Molecular Identification of Inulin-Degrading Thermophiles: A 16S rRNA Gene-Based Study

Poppy Mai Versiska[1], *Minda* Azhar[2], *Bahrizal*[2], *Toyokazu* Yoshida[3], *Mitsukura* Koichi[3]

[1]Department of Chemistry, Faculty of Mathematics and Natural Science, Bandung Institute of Technology, Bandung, Indonesia
[2]Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Padang, Padang, Indonesia
[3]Department of Biochemistry, Faculty of Engineering, Gifu University, Gifu, Japan

Abstract. Thermophilic bacteria are a potential source of thermostable enzymes capable of degrading inulin. One prominent habitat for these bacteria is hot springs. Despite their significance, many thermophilic bacteria remain unidentified at the molecular level. This study conducted to identify inulin-degrading thermophilic bacteria from the Sonsang hot spring in Solok, West Sumatera, using 16S rRNA gene analysis. Chromosomal DNA was extracted from the bacterial isolate, and the 16S rRNA gene was amplified through Polymerase Chain Reaction. The amplified gene products were purified, visualized using agarose gel electrophoresis, and cloned into a pT7Blue T-Vector for sequencing. The 16S rRNA gene sequence, consisting of 1548 base pairs, was analyzed using BLASTn, revealing 99% similarity to *Bacillus smithii*. Consequently, the thermophilic bacterial isolate was identified as belonging to the species *Bacillus smithii*.

1. Introduction

Inulin is a carbohydrate composed of fructose units, commonly found in plants like chicory, Jerusalem artichokes, and dahlias [1,6]. It can be hydrolyzed into fructooligosaccharides (FOS) and fructose using inulinase enzymes, which are often derived from thermophilic bacteria. These bacteria, which thrive at temperatures of 50-70ºC [1,5], produce thermostable inulinase enzymes ideal for industrial applications. The production of inulinase by thermophilic bacteria offers a promising approach for various biotechnological applications, particularly in the hydrolysis of inulin to fructooligosaccharides (FOS) and fructose [1,2,10]. Fructose serves as a widely used sweetener in the food industry, while FOS functions as a prebiotic. Prebiotics are defined as non-digestible dietary components that selectively stimulate the growth and activity of beneficial gut microorganisms, thereby contributing to host health [2,3,4]

Genotypic identification of inulin-degrading bacteria, through sequencing the 16S rRNA gene, is an efficient method for determining species-level identification [6]. The

16S rRNA gene, approximately 1500 bp, can be easily amplified by PCR and cloned into vectors like pT7Blue for further analysis [7]. Previous studies have isolated thermophilic inulin-degrading bacteria from hot springs in Solok, West Sumatera [8], but molecular identification of several bacterial isolates has not been completed. This study aims to identify the 16S rRNA gene of inulin-degrading thermophilic bacteria isolated from the Sonsang hot springs in Solok Regency. This research will contribute to the molecular characterization and potential biotechnological applications of thermophilic inulin-degrading bacteria from this region.

2. Experimental Section

2.1 Materials

The equipment used in this study included an incubator, shaker, autoclave, analytical balance, micropipette, microtube, vortex mixer, Erlenmeyer flasks, pH meter, measuring cup, inoculating loop, collector tube, DNA purification silica column, water bath, centrifuge, PCR thermocycler, electrophoresis apparatus, DNA concentration measurement device (*Quantus Fluorometer*), and a sequencing machine (*CEQ 8000 Genetic Analysis System*). The bacterial isolates used were inulin-degrading thermophilic bacteria obtained from the Sonsang hot springs in Solok Regency, West Sumatra, Indonesia. The materials used included bacterial chromosomal DNA, inulin, *E. coli* JM109, pT7Blue T-Vector, and reagents for PCR, cloning, and sequencing.

2.2 Methods

2.2.1 Rejuvenation of Bacterial Isolates

Pure bacterial cultures in liquid media were diluted with 0.85% NaCl and spread onto solid media containing inulin as the sole carbon source. The cultures were incubated at 55°C for 48 hours. Single colonies from the solid media were transferred to liquid media using a sterile inoculating loop. The liquid media were then incubated at 55°C with shaking for 48 hours.

