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Screening of Cellulolytic Bacteria from Rotten Fruits and Optimization of Cellulase Production by *Paenibacillus dendritiformis* PQ845045 using the Response Surface Methodology

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Abstract

Bacterial cellulase production remains limited by suboptimal culture conditions, resulting in high production costs and restricting its industrial applications. This research aimed to determine γ -haemolytic degrading bacteria cellulose and to optimize cellulase production. Of 42 strains isolated on a reconstituted medium supplemented with carboxymethylcellulose used as the exclusive carbon source, only 19 showed significant cellulose hydrolysis capacity. Following morphological and metabolic characteristics analyses, together with haemolysis tests, 16S rRNA gene sequencing identified four promising strains belonging to three bacterial species: *Paenibacillus dendritiformis* (PQ845045) and *Bacillus altitudinis* (PQ845085 and PQ845089), all γ -haemolytic, as well as *Mammaliococcus lentus* (PQ845126), a β -haemolytic strain. Cellulase production by *Paenibacillus dendritiformis* was optimized using a single-factor approach and a central composite experimental design. The results showed optimal activity at pH 7, with this activity increasing as the concentrations of the various carbon and nitrogen compounds increased. The statistical model, which was generally significant, allowed for a maximum activity of 3.32 IU/mL, representing an approximately 6-fold increase compared to the non-optimized medium. This activity was obtained in a medium containing 25 g/L of carboxymethylcellulose, 3 g/L of yeast extract, and 2.5 g/L of NaCl at 32.5 °C. Response surface analysis identified temperature as the main factor significantly influencing cellulase production. These results highlight the cellulolytic potential of *P. dendritiformis* B17_c and position this strain as a promising candidate for the biotechnological production of cellulase.

Keywords: Cellulolytic Bacteria, Optimization, *Paenibacillus dendritiformis*, 16S rRNA Sequencing, Response Surface Methodology

INTRODUCTION

Cellulose is among the most abundant polysaccharides found in the natural environment, constituting the main structural element of cell walls of plants.¹ It provides cell rigidity, regulates intracellular water exchange, and forms a barrier against pathogens.² It consists of a linear chain of glucose monomers joined through β -(1 $\rightarrow$ 4)-glycosidic linkages. Its water-attracting and highly crystalline structure confers increased resistance and reduces its susceptibility to enzymatic hydrolysis compared to other cell wall polysaccharides.³ This resistance poses specific technological challenges in certain industries, particularly the fruit juice industry. Indeed, its presence in cell walls impedes juice release from cells, thereby affecting production yield, nutrient bioavailability, and the overall nutritional quality of the finished products. It can also cause persistent turbidity and alter the texture of fruit juices and purees.⁴ Other problems, such as the cellulosic nature of the fibers present in juices, can clog the production line and lead to significant industrial losses.⁵ To overcome these constraints, the food industry has traditionally used chemical agents such as benzoic acid, sodium benzoate, sorbic acid,

or sulfur dioxide for clarification and stabilization.⁶ However, prolonged use of these chemicals can lead to undesirable organoleptic changes, altered nutritional quality, and raise health concerns regarding their safety for human consumption.⁷ In contrast, the enzymatic degradation of cellulose by cellulase offers a more respectful and sustainable approach. Cellulases constitute a complex enzymatic system. These enzymes are unique in that they degrade an insoluble polysaccharide. They primarily include endoglucanases, exoglucanases (cellobiohydrolases), and β -glucosidases. Endoglucanases [EC 3.2.1.4] hydrolyze the internal β -(1 $\rightarrow$ 4)-glycosidic bonds of cellulose chains, generating new reducing and non-reducing ends. Exoglucanases act processively from these ends and primarily release cellobiose. They include cellobiohydrolases acting from the non-reducing end [EC 3.2.1.91] and those acting from the reducing end [EC 3.2.1.176]. Finally, β -glucosidases [EC 3.2.1.21] hydrolyze cellobiose and other glucose-soluble β -D-glucosides, thus completing the saccharification process of cellulose.^{8,9} Their use, particularly in food processing industries and more specifically in the fruit and vegetable juice industry, helps to improve extraction yield, clarify juices, stabilize turbidity and texture, decrease

the viscosity of fruit nectars, and influence other sensory characteristics, including aroma and flavor, in the final product.^{4,10-12} They are also used for cereal pretreatment and agricultural silage to enhance the nutritional value of livestock feed. They also find application for the extraction of olive oil and phenolic compounds derived from tea, as well as in other industries such as papermaking, biofuels, and textiles, where they play a fundamental role.^{9,11,13,14} Due to their numerous applications in various industrial fields, cellulases are the subject of intensive research. Although

fungi are extensively studied for their cellulases, their slow growth and high production cost limit their industrial use.¹⁵ Bacteria, on the other hand, are readily cultured and exhibit weak feedback inhibition. They produce enzymes more rapidly and can be genetically modified.¹⁶ Therefore, various cellulose-rich sources, such as soil, plant debris, and ruminant excrement, were explored to identify bacteria with remarkable properties.^{15,17-19} In the present study, four bacteria with good cellulose-hydrolysis capabilities were identified by 16S rRNA gene sequencing as *Paenibacillus*

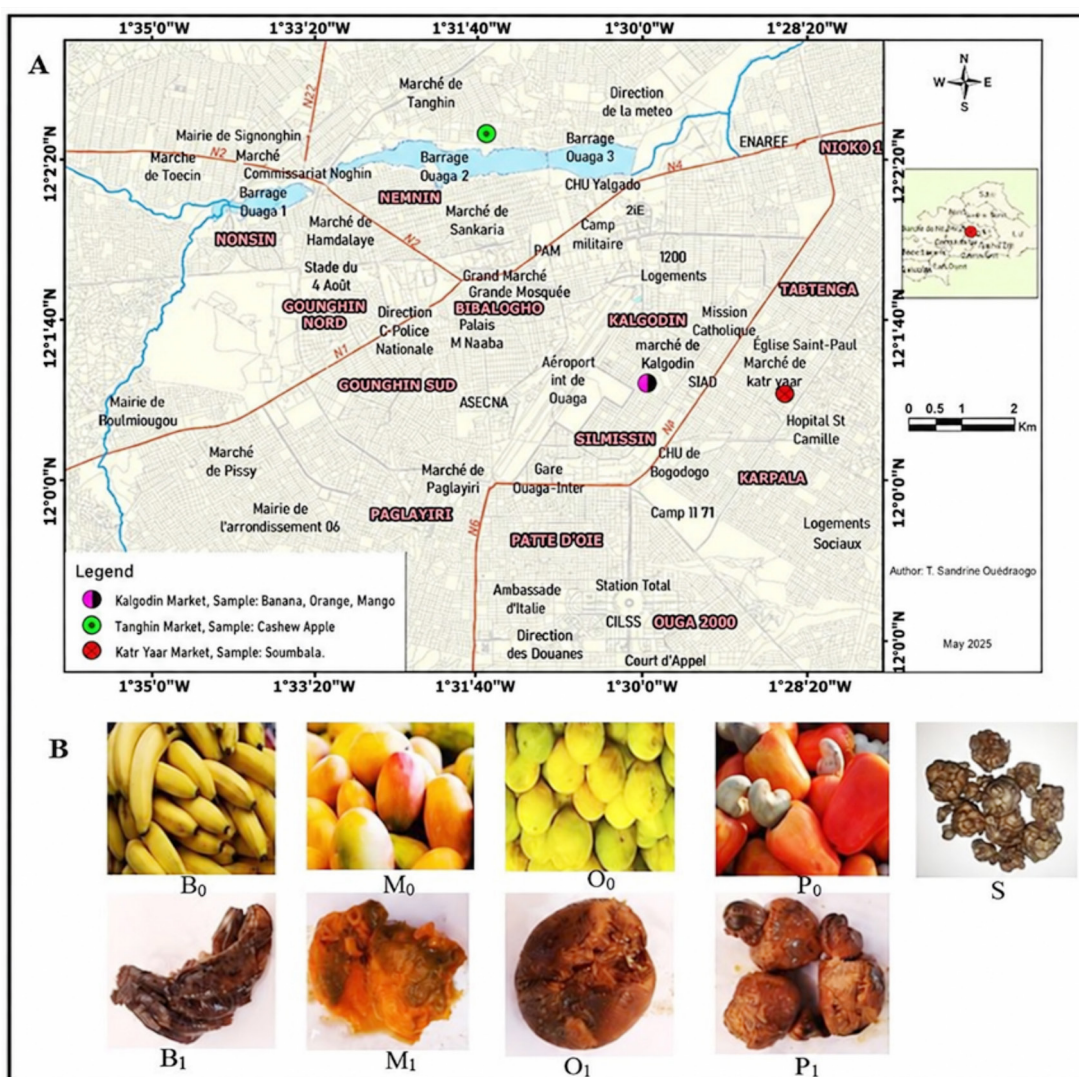


Figure 1. Localization of sampling site (A) and Samples (B) used for the isolation of cellulase-producing bacteria. B₀: Fresh bananas, B₁: Rotten bananas, M₀: Fresh mangoes, M₁: Rotten mangoes, O₀: Fresh Oranges, O₁: Rotten oranges, P₀: Fresh Cashew apples, P₁: Rotten cashew apples, S: Soubala²⁰

dendritiformis, *Bacillus altitudinis* (two strains), and *Mammaliicoccus lentus*. In addition to its high cellulolytic activity, *Paenibacillus dendritiformis* also exhibited a γ -haemolytic phenotype, considered a favorable preliminary indicator of its safety, although this alone does not guarantee its safety for food applications.

