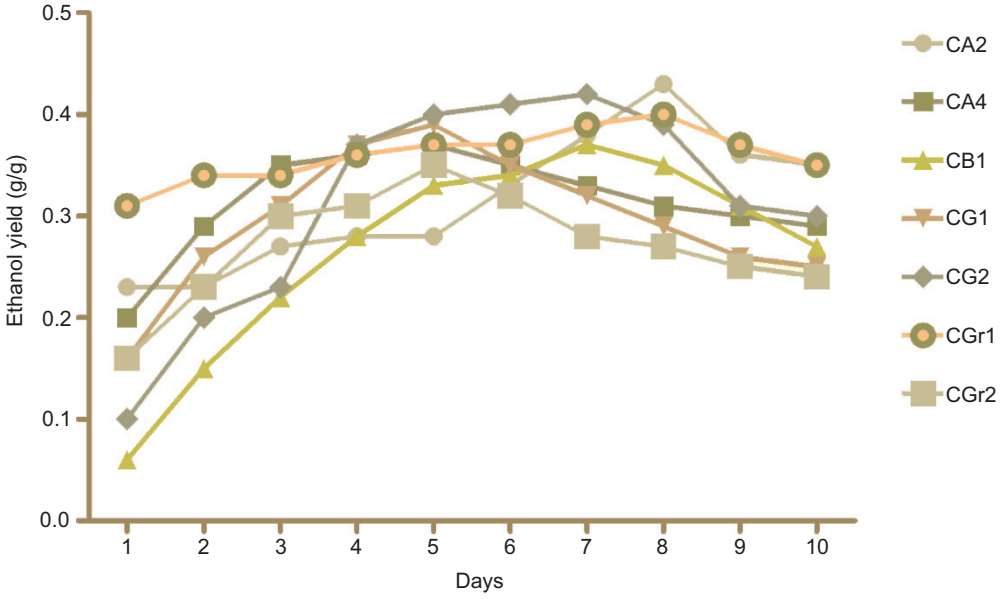

Figure 5. Ethanol yield (g of ethanol/ g of reducing sugar consumed) of cellulolytic bacteria.

the maximum production in different days by different isolates. CG2 and CA2 produced maximum ethanol; 2.2±0.138 and 2.12±0.142 g/L on days 7 and 8, respectively. Maximum ethanol in all other strains was seen between day 5 and 8 except CGr1. The highest ethanol on days 7 and 8 by CG2 and CA2, respectively, demonstrated the ethanol tolerance of these strains. So, CA2 and CG2 were termed as potential ethanol producing and tolerating strains.

The ethanol yield of selected bacterial isolates ranged from 0.35±0.013 to 0.43±0.011 g of ethanol per g of reducing sugar consumed. The highest ethanol yield was seen in CA2 at day 7 as 0.43±0.005 g of ethanol per gram of reducing sugars. Similarly, ethanol yield in CG2 was recorded on day 8 as 0.42±0.011 g/g. Values of ethanol yield are presented in Figure 5.

Overall percent fermentation efficiency was noted from 54.45±0.01 to 79.71±0.05. All isolates showed fermentation efficiency to some extent in 10 day experiment. The highest fermentation efficiency was recorded in CG2 at day 7 of fermentation as 79.71±0.059%. The second best fermentation efficiency was observed in CA2 as 75.58±0.011% at day 8. Detailed values of each isolate are given in Table 5.

Table 5. Fermentation efficiency (%) of all seven cellulolytic bacteria.