2.2.2 Chromosomal DNA Isolation, PCR Amplification, and Purification

Chromosomal DNA was isolated using the *QIAprep® Spin Miniprep Kit (QIAGEN)* following the manufacturer's protocol. The DNA was electrophoresed on agarose gel using a 500 bp DNA ladder as a marker. Subsequently, the 16S rRNA gene fragment was amplified by PCR using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAAGGAGGTGATCCAGCC-3'). The PCR reaction mixture (100 µL) consisted of 75 µL sterile distilled water, 10 µL 10× Ex Taq buffer, 8 µL 2.5 mM dNTP mix, 1 µL 1525R primer, 1 µL 27F primer, 4 µL chromosomal DNA, and 1 µL DreamTaq polymerase. The amplification protocol included initial denaturation at 94°C for 1 minute, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing and elongation at 68°C for 5

minutes. The PCR products were electrophoresed on agarose gel and purified using the *QIAprep® PCR Purification Kit (QIAGEN).*

2.2.3 Cloning and Cultivation of Recombinant DNA

The purified 16S rRNA gene fragment was ligated into the pT7Blue T-Vector and transformed into competent *E. coli* JM109 cells using the heat shock method. Successful cloning was confirmed by screening for blue and white colonies. White colonies were selected and cultured in Luria Bertani (LB) medium, followed by incubation at 37°C for approximately 15 hours. Recombinant DNA was isolated using the *QIAprep® Spin Minikit (QIAGEN)* according to the manufacturer's protocol.

2.2.4 Sequencing of 16S rRNA Gene Fragments and Bioinformatics analysis

The recombinant DNA was sequenced using the dideoxy-Sanger method with the CEQTM 8000 Genetic Analysis System. Sequencing primers included U19, R20, 518F, and 518R. The DNA electropherograms were analyzed using the DNAStar program. The 16S rRNA gene sequence was aligned with sequences in the GenBank database using the BLASTn tool. A phylogenetic tree was constructed to determine the evolutionary relationship of the bacterial isolate with other bacterial species using the MEGA6 software.

3. Results and Discussion

3.1 Isolation of Bacterial Chromosomal DNA

Chromosomal DNA was successfully isolated from inulin-degrading thermophilic bacterial isolates cultivated in liquid media containing inulin as the sole carbon source. The isolation process involved three main stages: bacterial cell wall lysis, chromosomal DNA extraction, and purification[9]. Electrophoresis was performed to evaluate the quality and integrity of the isolated DNA[10]. A 500 bp DNA Ladder marker was used as a reference for size determination during electrophoresis. The marker spans a range from 500 bp to 6,000 bp, providing a reliable standard for estimating DNA fragment sizes. The electrophoresis results, shown in Figure 1A, revealed a single distinct band for bacterial chromosomal DNA positioned above the 6,000 bp band of the marker. This indicates that the isolated chromosomal DNA exceeds 20,000 bp in size. Typical bacterial chromosomal DNA sizes range from 22–23 kb [9], aligning with the observed results. The presence of a single, high-molecular-weight DNA band confirms the successful isolation of intact chromosomal DNA with minimal degradation, ensuring its suitability for molecular applications such as PCR amplification and sequencing. High-quality DNA with sufficient concentration and purity minimizes amplification errors, reduces sequencing biases, and supports reproducible experimental replicates, alignment accuracy, and broad applications like whole-genome sequencing, thereby strengthening the foundation for comprehensive molecular studies and reliable downstream analyses.

3.2 Amplification of the 16S rRNA Gene and Amplicon Purification

The 16S rRNA gene of inulin-degrading thermophilic bacteria was amplified using the primers 27F and 1525R. Primer 27F, a forward primer, binds to the sense strand, while primer 1525R, a reverse primer, binds to the antisense strand. These primers were designed based on the conserved regions of the 16S rRNA gene in *E. coli*, with 27F targeting nucleotides 8–28 and 1525R targeting nucleotides 1534–1525 [11].

The PCR amplification of the 16S rRNA gene resulted in a single DNA band at approximately 1500 bp, as shown in Figure 1B. This band corresponds to the expected size of the 16S rRNA gene, further confirming the success of the amplification process [6]. The observed amplicon size aligns with the expected length of the 16S rRNA gene, reflecting the effectiveness of the primer design and the optimized PCR conditions.