The BRENDA database separately lists the different cellulase groups and provides information on substrate specificity, kinetic parameters, and optimal pH and temperature conditions for many producing bacteria.²¹ Although this database and the scientific literature document cellulase production in several species of the genus *Paenibacillus*, specific studies on *Paenibacillus dendritiformis* cellulases remain limited.^{10,22-24} Previous work on this species has mainly focused on other enzymatic activities.^{25,26} *Paenibacillus dendritiformis* could thus constitute a promising source of bacterial cellulases, the production conditions and properties of which require in-depth characterization. Moreover, the low enzyme yields frequently observed in natural strains necessitate the optimization of culture parameters. Statistical approaches, such as response surface methodology, allow for the simultaneous study of the effects of multiple factors and their interactions, while reducing the number of experiments required.²⁷ They thus constitute a relevant strategy for improving enzyme production without necessarily resorting to genetic modification of the microorganism.¹⁹

In this context, the present study aims to contribute to the valorization of microbial resources from tropical niches for potential biotechnological applications. Specifically, it consisted of isolating cellulolytic bacteria from rotten fruit and sombala, characterizing the selected strains at the molecular level, identifying the strain exhibiting both a γ -haemolytic phenotype and the highest cellulolytic activity, and then optimizing the enzymatic production conditions of *P. dendritiformis* using response surface methodology.

MATERIALS AND METHODS

Research site

The present study was carried out at the Laboratory of Biochemistry, Biotechnology, Food Technology, and Nutrition (LABIOTAN) and the Laboratory of Microbiology and Microbial Biotechnology (LAMBM) at Joseph KI-ZERBO University in Ouagadougou, Burkina Faso, where all microbiological analyses were performed.

Collection of samples

The samples used in this study were sourced from multiple local markets in Ouagadougou (Burkina Faso), with approximately 1 kg collected for each sample type (Figure 1A). The investigated substrates included banana, mango, orange, cashew apple, and Soumbala (Figure 1B). For each fruit type, as well as Soumbala, a single

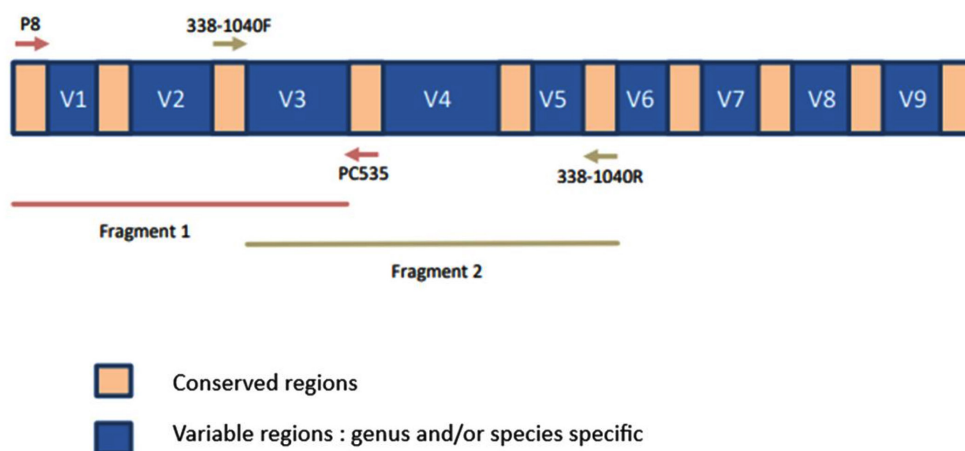


Figure 2. 16S rRNA gene sequence showing primer binding sites and amplified regions in fragments 1 and 2

composite sample was prepared from healthy fruits randomly collected from 5-7 vendors across different markets in Ouagadougou, the capital of Burkina Faso. After collection, the samples were aseptically packed in sterile polyethylene bags and transferred to the laboratory. Fruits were stored at room temperature for 21 days, until visible signs of deterioration appeared, before being used to isolate cellulolytic bacteria. Soumbala, being a traditionally fermented product, was used directly after random collection from five vendors.

Isolation of bacteria and screening for cellulase-producing strains

Cellulolytic bacteria were isolated on reconstituted medium containing CMC as the sole carbon source, according to the method

described by Kasana et al.²⁸ For sample processing, 10 g of each decomposed fruit or Soumbala sample was aseptically transferred into 90 mL of sterile sodium chloride solution 0.9% (w/v) and subjected to thorough homogenization. Tenfold serial dilutions were then prepared, and 100 μ L aliquots from each dilution were spread onto CMC agar plates. The plates were incubated at 37 °C for 96 hrs. Distinct colonies were selected and purified through repeated subculturing on CMC agar plates. Pure isolates were preserved at 4 °C in nutrient broth supplemented with 15% (v/v) glycerol for subsequent analyses. To detect cellulase activity, pure bacterial isolates were inoculated by spot deposition on the surface of CMC agar. After incubation at 37 °C for four days, Congo red solution (0.1% w/v) was poured over

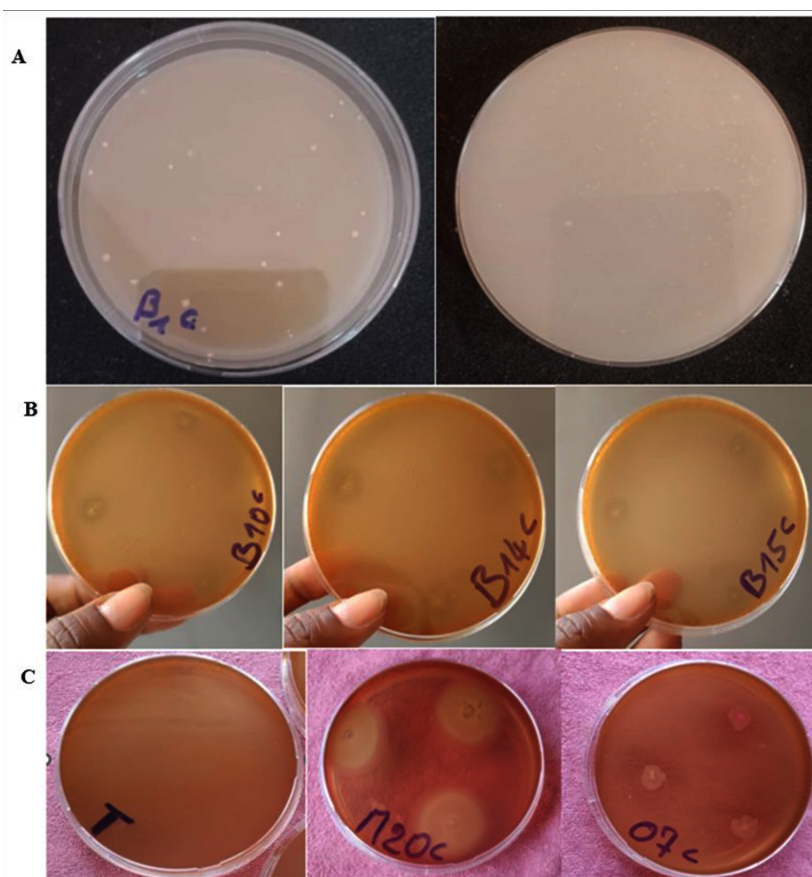


Figure 3. Macroscopic aspects of cellulase-producing bacterial colonies. (A) on reconstituted medium (agar, carboxymethylcellulose, yeast extract, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, K_2HPO_4 , NaCl); (B) on reconstituted medium, then flooded with Congo red; (C) on Columbia agar containing 5% sheep blood. Legend: T = negative control, M20_c = Example of a γ -haemolysis strain, O7_c = example of a β -haemolysis strain

the plates for several minutes and subsequently washed with NaCl, 1 M, for 15-20 minutes to eliminate excess stain. Clear halo formation surrounding bacterial colonies revealed cellulose hydrolysis and, therefore, positive cellulolytic activity, according to the method described by Gautam et al.²⁹ The diameters of the hydrolysis zones were measured using a vernier caliper, and the cellulolytic index (IC) was determined using the following equation:

$$\text{Cellulolytic index} = (\text{Clear zone diameter} - \text{Bacterial colony diameter}) / (\text{Bacterial colony diameter})$$

The different cellulolytic activities are classified according to the value of the cellulolytic index : if the IC < 1: low activity, if 1 < IC < 2, moderate activity, if the IC > 2, high activity

Phenotypic and biochemical characterization of isolates

Purified bacterial isolates were subjected to morphological, phenotypic, and biochemical analyses in order to achieve a preliminary classification. Morphological characterization involved macroscopic observation of colony features, including shape, color, surface appearance, and edge morphology. Microscopic examination was carried out on fresh preparations as well as after Gram staining, using a light microscope, following standard procedures.³⁰ Biochemical profiling included catalase and oxidase activity assays conducted according to established protocols. In addition, the carbohydrate fermentation characteristics of the isolates were assessed using the API 20E identification system (bioMérieux, Marcy letoile, France), following the manufacturer's instructions. Preliminary identification was achieved by interpreting the