Days	CA2	CA4	CB1	CG1	CG2	CGr1	CGr2
1	27.34±0.009	39.83±0.004	11.68±0.034	22.63±0.013	31.07±0.019	38.42±0.009	30.82±0.002
2	31.17±0.002	57.33±0.008	28.47±0.005	36.9±0.005	38.3±0.006	40.38±0.007	44.91±0.008
3	37.74±0.006	68.71±0.005	43.77±0.005	41.46±0.004	55.68±0.006	40.87±0.008	59.53±0.017
4	45.53±0.07	71.21±0.009	54.36±0.007	51.73±0.008	67.36±0.005	43.23±0.012	60.56±0.013
5	50.38±0.004	72.77±0.017	65.25±0.003	54.45±0.016	74.68±0.004	45.7±0.015	69.09±0.013
6	61.94±0.042	69.41±0.006	66.36±0.034	44.75±0.016	77.47±0.068	46.99±0.007	62.87±0.013
7	70.05±0.014	63.84±0.005	72.83±0.007	39.78±0.093	79.71±0.059	54.33±0.002	55.46±0.006
8	75.58±0.011	60.78±0.002	68.82±0.002	33.94±0.029	70.86±0.089	58.18±0.001	53.26±0.002
9	62.06±0.028	59.37±0.017	60.13±0.004	33.66±0.026	60.63±0.09	52.77±0.003	48.18±0.006
10	53.8±0.017	56.22±0.009	53.62±0.011	20.86±0.028	56.9±0.073	47.63±0.006	47.4±0.009

Growth tendencies of bacterial isolates

Growth tendencies of all bacteria were evaluated daily at 600 nm wavelength by spectrophotometer (Figure 6). CA2 showed lag phase (day 1–4), log phase (day 5–8) and decline phase (day 9 and 10). CB1, CG1 and CGr2 exhibited lag phase (day 1–3), exponential phase (4–6) and decline phase (7–10).

CA4 and CG2 exhibited a lag phase (day 1 and 2), exponential phase (day 3–7) and decline phase (day 8–10). CGr1 underwent lag phase (day 1–2) and exponential phase (day 3–10). There was no decline phase in this.

From the measurement of growth, it was clearly seen that all bacteria produced maximum ethanol in their exponential phase except CGr1. Bacterial cells were actively dividing and producing ethanol by consuming cellulose as substrate.

Evaluation of cellulolytic activity of selected bacterial isolates

Besides the production of ethanol, bacterial isolates were also subjected to the evaluation of their cellulase production for the conversion of cellulose into simpler sugar glucose. Enzyme activity was determined by the DNS method.

Figure 7 and Table 6 show that all cellulolytic bacteria released enzymes that would be responsible for the