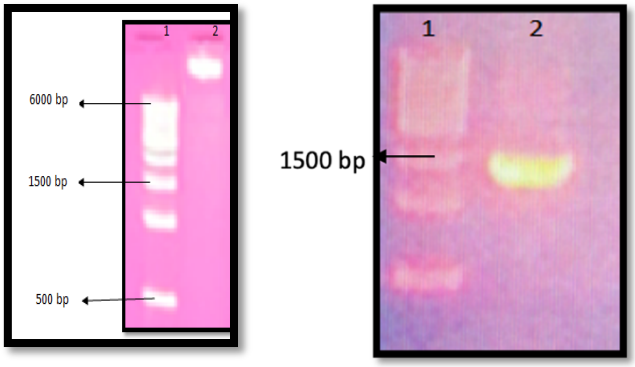

Fig. 1. A) Chromosomal DNA of Inulin-Degrading Thermophilic Bacteria (1)500bp marker DNA ladder (2) Bacterial Chromosomal DNA. B) Thermophilic Bacterial Amplicons (1) 500 bp DNA Ladder marker (2) Amplicon of the 16S rRNA gene of thermophilic bacteria

The amplicon was subsequently excised from the agarose gel and purified to remove impurities such as excess primers, nucleotides, and agarose residues. The purified amplicon had a concentration of 16 ng/μL, which was sufficient for downstream processes such as cloning and sequencing. This concentration ensures sufficient template quantity for high-fidelity downstream applications without requiring additional amplification steps. The successful amplification and purification of the 16S rRNA gene fragment provide a robust basis for further molecular characterization and phylogenetic analysis. These processes are pivotal for identifying and classifying the thermophilic bacterial isolate, contributing to a deeper understanding of its taxonomic placement and potential applications.

3.3 Cloning of the 16S rRNA Gene into pT7Blue T-Vector

The 16S rRNA gene fragment was successfully cloned into the pT7Blue T-Vector. The vector was first linearized using the Eco-RV restriction enzyme, followed by the addition of thymine (T) residues at the 3' end of the EcoRV cut site [7]. The ampicillin resistance gene enables the selection of transformants by growth on media containing ampicillin, while the lacZ gene encodes β-galactosidase, which can be used to verify successful DNA recombination. The successful cloning of the 16S rRNA gene fragment from inulin-

degrading thermophilic bacteria was confirmed by screening for blue and white colonies. Blue colonies indicated the presence of *E. coli* transformants that did not contain the inserted DNA, while white colonies corresponded to transformants that harbored the recombinant plasmid [12].

The results of the recombinant DNA screening are shown in Figure 2. Recombinant DNA was isolated from white colonies, specifically from isolate SN01, and the concentration of the purified DNA was measured at 170 ng/μL. The quality of the isolated DNA was further confirmed by agarose gel electrophoresis, which showed a distinct, high molecular weight band corresponding to the expected size of the recombinant plasmid. The purified DNA was then used for subsequent downstream applications, including sequencing to confirm the correct insertion of the 16S rRNA gene fragment into the pT7Blue T-Vector. These steps ensure the accuracy and integrity of the cloned gene for further molecular characterization.

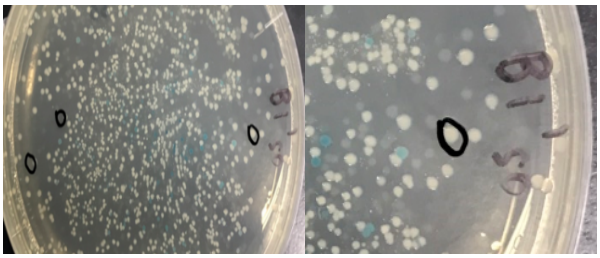

Fig. 2. Transformant Colonies of blue white screening

3.4 Analysis of the Nucleotide Base Sequence of the 16S rRNA Gene

The nucleotide base sequence of the 16S rRNA gene from thermophilic inulin-degrading bacteria Sequencing was carried out using four primers: U-19 (forward), R-20 (reverse), 518F (forward), and 518R (reverse). The U-19 and 518F primers are forward primers that bind to the sense strand of DNA, which is sequenced. The U-19 primer is located at nucleotides 50-68 in the pT7Blue T-Vector [7], while the 518F primer binds to the nucleotide region 518-538 in the E. coli 16S rRNA gene sequence. The R-20 and 518R primers are reverse primers that bind to the antisense strand of the DNA. The R-20 primer is located at nucleotides 2793-2814 in the pT7Blue T-Vector, whereas the 518R primer corresponds to nucleotides 518-538 in the E. coli 16S rRNA gene[11].