Table 1. Coded levels and actual values of independent variables

Independent variables	Symbol code	-α	Low level (-1)	Level (0)	High level (+1)	+α
CMC (g/L)	X ₁	15	20	25	30	35
Yeast extract (g/L)	X ₂	1	2	3	4	5
Temperature (°C)	X ₃	17.5	25	32.50	40	47.5
NaCl (g/L)	X ₄	2.5	5	7.5	10	12.5

CMC: Carboxymethylcellulose

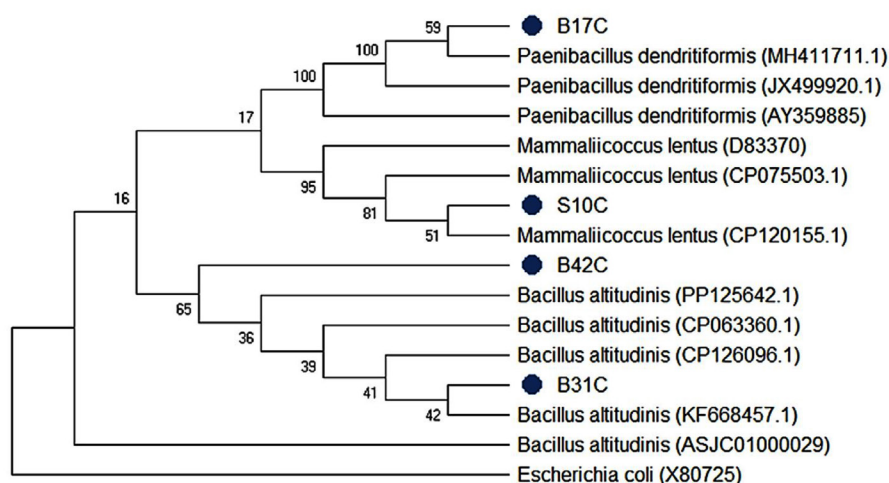


Figure 4. Phylogenetic tree using the neighbour-joining method

biochemical profiles through a digital probabilistic approach, as described by Hudson et al.³¹

Assessment of haemolytic activity

The haemolytic properties of the bacterial isolates were examined using Columbia blood agar medium (OXOID Ltd., Wesel, Germany); pH 7.5 supplemented with 5% (v/v) defibrinated sterile sheep blood. Bacterial suspensions were inoculated by spot inoculation onto the surface of the agar, then incubated at 37 °C for 48 hrs.³²

Haemolytic activity was determined according to the appearance of halos surrounding the colonies. The appearance of a clear zone indicated β -haemolysis, whereas a greenish discoloration corresponded to α -haemolysis. Isolates showing no visible halo were classified as γ -haemolytic (non-haemolytic), in accordance with previously described criteria.³³ These non-haemolytic isolates were considered potentially safe and were therefore selected for subsequent analyses.

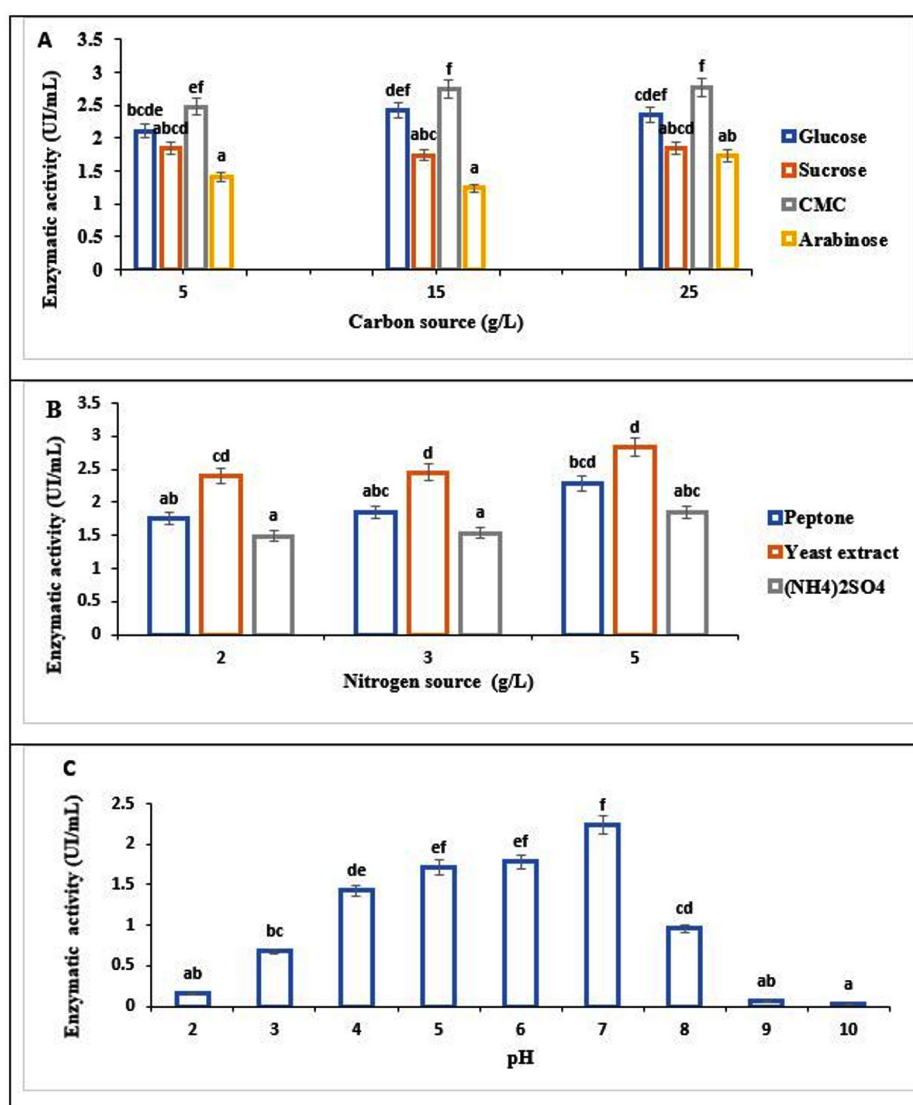


Figure 5. Influence of different carbon sources (A), different nitrogen sources (B) and different pH values (C) on cellulase activity. The data were analyzed by ANOVA, and within a group, values followed by different lowercase letters indicate significant differences at $P < 0.05$

Table 2. Cellulolytic index, hemolytic property, and morphological characteristics of isolated cellulolytic bacteria

Isolated strains	Bacterial colonies with clear areas	Colony diameter (mm)	Diameter of cellulolytic zone (mm)	Cellulolytic index	Haemolytic property	Shape	Grouping mode	Color	Size
B1 _c to B45 _c	B6 _c	3.333 ± 0.47 ^b	4.667 ± 0.47 ^f	0.444 ± 0.31 ^b	β-haemolysis	Stick	Isolated	White	Small
	B10 _c	4.000 ± 0.00 ^{ab}	5.333 ± 0.47 ^{ef}	0.333 ± 0.11 ^b	β-haemolysis	Circular	Chain	White	Small
	B14 _c	4.000 ± 0.00 ^{ab}	5.667 ± 0.94 ^{def}	0.416 ± 0.23 ^b	β-haemolysis	Stick	Isolated	White	Small
	B15 _c	3.333 ± 0.47 ^b	5.000 ± 0.00 ^{ef}	0.527 ± 0.19 ^b	β-haemolysis	Stick	Chain	Yellowish	Small
	B17 _c	4.333 ± 0.47 ^{ab}	14.333 ± 0.47 ^a	2.333 ± 0.23 ^a	γ-haemolysis	Circular	Chain	White	Medium
	B18 _c	6.000 ± 2.16 ^{ab}	9.333 ± 1.89 ^{bcd}	0.644 ± 0.27 ^b	β-haemolysis	Oval	Isolated	White	Medium
	B25 _c	7.333 ± 2.05 ^{ab}	10.000 ± 1.63 ^{abcd}	0.409 ± 0.16 ^b	β-haemolysis	Stick	Isolated	White	Large
	B31 _c	7.667 ± 0.94 ^{ab}	10.667 ± 0.94 ^{abc}	0.397 ± 0.04 ^b	γ-haemolysis	Stick	Isolated	White	Large
M1 _c to M30 _c	B42 _c	4.667 ± 1.25 ^{ab}	13.667 ± 0.94 ^{ab}	2.144 ± 0.84 ^a	γ-haemolysis	Stick	Isolated	White	Medium
	M10 _c	6.333 ± 0.94 ^{ab}	9.333 ± 1.70 ^{bcd}	0.467 ± 0.07 ^b	β-haemolysis	Circular	Chain	White	Medium
	M18 _c	7.667 ± 0.94 ^{ab}	11.000 ± 1.41 ^{abc}	0.434 ± 0.01 ^b	β-haemolysis	Circular	Chain	White	Large
	M19 _c	7.333 ± 0.47 ^{ab}	10.000 ± 0.00 ^{abcd}	0.369 ± 0.08 ^b	β-haemolysis	Stick	Isolated	White	Large
	M20 _c	7.833 ± 0.62 ^a	10.000 ± 0.00 ^{abcd}	0.285 ± 0.10 ^b	γ-haemolysis	Oval	Isolated	Yellowish	Large
	M27 _c	6.667 ± 0.62 ^{ab}	10.000 ± 0.00 ^{abcd}	0.513 ± 0.13 ^b	γ-haemolysis	Stick	Chain	White	Medium
	O1 _c	5.500 ± 1.87 ^{abcd}	8.000 ± 2.16 ^{abc}	0.500 ± 0.13 ^{ab}	β-haemolysis	Stick	Isolated	White	Medium
	O7 _c	5.667 ± 1.89 ^{abcd}	8.000 ± 2.16 ^{abc}	0.460 ± 0.15 ^{ab}	β-haemolysis	Ovale	Isolated	White	Medium
S1 _c to S15 _c	S4 _c	3.667 ± 1.43 ^{abcd}	5.667 ± 0.94 ^{bc}	0.734 ± 0.54 ^{ab}	γ-haemolysis	Stick	Isolated	White	Small
	S10 _c	6.833 ± 0.84 ^{abc}	12.333 ± 1.25 ^a	0.810 ± 0.04 ^{ab}	β-haemolysis	Circular	Isolated	White	Medium
	S12 _c	3.667 ± 0.94 ^{abcd}	5.667 ± 0.94 ^{bc}	0.577 ± 0.12 ^{ab}	γ-haemolysis	Stick	Chain	White	Small
	Pr > F	0.000	0.000	0.015	-	-	-	-	-
	Significatif	Yes	Yes	Yes	-	-	-	-	-

Legend: B = Bananas; M = Mangoes; O = Oranges; S = Sombala.