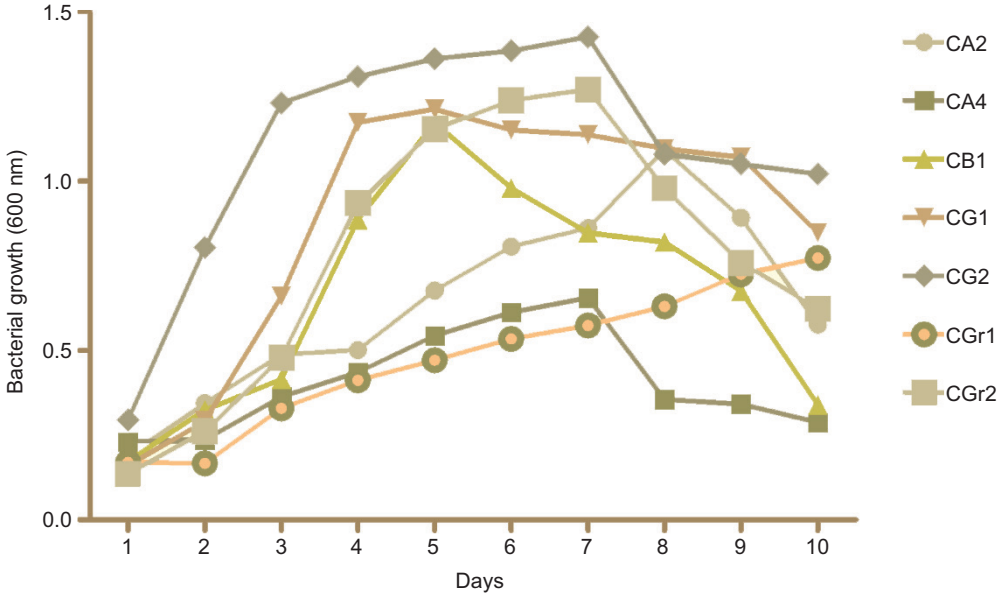

Figure 6. Bacterial growth of CA2, CA4, CB1, CG1, CGr1 and CGr2 in fermentation medium supplemented with cellulose measured at 600 nm on spectrophotometer.

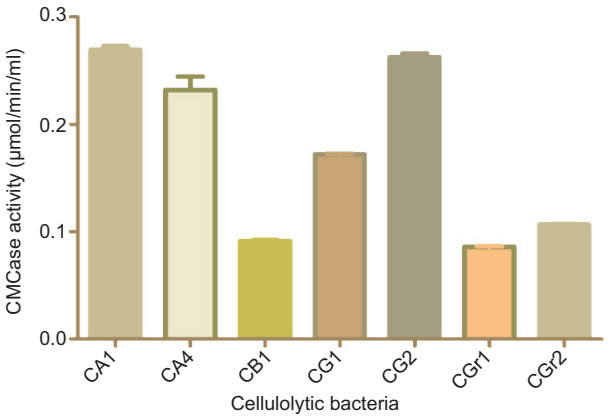

Figure 7. Enzyme activity (umol/min/mL) of CA2, CA4, CG1, CG2, CGr1 and CGr2 to hydrolyze cellulose into glucose.

Table 6. Estimation of enzyme activity (umol/min/mL) released from different bacterial isolates to hydrolyze cellulose

Bacteria	CM Case Activity (µmol/min/mL)
CA2	0.26±0.05
CA4	0.23±0.05
CB1	0.09±0.001
CG1	0.17±0.024
CG2	0.27±0.011
CGr1	0.09±0.011
CGr2	0.11±0.002

degradation of cellulose. CB1 and CGr1 showed enzyme activity but not up to the mark. CG1, CGr1 and CGr2 showed good enzyme activity. CA2 and CG2 showed maximum enzyme activity, i.e., 0.265±0.05, 0.27±0.011 (umol/min/mL).

Molecular characterization of selected bacterial isolates

The accession numbers ON324120 and OM974175 were assigned to the isolates *Bacillus manliponensis* (CA2) and *Bacillus* sp. (CG2) by database of GenBank (http://www.ncbi.nlm.nih.gov/blast) with 98% similarity. The phylogenetic tree was made using the software MEGA X version 10 based on the phylogenetic relationship of nucleotide sequences (Figures 8–9).

Discussion

Recently, rapid growth has been seen in the human population. With increasing human population, the demand of luxuries and consumption of energy is becoming proportional to it. The major sources of energy, fossil fuels, are diminishing at the same speed (Ullah *et al.*, 2023; Aziz *et al.,* 2023). Furthermore, fossil fuel usage fuels up the GHG emission in the environment. With this ambiguous situation, clearer strategies should be adopted that would yield both energy as well as a clean environment and cheap price. There are routes to produce bioenergy by consuming atmospheric carbon in a system that is carefully designed for sustainability. The most excessive biomass present on earth is cellulose which is available to the microbes to be fermented into simpler sugar substances and hence yielding bioethanol or we can term it biofuel. It can also be renewed. Now, we need to move from non-renewable, non-environment friendly and costly fuel sources to renewable, environment-friendly, and cheaper ones (Khan *et al.*, 2023). Biomass as a biofuel source is important not only for bioethanol production but also for mitigating the negative changes in the environment. Different microorganisms reside in waste like bacteria, protozoa and fungi. Those microbes that degrade cellulose are known as cellulolytic bacteria present in different products and waste (Narjis *et al.*, 2023; Li *et al.*, 2023; Arooj *et al.*, 2023; Shah *et al.*, 2023; Najeeb *et al.*, 2022).