The nucleotide sequence obtained from the four primers extends to 1548 base pairs. The full nucleotide base sequence of the 16S rRNA gene from inulin-degrading thermophilic bacteria is shown in Figure 3. This sequencing analysis provides critical insights into the genetic composition of thermophilic bacteria capable of degrading inulin, a process that could have significant implications for industrial applications, particularly in the field of biotechnology and environmental sustainability. The sequence highlighted in purple corresponds to the 27F forward primer, which was used during PCR amplification, indicating the 5' end of the target 16S rRNA gene. This region is essential for the initial identification and classification of the bacterial strain. The sequence highlighted in greenish-blue corresponds to the region amplified by the 1525R reverse primer during PCR, confirming that the 16S rRNA gene of the thermophilic

bacteria starts from this nucleotide position in the reverse direction, ensuring accurate gene orientation. Additionally, the nucleotide bases in green correspond to the regions amplified by the 518F and 518R primers, providing further validation of the sequence and its accuracy. These findings not only confirm the successful cloning of the 16S rRNA gene from thermophilic inulin-degrading bacteria but also highlight the potential utility of the sequence data as a valuable resource for future phylogenetic studies and the development of targeted strategies in metabolic engineering to optimize the inulin degradation process.

```
AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG  050
CAAGTCGAGC GGACTTTCAA GAAGCTTGCT TTTTGAAAGT CAGCGGCGGA  100
CGGGTGAGTA ACACGTGGGC AACCTGCCTG CAAGACGGGG ATAACTCCGG  150
GAAACCGGGG CTAATACCGG ATAATATCTT CCTTCGCATG AAGGAAGGTT  200
GAAAGGCGGC GCAAGCTGCC GCTCGCAGAT GGGCCCGCGG CGCATTAGCT  250
AGTTGGTGAG GTAACGGCTC ACCAAGGCGA CGATGCGTAG CCGACCTGAG  300
AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA  350
GGCAGCAGTA GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG  400
CCGCGTGAGC GAAGAAGGTC TTCGGATCGT AAAGCTCTGT TGTCAGGGAA  450
GAACAAGTAC CGTTCGAACA GGGCGGTACC TTGACGGTAC CTGACCAGAA  500
AGCCACGGCT AACTACGTGC CAGCAGCCGC GGTAATACGT AGGTGGCAAG  550
CGTTGTCCGG AATTATTGGG CGTAAAGCGC GCGCAGGCGG TCTCTTAAGT  600
CTGATGTGAA AGCCCACGGC TCAACCGTGG AGGGTCATTG GAAACTGGGA  650
GACTTGAGTG CAGAAGAGGA GAGCGGAATT CCACGTGTAG CGGTGAAATG  700
CGTAGAGATG TGGAGGAACA CCAGTGGCGA AGGCGGCTCT CTGGTCTGTA  750
ACTGACGCTG AGGCGCGAAA GCGTGGGGAG CGAACAGGAT TAGATACCCT  800
GGTAGTCCAC GCCGTAAACG ATGAGTGCTA AGTGTTAGAG GGCTTCCACC  850
CTTTAGTGCT GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGGCC  900
GCAAGGCTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG  950
CATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT 1000
CCTTCGCTAC CCCTAGAGAT AGAGGGTTCC CCTTCGGGGG ACGGAGTGAC 1050
AGGTGGTGCA TGGTTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT 1100
CCCGCAACGA GCGCAACCCT TGACCTTAGT TGCCAGCATT CAGTTGGGCA 1150
CTCTAAGGTG ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA 1200
AATCATCATG CCCCTTATGA CCTGGGCTAC ACACGTGCTA CAATGGATGG 1250
TACAAAGGGT CGCGAAACCG CGAGGTGGAG CCAATCCCAA AAAACCATTC 1300
TCAGTTCGGA TTGCAGGCTG CAACTCGCCT GCATGAAGCC GGAATCGCTA 1350
GTAATCGCGG ATCAGCATGC CGCGGTGAAT ACGTTCCCGG GCCTTGTACA 1400
CACCGCCCGT CACACCACGA GAGTTTGCAA CACCCGAAGT CGGTGAGGTA 1450
ACCCTTACGG GAGCCAGCCG CCGAAGGTGG GGCAGATGAT TGGGGTGAAG 1500
TCGTAACAAG GTAGCCGTAT CGGAAGGTGC GGCTGGATCA CCTCCTTT   1548
```