Values on the same line with different lowercase letters in superscript are significantly different at P < 0.05

Molecular characterization of strains

Extraction and preparation of genomic DNA for sequencing

A cell culture of each isolate was obtained by streak plating on CMC agar medium and incubating under aerobic condition at 37 °C for 24 hrs. From a single colony per isolate, total genomic DNA was extracted using the heat shock technique, according to the method described by Dashti et al.³⁴ The extracted DNA was then amplified by PCR, followed by Sanger sequencing according to the chain-termination methodology described by Sanger et al.³⁵ This service was provided by Genoscreen, Lille, France. PCR amplification of the 16S rRNA gene was included in this service, targeting the region encoding the 30S subunit of the bacterial ribosome. The variable regions V1-V5 (Figure 2) were specifically targeted to discriminate between different bacterial populations by sequence alignment with a global reference database. This approach allows reliable molecular identification of isolates at the genetic level.

PCR amplification of bacterial 16S rRNA genes

The amplification of 16S ribosomal RNA gene sequences was carried out for all bacterial isolates using two sets of universal primers targeting conserved regions of the bacterial genome. The primer pairs P8 (5'-AGAGTTTGTATCCTTGGCTCAG-3')/P535 (5'-GTATTACCGCGGCTGCTGGCAC-3') and 338 – 1040R (5'-GACACGAGCTGACGACA-3') were employed, allowing the amplification of fragments of about 550 bp and 750 bp, respectively. Together, the oligonucleotide primers target highly conserved regions and enable the amplification of nearly the full-length 16S rRNA gene from the genomic DNA of each isolate. PCR reactions were prepared separately for each primer set in a final volume of 50 µL containing 0.3 mM of each deoxynucleotide triphosphate (dNTPs; Fermentas, Germany), 0.5 mM MgCl₂, 0.5 pmol·µL⁻¹ of each primer, 0.24 µL of DreamTaq™ DNA polymerase (1.25 U·µL⁻¹), and 2 µL of genomic DNA template. The reaction volume was adjusted with sterile Milli-Q water. Each PCR run included both positive

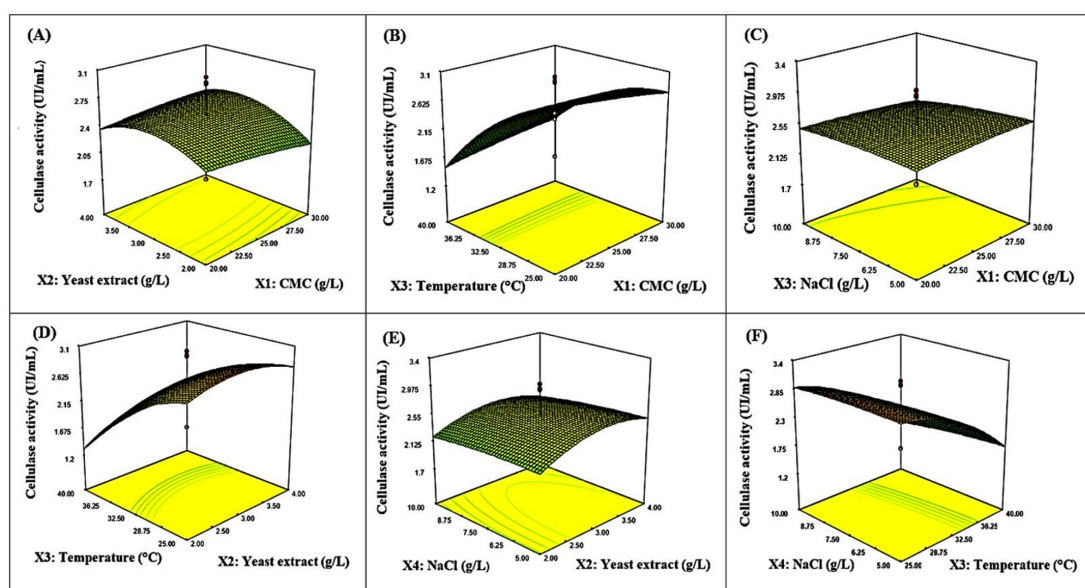


Figure 6. Three-dimensional response surface plots showing the interaction effect of variables for cellulase activity. (A) Effect of the interaction between carboxymethylcellulose and yeast extract on enzyme activity; (B) Effect of the interaction between carboxymethylcellulose and temperature on enzyme activity; (C) Effect of the interaction between carboxymethylcellulose and NaCl on enzyme activity; (D) Effect of the interaction between temperature and yeast extract on enzyme activity; (E) Effect of the interaction between NaCl and yeast extract on enzyme activity; (F) Effect of the interaction between NaCl and temperature on enzyme activity

and negative controls to confirm the reliability of the reaction. PCR amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 95 °C for 12 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, primer annealing at 58 °C for 30 sec, and extension at 72 °C for 10 min. A final extension was performed at 72 °C for 10 min. The amplified products were subsequently maintained at 4 °C until further analysis. The success of PCR amplification was initially verified by electrophoretic separation with 10 µL of each PCR product on a agarose gel, 1.5% (w/v), prepared in 1x TBE buffer and run at 100 V for 1 hrs. Amplicon sizes were estimated using a 50-1500 bp DNA ladder (QIAGEN). The amplified PCR products were purified with the GFX™ PCR DNA purification and Gel Band Kit following the manufacturer's protocol. The efficiency of the amplification and the quality of the fragments were confirmed by loading 1 µL of each purified PCR product onto a QIAxcel capillary electrophoresis system (QIAGEN) using the rapid DNA analysis parameters. The purified and validated PCR products were then sequenced.

Sequencing of the 16S rRNA gene

Following membrane-based PCR product purification amplicons using a commercial kit (Macherey-Nagel), purified products were assessed for quality and quantity via a fluorescence-based assay with SYBR Green dye. Bidirectional sequencing was subsequently performed using the same primers employed for PCR amplification, in accordance with an optimized sequencing protocol. Sequencing reaction products were purified via Sephadex G-50 columns, followed by analysis using an ABI 3730XL capillary DNA sequencer.

Bioinformatics data analysis and processing

Sequencing chromatograms were first analyzed and optimized with Auto Peak Trace 6 RP software. High-quality sequences were then assembled into consensus contigs with Sequencher version 4.9 (Gene Codes Corporation). The 16S rRNA sequences obtained were analyzed by comparison with NCBI GenBank reference sequences via the BLASTn tool.³⁶ Additional

taxonomic validation was conducted using the EZBioCloud database which is specialized in bacterial 16S rRNA gene sequences,³⁷ to accurately determine the phylogenetic assignment of the isolates. The sequences obtained were carefully checked and edited in BioEdit version 7.2, followed by alignment in MEGA X with the muscle algorithm.³⁸

Cellulase production: Modeling and optimization

Given its cellulolytic index and lack of haemolytic activity, isolate B17_C, identified as *Paenibacillus dendritiformis* PQ845045, isolated from rotting banana, was used to optimize cellulase production.

Optimization of cellulase production through one factor on time

The effect of different carbon and nitrogen sources, as well as pH, on cellulase production was evaluated in a liquid culture medium containing MgSO₄, K₂HPO₄, KH₂PO₄, and NaCl as basic mineral constituents. This enabled us to determine the preferred carbon and nitrogen sources and the pH conditions for optimal performance. The different media were prepared in 250 mL Erlenmeyer flasks, each containing 100 mL of culture broth, to promote bacterial growth and cellulase production, as described by Bhati et al.¹⁷

Effect of various carbon sources

To the basal medium, individual carbon sources, namely glucose, sucrose, carboxymethylcellulose (CMC), or arabinose, were added separately at concentrations of 5.00, 15.00, and 25.00 g/L. As described previously, yeast extract was employed as the nitrogen source.^{39,40} The pH of the medium was adjusted to 7 before sterilization. Each flask was inoculated with 5% (v/v) of a bacterial suspension standardized to an optical density of 0.6 at 600 nm and then incubated at 37 ± 2 °C for 72 hours under continuous agitation at 200 RPM (revolution per minute). At the end of the incubation period, cellulase activity was measured, and the carbon source yielding the highest enzymatic activity was selected for subsequent experiments.