To evaluate carboxy methyl cellulose's fermentative and cellulolytic potential, various biochemical tests were carried out. According to Hidayat (2021), cellulose microfibril is broken down or digested into oligosaccharides and monosaccharides by a process called cellulolysis. According to Peristiwati and Herlini (2018), bacterial cellulases facilitated the breakdown of complicated substrates into monomers through cellulolysis. The amount of carbon dioxide produced during the fermentation of monomeric sugars in Durham tubes was measured as bubbles. Durham tube testing is not widely used since it is ineffective in detecting slow-fermenting bacteria (Scheffers, 1987). Tetrazolium chloride (TTC) and Congo red stain are two other indicators that were used to identify the hydrolysis of sugar.

Congo red staining is a quick and sensitive method for identifying bacteria that break down cellulose (Lu *et al.*, 2004). Every bacterial isolate created a different clearance zone surrounding its colony. The bacterial cell's lysis or age caused the enzymes to migrate into the medium and interact with the dye, reducing the dye's hue (Jalandoni-Buan *et al.*, 2010). Sulfonated azo dye, or Congo red stain, is unable to pass through bacterial plasma membranes. High zones of clearance and low dye retention were the outcomes of the high cellulolytic activity (Stolz, 2001; Fujimoto *et al.*, 2011; Florencio *et al.*, 2012, Suharti *et al.*, 2023). When evaluating the cellulolytic potential and, thus, the cellulolytic index, the hallow development surrounding the bacterial colonies by Congo red is useful.

With TTC, all isolates exhibited favorable color development. TTC, which manifested as brightly colored

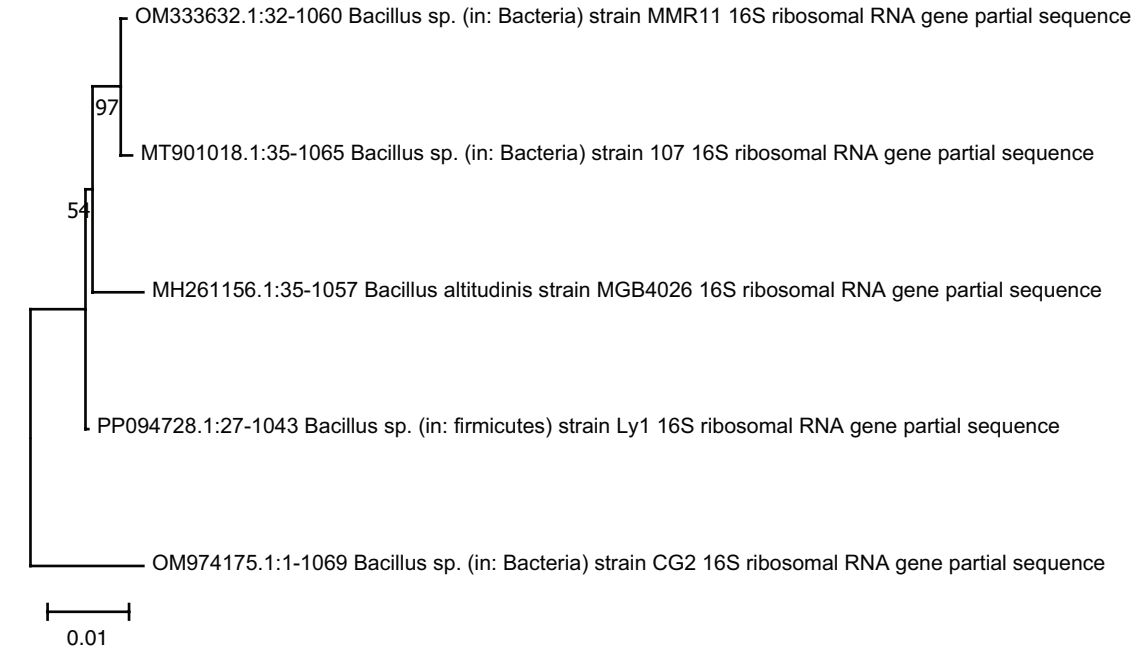

Figure 8. Phylogenetic tree representing CG2 (*Bacillus* sp. OM974175) cellulolytic isolate showing their evolutionary relationship with closely related species.