Fig. 3. Nucleotide sequence of the 16S rRNA gene of thermophilic bacteria isolate SN01

3.5 Molecular Identification of Inulin-Degrading Thermophilic Bacteria

The nucleotide base sequence of the 16S rRNA gene fragment from the thermophilic bacterial isolate SN01 was compared for similarity to sequences in the GenBank database using BLASTn. The analysis revealed that the 16S rRNA gene of the thermophilic bacteria exhibited 99% sequence similarity to *Bacillus smithii*. According to taxonomic classification standards, a sequence similarity greater than 97% typically indicates that the isolated bacteria are a closely related species [12].

Phylogenetic analysis, as shown in Figure 4, revealed that the thermophilic bacterial isolate SN01 shares a close evolutionary relationship with *Bacillus smithii*, while being more distantly related to other *Bacillus* species, including *Bacillus alcalophilus, Bacillus acidicola, Bacillus firmus, Bacillus aquimaris,* and *Bacillus foraminis*. Although SN01 and *Bacillus smithii* exhibit close evolutionary similarities, potential differences in their genetic composition or metabolic pathways may contribute to distinct thermophilic behavior and substrate utilization. These unique characteristics highlight the potential of

SN01 for innovative industrial applications, particularly in processes requiring high-temperature resilience and efficient substrate conversion. Thermophilic Bacillus species are promising microorganisms for food industry applications due to their thermal stability, probiotic properties, and ability to produce beneficial metabolites such as extracellular enzymes and bacteriocins[13,14]. In this context, SN01 may represent a subspecies or unique strain of *Bacillus smithii* with distinct capabilities, including its potential for inulin degradation. To fully harness its industrial potential, further research is required to screen additional strains, elucidate its probiotic mechanisms, and establish a standardized safety evaluation system for practical applications in the food industry.

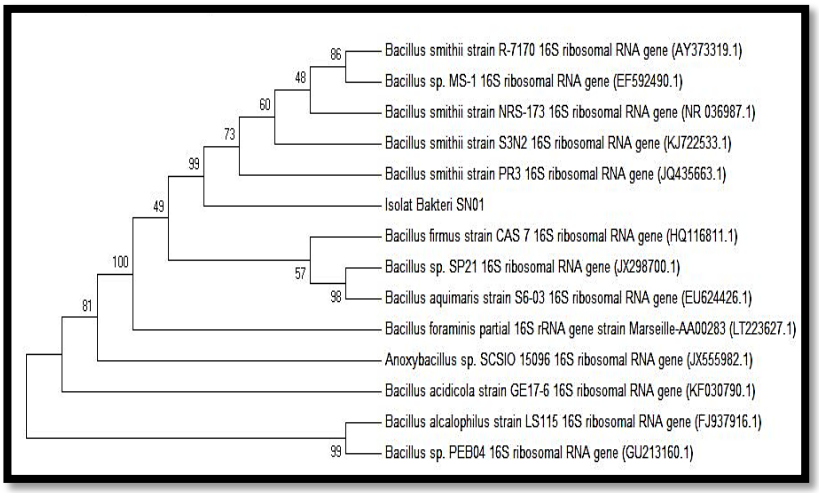

Fig. 4. Phylogenetic of Nucleotide Sequences of the 16S RNA Gene SN01

These molecular identification results not only confirm the taxonomic classification of the bacterial isolate but also provide a basis for further studies aimed at exploring the metabolic pathways involved in inulin degradation. The close relationship with *Bacillus smithii* highlights the potential for this bacterium to be utilized in biotechnological applications, including industrial processes that require high-temperature inulin hydrolysis for the production of biofuels, prebiotics, and other value-added products [13,15].