Effect of various nitrogen sources

To identify the most suitable nitrogen source for cellulase production, an experimental design similar to that described above was employed. Various nitrogen source, namely yeast extract, peptone and ammonium sulfate ((NH₄)₂SO₄), were individually incorporated into the basal medium at concentrations of 2.00, 3.00, and 5.00 g/L. In this assay, CMC was used as the sole carbon source, and the medium was buffered at pH 7, following previously established protocols.⁴¹ Cultures were incubated under shaking conditions for three days, after which cellulase activity was evaluated.

Effect of pH

The effect of pH on cellulase production was determined by culturing the bacterial strains in media adjusted to different pH values using appropriate buffer systems. Citrate-phosphate buffers were used to adjust the medium to pH 2-6, phosphate buffer to pH 7 and Tris-HCl buffer to pH 8-9. The culture media were prepared using the previously optimized sources of carbon and nitrogen, along with the basal components MgSO₄ and NaCl. The cultures were maintained at 37 °C for 72 hrs under continuous agitation. Following incubation, all culture broths were centrifuged at 6,000 RPM for 30 min at 4 °C. Cell-free supernatants, containing extracellular enzymes, were harvested and used to assess cellulase activity.

Optimizing culture conditions using an experimental design approach

Based on single-factor experiments, carboxymethylcellulose and yeast extract were chosen as the most suitable carbon and nitrogen sources, respectively. A central composite experimental design with four independent variables—carboxymethylcellulose (X₁), yeast extract (X₂), temperature (X₃), and sodium chloride (X₄) was used to evaluate the combined effects of these variables on bacterial cellulase production. Each variable was associated with five distinct levels (Table 1) for optimization.⁴² The design was developed using a factorial approach with 30 experiments. Each experiment was conducted in triplicate, and the mean values were used for data analysis. Response surface methodology

was employed to evaluate interactions among independent variables and identify optimal conditions for cellulase production using a minimal number of experiments. The results were fitted to a second order polynomial model, yielding regression coefficients.

$$Y1 = a_0 + \sum_{i=1}^n a_i X_i + \sum_{j=1}^{n-1} \sum_{j=2}^n a_{ij} X_i X_j + \sum_{i=1}^n a_{ii} X_i^2$$

Y1 represents the response variable, X_i and X_j denote the independent variable and the total number of variables tested was 4. The coefficient a₀ corresponds to the model constant, while a_i, a_{ij} and a_{ii} represent the coefficients associated with the linear, quadratic, and interaction effects, respectively. The significance of the model and its goodness of fit were evaluated by an analysis of variance (ANOVA). Statistical analyses, three-dimensional response surface representations, and contour lines were generated using Design-Expert software, version 7.0.

The cellulase activity values obtained from 30 experiments were used in the equation, and predicted values were calculated.⁴³

Cellulase activity determination

Following each culture performed as part of the single-factor and multi-factor optimization trials, the culture media were centrifuged at 6,000 RPM for 30 min at 4 °C. The various supernatants were then collected and used as crude enzyme extracts containing the extracellular cellulases of isolate B17_c, identified as *Paenibacillus dendritiformis* PQ845045.

Cellulolytic activity was determined by colorimetry using carboxymethylcellulose (CMC) as the substrate. The reaction mixture contained 1 mL of enzyme solution (diluted or undiluted) and 1 mL of 1% (w/v) CMC prepared in 50 mM citrate phosphate buffer (pH 5). The mixture was incubated at 45 °C for 30 min. Following incubation, 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added, and the mixture was heated in a boiling water bath at 100 °C for 10 min. After cooling, the amount of reducing sugars released was measured at 540 nm. Glucose concentration was determined using a Biotek Instruments Inc. Epoch

TM spectrophotometer (Highland Park, Winooski, VT 05404-0998, USA). Carboxycellulolytic activity was expressed as the amount of enzyme required to release 1 μ mol of glucose per mL per minute under standard assay conditions.⁴⁴

Statistical analysis

Statistical analyses were performed using XLSTAT software (version 2016.02.27444). One way analysis of variance (ANOVA) was applied to evaluate differences among the studied parameters, with statistical significance defined at $P < 0.05$. When significant effects were observed, mean values were compared using the Newman-Keuls post hoc test. Data obtained from the central composite design were further analyzed using RSM.

All experimental assays were performed in triplicate. Each isolate was cultured independently in three Petri dishes to determine the cellulolytic index, while isolate B17_c, identified as *Paenibacillus dendritiformis*, was cultured in three separate tubes in liquid medium for each experimental assay. A measurement was taken for each independent culture, and the results are expressed as mean $\pm$ standard deviation. Comprehensive data analysis, including ANOVA, regression modelling, response surface analysis, and optimization of experimental conditions, was conducted using Design Expert software (version 7.0.0)

RESULTS AND DISCUSSION

Isolation of cellulolytic bacteria from fruit and soumbala samples

From samples of decaying fruit (banana, mango, and orange) and soumbala seeds, 115 bacterial colonies were initially isolated on a reconstituted medium containing carboxymethylcellulose (CMC) as the sole source of carbon and energy (Table 2 and Figure 3A). Initial colony selection was based on morphological characteristics. After three successive subcultures on the same medium to obtain pure cultures, 42 bacterial isolates exhibiting distinct morphotypes were selected. Of these isolates, 18 originated from banana samples, 13 from mango, 6 from orange, and 5 from soumbala (Table 2). However, no strain was obtained from the cashew apple samples. After Congo red staining, 19 of them, or

approximately 45.23%, exhibited clear hydrolysis zones around their colony, indicating their ability to degrade cellulose (Table 2 and Figure 3B). This proportion suggests that decomposing fruit is a valuable source of cellulase-producing bacteria. Their presence could be explained by the high cellulose content of these matrices, which may favor the selection of microorganisms capable of using them as a carbon source. This rate of cellulolytic isolates is comparable to those observed in other environments rich in decomposing organic matter, where cellulase-producing bacteria are often found.^{24,45}

Screening of cellulase-producing bacterial isolates

A total of 19 isolates with positive cellulolytic activity were selected for further enzymatic characterization. The results in Table 2 reveal significant variations among these strains in terms of hydrolysis zone diameter, colony size and cellulolytic index (Table 2). These variations observed between strains reflect a heterogeneity in their growth capacity and their ability to degrade cellulose. Strains B17_c, B42_c, and S10_c exhibited the best hydrolysis zone diameters with values of 14.33 mm, 13.66 mm and 12.33 mm, respectively. The maximum diameter of the cellulose degradation zone observed in this study (14.33 mm) is slightly smaller than that previously reported which is 14.5 mm.⁴⁶ Conversely, the lowest cellulolytic zone diameter (4.66 mm) was recorded for strain B6_c, followed by B10_c (5.33 mm). Regarding colony size, the largest value (7.83 mm) was obtained with strain M20_c, followed by strains B31_c (7.66 mm) and M18_c (7.66 mm). In contrast, the smallest colony size (3.33 mm) was obtained with B6_c and B15_c, followed by B10_c and B14_c (4.00 mm). These values remain higher than those previously described, which range from 1.20 mm to 2.35 mm.¹⁸ The cellulolytic index (ICs) varied between 0.28 and 2.30, and strain B17_c presented the best index which was 2.30. The lowest cellulolytic index (0.28) was obtained with M20_c. However, these indices remain significantly lower than those reported in other studies, in which indices up to 8 have been observed for some bacterial strains.⁴⁵ Similarly, the cellulolytic activity measured in this study appears, overall, to be lower than that described in other comparable investigations.^{18,46} The statistically significant

Table 3. Biochemical tests of bacterial isolates and molecular identification of isolated strains based on 16S rRNA sequences similarity with EZBioCloud database sequences

Tests performed	Isolated cellulolytic bacteria																		
	B6 _c	B10 _c	B14 _c	B15 _c	B17 _c	B18 _c	B25 _c	B31 _c	B42 _c	M10 _c	M18 _c	M19 _c	M20 _c	M27 _c	O1 _c	O7 _c	S4 _c	S10 _c	S12 _c
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mobility test	+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	-	-	-	-
ONPG	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	+	+	+
ADH	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
LDC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	+	-	-	-	-	+	+	-	-	+	-	+	-	-	+	+	-	+	+
H ₂ S	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	-	-	-	-
Ureate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TDA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
V-P	-	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+	+
Gelatinase	-	+	+	+	+	+	-	+	+	-	+	+	-	+	-	-	+	+	+
Glucose	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	+	+	+
Mannitol	-	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-	+	+	+
Inositol	-	+	+	+	+	+	-	-	+	-	+	-	+	+	-	-	+	+	+
Sorbitol	-	+	+	+	+	+	-	-	-	-	+	-	-	-	+	-	+	+	+
Rhamnose	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	-
Saccharose	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	+	+	-
Melibiose	-	-	+	-	+	-	-	-	+	-	-	-	+	+	-	-	+	-	-
Amygdaline	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+
Arabinose	-	-	+	+	+	-	-	+	+	+	+	-	+	+	-	-	+	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Strain	Identified species										Identified species		Similarity (%)		Number base pairs (pb)		GenBank accession number of deposited sequences		
B17 _c	Paenibacillus dendritiformis										AY359885		100		1471		PQ845045		
B31 _c	Bacillus altitudinis										ASJC01000029		99.90		1934		PQ845085		
B42 _c	Bacillus altitudinis										ASJC01000029		100		1943		PQ845089		
S10 _c	Mammaliococcus lentus										D83370		100		1943		PQ845126		

where, ONPG = Ortho Nitro Phenyl Galactopyranoside, ADH = Arginine dihydrolase, LDC = Lysine decarboxylase, ODC = Ornithine decarboxylase, TDA = Tryptophan deaminase; H₂S = Hydrogen sulfide, V-P = Vogue-Proskauer