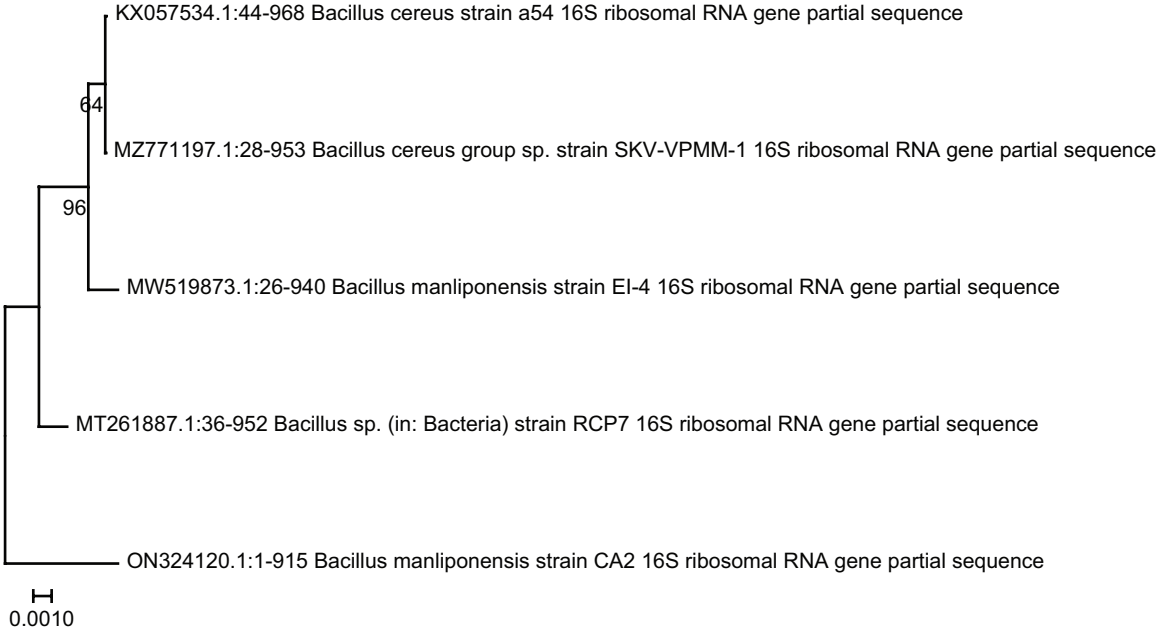

Figure 9. Phylogenetic tree representing CA2 (*Bacillus manliponensis* ON324120) cellulolytic isolate showing their evolutionary relationship with closely related species.

(dark maroon to light pink) product formazones, can be used to identify the hydrolysis of sugar by bacteria (Thom *et al.*, 1993; Caviedes *et al.*, 2002; Abate *et al.*, 2004). Because TTC is an excellent electron acceptor, it is changed from colorless to colored triphenyl formazon in living cells (Mshana *et al.*, 1998; Lee *et al.*, 2006). TTC is used extensively to detect biological reducing systems (Wardani *et al.*, 2023; Luo *et al.*, 2023; Riskawati *et al.*, 2023). TTC is a good indicator of sugar hydrolysis and antimicrobial activity. TTC is not a single compound but an enormous set of organic compounds primarily made in 1894 (Wu *et al.*, 2023; Chaudhary *et al.*, 2023;

Malik *et al*., 2023). The tetrazolium salts are hydrophilic, stable available in white color to various other shades (Lee *et al*., 2006).