4. Conclusion

The molecular identification of the 16S rRNA gene confirms that the inulin-degrading thermophilic bacterial isolate from Sonsang hot springs belongs to the *Bacillus smithii* species, with 99% sequence similarity in the GenBank database. This finding underscores the bacterium's potential for biotechnological applications in high-temperature inulin degradation. Further exploration of its metabolic pathways could significantly enhance industrial processes requiring efficient inulin hydrolysis.

5. Acknowledgement

The author would like to express sincere gratitude to the Indonesia Endowment Fund for Education (LPDP), Ministry of Finance, Republic of Indonesia, for funding this research. Special thanks are extended to Sayaka Degura, my laboratory tutor at Gifu University,and the Biochemistry Research Team at Padang State University for their invaluable support.

References

1. R. Tatini, K. L. Kauppi, Microbiological Analyses. In *Encyclopedia of Dairy Sciences*, edited by Hubert Roginski, Elsevier, Pages 74–79, (2002). ISBN: 9780122272356. https://doi.org/10.1016/B0-12-227235-8/00021-3.
2. A. Riva, H. Rasoulimehrabani, J. M. Cruz-Rubio, et al., Identification of inulin-responsive bacteria in the gut microbiota via multi-modal activity-based sorting. *Nat. Commun.* 14, 8210 (2023). https://doi.org/10.1038/s41467-023-43448-z.
3. N. T. Porter, E. C. Martens, The critical roles of polysaccharides in gut microbial ecology and physiology. *Annu. Rev. Microbiol.* 71, 349–369 (2017).
4. H. D. Holscher, Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut Microbes* 8, 172–184 (2017).
5. M. T. Madigan, *Biology of Microorganisms*, 13th ed., (Amerika Serikat, 2011).
6. A. A. Kulminskaya, M. Arand, E. V. Eneyskaya, D. R. Ivanen, K. A. Shabalin, S. M. Shishlyannikov, A. N. Saveliev, O. S. Korneeva, K. N. Neustroev, Biochemical characterization of *Aspergillus awamori* exoinulinase: substrate binding characteristics and regioselectivity of hydrolysis. *Biochim. Biophys. Acta* 1650, 22–29 (2003).
7. Novagen, *pT7Blue-T Vector Manual*. EMD Millipore, 2016. https://www.merckmillipore.com/ID/id/product/pT7Blue-T-Vector-Novagen.
8. M. Azhar, B. Oktavia, Eksplorasi bakteri pendegradasi inulin yang potensial untuk pembuatan fruktosa dari sumber air panas Solok dan rizosfer umbi dahlia. *Laporan Penelitian*, Universitas Negeri Padang (2014).
9. L. M. Rodriguez-R, C. Jain, R. E. Conrad, S. Aluru, K. T. Konstantinidis, Reply to: "Re-evaluating the evidence for a universal genetic boundary among microbial species". *Nat. Commun.* 12, 4060 (2021).
10. B. L. Zittan, B. Denmark, Enzymatic hydrolysis of inulin — an alternative way to fructose production. *Starch/Stärke* 33, 373–377 (1981).
11. G. C. Baker, J. J. Smith, D. A. Cowan, Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods* 55, 541–555 (2003).
12. E. Stackebrandt, B. M. Goebel, Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* 44, 846–849 (2015).
13. N. Zhao, T. Yu, F. Yan, Probiotic role and application of thermophilic *Bacillus* as novel food materials. *Trends Food Sci. Technol.* 138, 1–15 (2023). https://doi.org/10.1016/j.tifs.2023.05.020.
14. W. Gao, Y. Bao, Y. Liu, X. Zhang, Characterization of thermostable endoinulinase from a new strain *Bacillus smithii* T7. *Appl. Biochem. Biotechnol.*, DOI: 10.1007/s12010-008-8313-1 (2008).
15. N. Zhao, X. Huang, Z. Liu, Y. Gao, J. Teng, T. Yu, and F. Yan, Probiotic characterization of *Bacillus smithii*: Research advances, concerns, and prospective trends. *Compr. Rev. Food Sci. Food Saf.* **23**, e13308 (2024). https://doi.org/10.1111/1541-4337.13308.