Table 4. Design of experiments and results

Run	X ₁	X ₂	X ₃	X ₄	Predicted Value	Cellulase activity (IU/ml)
1	20	2	25	10	2.9499	3.21422
2	20	4	40	10	1.2910	1.40084
3	25	3	32.5	7.5	2.5585	1.70865
4	30	2	25	10	2.4355	2.79601
5	25	3	32.5	7.5	2.5585	2.94656
6	25	3	32.5	7.5	2.5585	2.92656
7	25	3	47.5	7.5	0.2708	1.22687
8	30	2	40	5	1.4519	1.083
9	30	2	40	10	1.1778	1.02947
10	35	3	32.5	7.5	2.4428	2.60865
11	30	4	25	5	2.6965	2.86627
12	20	4	25	10	2.7812	2.6856
13	25	3	32.5	2.5	2.5537	3.32463
14	15	3	32.5	7.5	2.4829	2.96329
15	30	4	40	5	1.9507	1.50456
16	25	5	32.5	7.5	1.9437	2.30753
17	20	2	25	5	2.8342	2.52835
18	25	3	32.5	7.5	2.5585	2.33095
19	20	2	40	10	1.1586	0.52427
20	30	4	25	10	2.5428	2.44805
21	25	3	32.5	7.5	2.5585	2.42798
22	30	2	25	5	2.4988	2.20716
23	20	4	25	5	2.7559	2.7224
24	25	3	32.5	12.5	2.3050	2.1804
25	25	1	32.5	7.5	1.6136	1.89601
26	25	3	17.5	7.5	2.8080	2.49824
27	20	4	40	5	1.4764	0.65141
28	30	4	40	10	1.5863	1.42761
29	20	2	40	5	1.2536	1.16664
30	25	3	32.5	7.5	2.5585	3.01013

variability ($P \leq 0.05$) between the ICs of the strains tested confirms the interest of targeted screening to select the most efficient strains, particularly for industrial applications such as juice clarification, the valorization of agriculture biomass or pilot scale enzyme production.

Morphological characteristics of cellulase-producing isolates

The morphological characteristics of the 19 cellulolytic bacterial isolates (Table 2) reveal that, among all the strains identified, 17 exhibited whitish pigmentation and 2 displayed yellowish coloration, indicating relative chromatic homogeneity among the isolates. Regarding cell morphology, microscopic observations revealed

that 5 isolates were circular, 11 *bacillus*-shaped, and 3 ovals. Colony size also revealed significant variability. Thus, 8 were medium-sized, 5 large, and 6 small. These observations are consistent with previously reported results, which describe a similar morphological diversity in terms of shape, color, and size of bacterial colonies isolated from rich organic substrates.¹⁸ Indeed, the prevalence of bacillary forms (11 isolates) has been reported by many authors, who indicate that rod-shaped bacteria are dominant among cellulolytic microorganisms because of their ability to efficiently secrete extracellular enzymes.^{47,48} Circular (coccoid) and oval shapes, although less frequent, demonstrate taxonomic diversity that may reflect variation in cellulase production or

regulation mechanisms.¹⁷ Colony size, on the other hand, provides indirect evidence of strain growth rate and vitality. Isolates from large colonies could reflect fast-growing bacteria, often adapted to environments rich in carbon substrates. Conversely, small colonies may correspond to slower-growing strains, but whose specific enzymatic activity could be higher, as observed in some cases.⁴⁹

Biochemical characteristics and presumptive identification of isolates

The results of the biochemical analyses of the 19 cellulolytic isolates (Table 3) revealed a common profile, characterized by Gram-positive staining and a positive catalase reaction. This phenotypic profile indicates that these isolates possess an enzymatic system enabling them to degrade hydrogen peroxide and constitutes a starting point for their taxonomic classification.

Furthermore, all isolates were negative for lysine decarboxylase (LDC), ornithine decarboxylase (ODC), and urease, indicating the absence of lysine and ornithine decarboxylation activity and urea hydrolysis. In contrast, 18 of the 19 isolates were positive in the tryptophan dehydration test. This high frequency indicates that the ability to deaminate tryptophan is a metabolic trait widely shared among the cellulolytic isolates studied. Beyond these common characteristics, some biochemical tests revealed differences between the isolates. Twelve isolates fermented glucose, while seven did not, reflecting the physiological diversity of the strains studied. Furthermore, ten isolates showed a positive result and nine a negative result in the arabinose fermentation test; thirteen isolates showed a positive result and six a negative result in the sucrose fermentation test. These differences in sugar utilization reflect metabolic heterogeneity and constitute useful criteria for their biochemical differentiation, suggesting physiological diversity that may influence cellulase production. The ability to ferment glucose observed in most isolates could indicate efficient exploitation of simple carbon sources derived from cellulose degradation. Differences in motility were also observed: eight isolates were motile and eleven were non-motile. The motility observed in eight isolates reflects the ability of some strains to

actively explore their environment, which can be advantageous on heterogeneous substrates such as decaying fruit. Conversely, the eleven non-motile isolates could belong to spore-forming species or species with slower metabolism. Based on all the observed morphological, physiological, and biochemical characteristics, a probabilistic numerical method was used to establish the presumptive identification of the 19 isolates. Comparison of their profiles with reference taxonomic characters allowed all the isolates to be assigned to the genus *Bacillus*, with varying degrees of similarity. To refine the taxonomic identity of the selected isolates, molecular identification and evaluation of their hemolytic activity were performed.

Evaluation of haemolytic activity

Although all 19 isolates exhibited cellulolytic activity, assessing their safety is a crucial step before any biotechnological application, particularly in the food industry. Indeed, some strains may also possess virulence factors. Haemolytic activity was studied as a preliminary phenotypic indicator of the isolates' ability to lyse erythrocytes and to assess their potential safety. The presence of partial (α -haemolysis) or complete (β -haemolysis) on blood agar suggests the possible production of toxic factors, particularly phospholipases, that are involved in cell membrane lysis. β -haemolysis reflects high enzymatic activity, which can compromise the strain's safe use in the agro-industry, particularly by depleting haemoglobin, a source of iron.^{50,51} In this study, the evaluation of the haemolytic activity of the isolates showed that out of the 19 isolates identified, 12 exhibited β -haemolytic activity, characterized by a clear zone around the colonies, indicating complete lysis of erythrocytes, while the other seven were γ -haemolytic showing no haemolytic activity (absence of halo), indicating that they do not read blood cells (Table 2 and Figure 3C). The high proportion of β -haemolytic isolates indicates that several cellulolytic strains can also lyse erythrocytes, a characteristic that may be associated with the production of virulence factors. Previous studies have also reported a high frequency of hemolytic activity among bacterial isolates exhibiting cellulolytic activity.⁵² Conversely, the absence of hemolysis observed in the seven

Table 5. Analysis of variance of independent variables and processing of experimental data

Source	Sum of squares	d_f square	Mean	F-value	P-value
Model	12.98	14	0.93	2.6318078	0.0365*
X_1 -CMC	0.0024	1	0.0024	0.0067516	0.9356
X_2 -Yeast Extract	0.16	1	0.16	0.465182	0.5056
X_3 -Temperature	9.66	1	9.66	27.415923	0.0001*
X_4 -NaCl	0.093	1	0.093	0.2642014	0.6147
X_1X_2	0.076	1	0.076	0.2155854	0.6491
X_1X_3	0.28	1	0.28	0.8072411	0.3831
X_1X_4	0.032	1	0.32	0.0904808	0.7677
X_2X_3	0.091	1	0.091	0.2566467	0.6198
X_2X_4	0.0082	1	0.0082	0.0229203	0.8817
X_3X_4	0.045	1	0.045	0.1255753	0.7280
X_1^2	0.016	1	0.016	0.0446147	0.8356
X_2^2	1.04	1	1.04	2.9615538	0.1058
X_3^2	1.78	1	1.78	5.0568631	0.0400*
X_4^2	0.028	1	0.028	0.0812482	0.7795
Residual	5.28	15	0.35		
Lack of fit	4.00	10	0.40	1.5613582	0.3253**
Pure error	1.28	5	0.26		
Cor total	18.26	29			

CMC: Carboxymethylcellulose; *Significant terms; **no significant

γ -haemolytic isolates constitutes a favorable phenotypic trait for potential biotechnological applications, particularly in the food sector. However, the absence of haemolytic activity alone is insufficient to demonstrate a strain's safety; further analyses are necessary before considering food applications.

Molecular characterization of isolates

To confirm and refine the presumptive identification obtained by phenotypic and biochemical methods, the selected isolates were subjected to molecular identification based on PCR amplification and sequencing of the 16S rRNA gene.