The goal of the current investigation was to screen bacterial strains that produce ethanol on a medium supplemented with 2% CMC. The ethanol titer demonstrated the highest amount of ethanol produced by several isolates on various days. On days 7 and 8, respectively, CG2 and CA2 produced the most ethanol, 2.2±0.138 and 2.12±0.142 g/L. On days 8 and 7, the ethanol yield of CA2 and CG2 was determined to be 0.43±0.005 and 0.42±0.011 g of ethanol per g of reducing sugar used, respectively. Between 0.35±0.013 and 0.43±0.011 g of ethanol were produced overall for every gram of reducing sugar that was consumed. It was seen that all bacteria were producing maximum ethanol content in their exponential growth phase. Actively growing bacteria produced ethanol as well as survived in it. From *S. cerevisiae* cultivated on sugarcane, Rudolf *et al*., (2005) reported an ethanol titer and yield of 26.7 g/L, 0.30 g/g. Abedinifar *et al*., (2009) described the ethanol production of rice straw that had been enzymatically processed, utilizing *S. cerevisiae* to produce 0.37–0.45 g/g and *Mucor indicus* to produce 0.36–0.43 g/g. These results were similar to the ethanol titer performed by bacterial isolates of the present study. Using *B. subtilis* in potato wastes, the results of the ethanol concentrations were confirmed to be 8.3 g/L (Ali *et al*., 2016). The ethanol assay was reported by Chandel *et al*., (2010) using enzymes produced from *P. stipitis*, *A. oryzae* MTCC 1846, and *S. cerevisiae* VS3, yielding corresponding results of 15.73 ± 0.44 g/L, 14.22 ± 0.15 g/L, and 17.73 ± 0.25 g/L. These findings varied from our ethanol titer values.

The isolated bacterial strains used in the study are capable to convert cellulose into glucose and other reducing sugars by releasing the enzyme cellulases. *Bacillus manliponensis* ON324120 (CA2) and *Bacillus* sp. OM974175 (CG2) showed efficient hydrolyzing potential as 0.26±0.05, 0.27±0.011 (µmol/min/mL) respectively. These results were in accordance with the findings of Juturu and Wu (2014), Sillu and Agnihotri, (2019), Rajnish *et al.,* (2021). These enzymes help produce ethanol from biomass that can be used in industries (Yamakawa *et al*., 2023). High reducing sugar content may hinder the enzymatic action and ethanol production. The reducing sugar contents in the fermentation medium were seen in decreasing order from the beginning till the end of the fermentation experiment because of its consumption by cellulolytic bacteria for bioethanol production. Gupta *et al*., (2012) stated that significant cellulose degradation can be obtained in mixed cellulolytic bacterial culture and non-cellulolytic yeast in which yeast uses reducing sugar derived from cellulose degradation and converts it into ethanol. Our findings were in agreement with the results described by Darwesh *et al*., (2020), and Ma *et al*., (2020).

Conclusion

The present study revealed the competency of two bacterial strains *Bacillus* sp. CG2 (OM974175) and *Bacillus manliponensis* CA2 (ON324120) screened from fruit waste with cellulolytic index 19, 16.3 and cellulase activity 0.27±0.011, 0.26±0.05 µmol/min/L respectively. Maximum ethanol yield of 0.42±0.005, 0.43±0.011 (g of ethanol/ g of reducing sugar consumed) as well as percent fermentation efficiency 79.71±0.059, 75.58±0.011 of CG2 and CA2 were evaluated. Hence, these bacterial isolates will be proven as promising candidates to convert cellulosic biomass into bioethanol at the commercial level.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors. Therefore, as an observational study, it doesn't require any ethical approval.

Competing interests

The authors declare no conflict of interest.

Availability of data and materials

All the data has been included in the manuscript.

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