In the present study, the 16S rRNA gene of strains B17_c, B31_c, B42_c, and S10_c was sequenced. Comparison of the obtained sequences with those available in the EZBioCloud and NCBI databases revealed similarity levels ranging from 99.81%-100% with the corresponding type species (Table 3). Isolate B17_c has a maximum identity of 100% with *Paenibacillus dendritiformis* (MT256092.1). As for isolates B31_c and B42_c, they have a maximum identity of 99.90% and 100%, respectively, with *Bacillus altitudinis*

(PP818882.1). Isolate S10_c, a β -hemolytic strain, has a similarity of 99.81% with *Mammaliicoccus lentus* (MK439492.1). The phylogenetic history of the isolates was reconstructed using the Neighbor-Joining method,⁵³ which is known for its speed and ability to produce reliable trees from large distance matrices. The optimal phylogenetic tree obtained has a sum of branch lengths equal to 144.28, reflecting the genetic divergence between the different sequences analyzed. Evolutionary distances, expressed as the number of base substitutions per site, were estimated using the composite maximum likelihood method. The analysis included 39 nucleotide sequences, after removing ambiguous positions using the pairwise deletion method. Phylogenetic analysis revealed that several isolates exhibited a close evolutionary relationship to known species. Strain B17_c (PQ845045) was phylogenetically related to *Paenibacillus dendritiformis*, B31_c (PQ845085) to *Bacillus altitudinis*, B42_c (PQ845089) to *Bacillus altitudinis* and S10_c (PQ845126) to *Mammaliicoccus lentus* (Figure 4).

Our results are consistent with those of other studies that have highlighted the toxonomic diversity of cellulolytic bacteria. Cellulolytic strains

belonging to the genus *Bacillus*, *Priestia*, and *Lysinibacillus* have notably been identified by 16S rRNA gene sequencing in the rumen of the sheep *Ovis aries*.⁴⁷ Similarly, cellulase-producing bacterial species, such as *Bacillus megaterium* and *Paracoccus yeei*, have been described in the gut flora of termites.⁴⁶ These observations show that cellulolytic activity is widely distributed among different bacterial groups colonizing environments rich in plant substrates. Although 16S rRNA gene sequencing is considered a reliable identification tool, the strains exhibited high similarity to a different type species in GenBank.⁵⁴ In addition, some strains were assigned to *Bacillus altitudinis* due to the relatively low similarity levels (ranging from 99.81%-100%). These observations highlight the limitation of 16S rRNA gene sequencing for accurate microbial identification. Currently, microorganism identification from diverse sources is enhanced by combining 16S rRNA gene sequencing with techniques such as MALDI-TOF-MS, chemometric analysis, or whole genome sequencing. Accordingly, the identification of the strains in this study could be refined through such multifactorial approaches.

Cellulase production: Modeling and optimization Optimization of culture conditions through a single factor approach

Culture parameters, including carbon and nitrogen sources and pH, were varied to determine their effects on cellulase activity. Figure 4 shows significant variation in activity across the different media. This demonstrates a significant influence of each factor on enzymatic activity.

Influence of different carbon sources

Paenibacillus dendritiformis PQ845045 showed strong cellulolytic activity in the medium containing carboxymethylcellulose (CMC), followed by glucose (Figure 5A). The lowest activity was observed with arabinose. Enzyme activity also increased with rising concentrations of each carbon source. The maximum activity (2.77 IU/mL) was obtained in the medium containing 25 g/L CMC. These results are consistent with those reported by several authors, who found that CMC is the best carbon source for optimal cellulase

production.^{10,55,56} This is thought to be because this carbon compound is highly soluble in water, thus facilitating access to the enzymes.¹⁰ In contrast, previous studies have reported higher activity with glucose as the carbon source.^{57,58} Other studies have reported significant cellulase production by *Aspergillus terreus* when CMC is replaced with maize cobs.⁵⁹

Influence of various nitrogen sources

The influence of yeast extract, peptone, and ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), three nitrogen compounds, was investigated to identify the best nitrogen source. As illustrated in Figure 5B, optimal cellulase activity (2.24 IU/mL) was observed at a yeast extract concentration of 5 g/L. At the same time, low production was observed in the medium where ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) served as the nitrogen source. This study shows that organic nitrogen sources are more effective than inorganic ones. These results corroborate those reported on the mushrooms *Pleurotus ostreatus* and *Phanerochaete chrysosporium*.⁵⁵ This stimulating effect of the yeast extract may be attributable to its abundance of amino acids and vitamins.⁶⁰ Conversely, other studies suggest higher activity with peptone than with yeast extract.⁴⁸

Influence of pH

In this study, different pH values were tested. As illustrated in Figure 5C, pH influenced cellulase activity. Maximum activity was recorded at neutral pH. Furthermore, the strain is effective within a pH range of 4-6. However, above pH 7, activity was reduced by more than 60%. The maximum activity at neutral pH is advantageous because many industrial processes are conducted at this pH. Our results corroborate those of several authors who have reported 7 as the optimum pH.^{10,13,58,61,62} Previous studies have reported optimal activity at pH 8⁴⁸ and pH 9.⁶³

Therefore, in submerged fermentation, a medium containing CMC as the carbon source and yeast extract as the nitrogen source, at pH 7 used, may be suitable for good cellulase production. These variables were incorporated into the culture medium formulation to assess their cumulative effect.

Optimization of cellulase production using response surface methodology

MSR coupled with a centered composite design was used to study the combined effects of four independent factors, the amount of CMC (X_1), the amount of yeast extract (X_2), the temperature (X_3), and the amount of NaCl (X_4), with their competence levels ($-\alpha$, -1 , 0 , $+1$, $+\alpha$) on cellulase activity. These components were selected based on a one-way analysis. A series of 30 experiments was conducted, and the results highlighted the conditions that significantly optimized cellulase production. Table 4 presents the composition of the experimental designs and the observed and predicted responses. Enzyme activity varied from 0.52-3.32 IU/mL (Table 4) depending on the combination, corresponding to an optimization factor of 6.16. This result demonstrates the influence of the different factors and their levels of competence on activity. Slight variations were observed between the observed and predicted data. This once again demonstrates the model's accuracy. An optimization factor of 2.43, lower than that obtained in the present study, has been previously reported for *E. faecium*, where authors considered pH, temperature, inoculum volume, and incubation time as the main parameters influencing enzyme production.⁶⁴ Furthermore, activity increases of up to a factor of 60 compared to non-optimized conditions have also been described following appropriate optimization strategies.⁵⁸

Experimental results were modeled using a multiple Y-axis regression approach with Design Expert 7.0.0, including linear and quadratic effects and interactions between factors.

$$Y = +2.56 - 0.010X_1 + 0.082X_2 - 0.63X_3 - 0.62X_4 + 0.69X_1X_2 + 0.13X_1X_3 - 0.045X_1X_4 + 0.075X_2X_3 - 0.023X_2X_4 - 0.053X_3X_4 - 0.024X_1^2 - 0.19X_2^2 - 0.25X_3^2 - 0.032X_4^2$$

The model fit was evaluated using analysis of variance (ANOVA), and the results are shown in Table 5. The overall model showed a significant effect ($P < 0.05$; $F = 2.63$) on the evaluated response, namely, cellulase activity. The significant effect of the model ($P = 0.0365$) provides confidence, as there is only 3.65% probability that such a large F value occurred by

chance. Furthermore, the non-significant lack-of fit statistic (0.3253), along with the R^2 (0.71) and adjusted R^2 (0.44) values, confirms that the mathematical model (Y) was adequate for predicting cellulase activity across different combinations of variable values. A non-significant lack of fit (0.57) has also been previously reported, highlighting the relevance of this approach for optimizing experimental conditions.⁶⁴ The linear effect of X_3 , representing temperature, is also significant, indicating that temperature has a major impact on cellulase production by the bacterium. However, the quadratic model (X_3^2) and their interaction coefficients did not show a significant effect under the conditions studied. In contrast, significant effects of carboxymethylcellulose (CMC) on cellulase production have been reported in other studies on cellulolytic yeasts.⁶⁵ Similarly, the determining influence of the carbon source on the optimization of cellulase production has been demonstrated in certain bacterial species.⁵⁸ Furthermore, a significant effect of yeast extract on enzymatic activity has also been reported in several previous studies. The negative coefficient associated with indicates that an increase in temperature decreases the response. X_3^2 is also significant ($P < 0.05$). The negative coefficient of this quadratic term demonstrates a curvature that reflects an optimum temperature beyond which activity decreases. These results align with previous observations highlighting a comparable effect of temperature on cellulase production in *Enterococcus faecium*.⁶⁴ The highest activity was obtained with combination N°13. This medium consisted of 25 g/L CMC, 3 g/L yeast extract, and 2.5 g/L NaCl and the culture was maintained at 32.5 °C. The production predicted under this condition was nearly identical to the observed value, demonstrating the model's accuracy. Combinations N°1 and N°30 also yielded relatively high activities of 3.21 IU/mL and 3.01 IU/mL, respectively. Trial 30 differs from trial 13 only in its NaCl concentration, which is 7.5 g/L. Trial 1 consisted of 20 g/L of CMC, 2 g/L of yeast extract, 10 g/L of NaCl, and an incubation temperature of 25 °C. The lowest activities, below 0.70 IU/mL, were observed in trials 19 and 27, which used media containing 20 g/L of CMC and were incubated at 40 °C. All other media incubated at 40 °C also exhibited relatively low enzymatic

activities. In contrast, maximum cellulase productivity has been reported at temperatures above 40 °C in other studies.⁶⁴ This difference could be explained by genetic factors influencing thermotolerance and the ability of the bacteria to adapt to temperature fluctuations in the culture medium. Previous work has also demonstrated the value of statistical approaches in optimizing enzyme production by various microorganisms. Among these, the centred composite design has been successfully used to determine the optimal conditions for enzyme production by different bacteria.^{48,64,66} Several studies have highlighted the crucial role of temperature as a parameter influencing the statistical optimization of cellulase production.⁶⁴ Furthermore, Significant optimization of cellulase production was achieved in *Trichosporum insectorum* by the combined variation of three influencing factors: Carbon source concentration, incubation temperature and pH.⁶⁵ Other statistical optimization designs conducted with various influencing have enabled the optimization of cellulase production from several microorganisms, such as *Aspergillus flavus*,⁶⁷ *Trichoderma viride*,⁶⁸ and *Enterococcus faecium*.⁶⁴ An activity of 170.55 IU/ml has been reported for *Bacillus cereus* cellulase after optimization under the following four experimental conditions: culture time, temperature, pH, and inoculum volume.⁶⁹ Three dimensional response surface plots were constructed to evaluate the combined effects of two independent variables on enzyme production, with the remaining factors held at their median values. Figure 6 presents 3D response surfaces generated to evaluate the combined effect of two variables and the possible interactions between these variables on enzyme activity. Figure 6A illustrates the evolution of cellulolytic activity as a function of carboxymethylcellulose and yeast extract concentrations, at a temperature maintained at 32.5 °C and a NaCl concentration of 7.5 g/L. The response surface exhibits a curvature reflecting a non-linear variation of cellulolytic activity as a function of CMC and yeast extract concentrations within the experimental range studied. Furthermore, the contour lines show a nearly parallel arrangement, suggesting the absence of a marked synergistic or antagonistic interaction between CMC and yeast extract. This observation

is consistent with the non-significant nature of the interaction term ($P > 0.05$). Similarly, the linear effects of CMC (X_1) and yeast extract (X_2), as well as their respective quadratic effects (and), were not statistically significant ($P > 0.05$). Thus, within the studied concentration range, variations in CMC and yeast extract did not result in statistically significant changes in activity. The visible variations observed on the contour lines and response surface should be considered as trends predicted by the model and do not, on their own, allow us to conclude that each factor has an independent effect or that there is a significant optimum. Comparable observations were reported by Irfan et al.⁷⁰ who noted the non-significance of several linear terms despite the overall significance of the model. Figure 6B illustrates the combined effect of CMC concentration (X_1) and temperature (X_3) on activity, with yeast extract (3 g/L) and NaCl (7.5 g/L) concentrations held constant. The response surface shows an upward slope as the CMC concentration increases from 20-30 g/L, indicating an increase in cellulolytic activity within the studied concentration range. However, since the linear (X_1) and quadratic (X_1^2) effects of CMC are not statistically significant ($P > 0.05$), this trend suggests a prediction of the statistical model rather than a true effect of CMC concentration. The response surface shows a decrease in activity with increasing temperature from 25-40 °C. This trend is consistent with the significance of the linear term; $P < 0.05$). Furthermore, the absence of a significant quadratic effect of temperature (X_3^2 $P > 0.05$) indicates that this variation is primarily linear and does not allow for the identification of a significant thermal optimum. The contour lines are nearly parallel and closely spaced. This presentation is consistent with the lack of significance of the interaction terms between CMC and temperature. Thus, despite the overall significance of the model, analysis of the individual coefficients shows that only temperature exerts a statistically significant influence on activity within the experimental ranges studied. The decreases in predicted activity with increasing temperature suggest that lower temperatures are more favorable for cellulase production. A similar trend has been reported by Steiner and Margesin,⁷¹ where optimal activity was obtained between 20 and 25 °C. Figure 6C shows the effect of CMC and NaCl concentrations on

cellulolytic activity. The response surface is relatively flat, indicating little variation in activity as CMC and NaCl concentrations increase. The contour lines are gently curved and nearly parallel. The linear effects X_1 and X_4 , the quadratic effects X_1^2 and X_4^2 , and the $X_1 X_4$ interaction were not statistically significant, suggesting the absence of an optimal concentration and interaction between the two variables. Similar results were obtained by Waheeb et al.¹⁹ who showed that NaCl concentration did not have a statistically significant effect on cellulase production in *S. thermodiastaticus*. Figure 6D shows the combined effect of yeast extract concentration (X_2) and temperature (X_3) on cellulolytic activity. Unlike the previous surfaces, it exhibits a more pronounced curvature, indicating a greater variation in activity. A decrease in activity is observed with increasing temperature, consistent with the results of the analysis of variance showing a significant linear effect of X_3 . The response surface also shows an increase in activity as the yeast extract concentration increases to intermediate levels. However, since the linear (X_2) and quadratic (X_2^2) effects are not statistically significant, this trend should be considered a predicted trend. Furthermore, the contour lines have a distinctly curved shape, potentially suggesting an interaction between the two variables. However, this interaction is not statistically significant due to the lack of significance of the interaction term. Thus, among the effects represented, only the linear influence of temperature on activity is statistically established. Figure 6E shows the variation of activity as a function of combined variations in NaCl (X_4) and yeast extract (X_2) concentrations. The response surface exhibits moderate curvature, with an apparent nonlinear evolution of activity. Activity increases up to the intermediate level of the two factors, then decreases slightly to the highest concentrations. This pattern suggests the presence of optimum concentration. However, this trend is not statistically significant, as neither the linear nor the quadratic terms are significant. The contour lines are relatively spaced and not very concentric, indicating low sensitivity of the activity to concentration variations, as well as a moderate interaction between the two variables. Figure 6F shows the evolution of activity under the combined effect of temperature (X_3) and NaCl concentration

(X_4). The response surface shows a downward trend in activity as the temperature increases from 25-40 °C, consistent with the significant linear effect of X_3 ($P < 0.05$). In contrast, variations in NaCl concentration have little influence on the response, which is consistent with the non-significant linear effect of X_4 . The contour lines are almost parallel, indicating that the effect of temperature varies very little with NaCl concentration, consistent with the absence of a significant $X_3 X_4$ interaction ($P > 0.05$).

The statistical model obtained in this study was globally significant, although the linear effects of CMC, yeast extract, and NaCl, as well as the quadratic and interaction terms between factors, were not significant. Only the linear effect of temperature proved significant, indicating that this parameter was the main factor influencing cellulase production in *P. dendritiformis* B17_c under the conditions studied. A similar situation was reported by Wang et al.,⁶² in which the overall model was significant, whereas the interactions among the factors studied were not. The importance of temperature in cellulase production has also been reported in cellulase production in *Bacillus subtilis*.⁷⁰ Thus, the identification of temperature as a significant factor in the present study, combined with the significant improvement in activity after optimization, reinforces the cellulolytic potential of *P. dendritiformis* B17_c.

CONCLUSION

At the end of this study, nineteen cellulolytic bacterial strains were isolated from rotten fruit and soumbala. Among them, three strains, named B17_c, B31_c, and B42_c, all from rotten bananas, stood out due to their high cellulolytic activity and γ -haemolytic phenotype. Molecular identification of these strains allowed them to be assigned to *Paenibacillus dendritiformis* (PQ845045), *Bacillus altitudinis* (PQ845085), and *Bacillus altitudinis* (PQ845089), respectively. A fourth strain with high cellulolytic activity, S10_c, isolated from soumbala (fermented carob paste), was identified as *Mammaliicoccus lentus* (PQ845126) but was distinguished by a β -haemolytic phenotype. The statistical optimization model for the culture conditions of *Paenibacillus dendritiformis* was found to be

globally significant ($P < 0.05$) and resulted in a maximum activity of 3.32 IU/mL, representing an approximately 6-fold increase compared to the non-optimized medium. Response surface analysis identified temperature as the main factor influencing cellulase production. In contrast, variations in the concentrations of CMC, yeast extract, and NaCl did not significantly affect activity, and no statistically significant synergistic or antagonistic interactions were observed between the variables studied. These results highlight the ability of *P. dendritiformis* B17_c to efficiently produce cellulase and support its interest as a particularly promising candidate for the development of biotechnological processes for cellulase production. Further research is recommended, focusing on the purification and biochemical characterization of the cellulase produced by this strain, particularly by evaluating its thermal stability, pH stability, and kinetic properties. Pilot and larger-scale trials using real-world matrices, especially for juice clarification and the valorization of plant residues, are recommended to determine the conditions for potential biotechnological applications. Finally, a thorough safety assessment of the strain, including screening for virulence factors, should be conducted before any food application.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

TSO, MD, YD and IM conceptualized and designed the study. TSO and IM collected the samples. TSO, HS, YD, SG and IM carried out the experimental work. MD, IM, AS, and SNG performed data analysis and interpretation of results. OTS, MI and KaK wrote the manuscript. CID, MD., KiK., and MHD reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available in the NCBI GenBank database and are accessible under the following accession numbers PQ845045, PQ845085, PQ845089 and PQ845126.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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