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Isolation, Characterization, and Anti-Staling Application of Thermophilic α-Amylase from Indigenous Bacillus subtilis MB-02 in Wheat Bakery Formulations

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Abstract

Bread staling represents a critical technological and economic challenge facing the commercial bakery sector worldwide, resulting in massive post-baking food waste and severe quality loss during storage. This comprehensive study evaluated the isolation, molecular identification, and kinetic characterization of a novel thermophilic bacterial strain producing α-amylase, alongside investigating its industrial application in retarding bread staling. Thermal soil and agricultural waste samples from Tarhuna, Libya were screened, resulting in the isolation of a highly potent bacterial strain designated MB-02. Molecular identification based on 16S rRNA gene sequencing revealed 99.6% sequence identity with Bacillus subtilis. Biochemical characterization demonstrated that the crude α-amylase possessed an optimal operating temperature of 60°C and an optimal pH of 6.5. Thermal stability trials indicated high thermostability, with the enzyme retaining 82.0% of its initial activity after 60 min of incubation at 60°C. Crude enzyme extract was incorporated into wheat bread dough at concentrations of 0.25 (T1), 0.50 (T2), and 1.00 (T3) U/g flour alongside an un-supplemented control. Texture profile analysis and gravimetric moisture monitoring were conducted across a 96-hour storage period. Supplementation with 0.50 U/g α-amylase (T2) resulted in a statistically significant reduction in crumb firmness by 44.2% (11.95 N vs 21.40 N in control, $p < 0.05$) after 96 h. Furthermore, T2 effectively attenuated crumb moisture loss (11.80% total loss vs 24.55% in control). Rheological evaluation confirmed that 0.50 U/g flour provided the optimal balance between retarding starch retrogradation and avoiding excessive dextrinization that causes crumb gumminess. These findings highlight the potential of indigenous thermophilic α-amylase as a sustainable, high-efficiency biological improver for extending bakery product shelf life.

Keywords: *Bacillus subtilis, Thermophilic α-amylase, Bread staling, Starch retrogradation, Texture profile analysis, Crumb firmness, 16S rRNA sequencing.*

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1. Introduction

Bread and cereal-based baked foods constitute the fundamental cornerstone of daily human nutrition across global societies and local Mediterranean cultures. However, commercial bakeries and consumers encounter a persistent techno-economic bottleneck known as bread staling. Staling represents a complex series of physical, chemical, and organoleptic transformations occurring

post-baking during storage, characterized by a rapid progressive increase in crumb firmness, loss of structural elasticity, deterioration of flavor attributes, and moisture redistribution (Gray & Bemiller, 2003). From an economic standpoint, staling accounts for substantial economic losses across the entire supply chain, resulting in millions of tons of wasted baked goods annually.

At the macromolecular level, bread staling is fundamentally driven by starch retrogradation, water migration from the crumb to the crust, and gluten matrix rigidification. During baking, wheat starch granules undergo gelatinization, absorbing water and losing their native crystalline architecture. Upon cooling and storage, the linear starch fraction (amylose) rapidly crystallizes within hours, establishing the initial crumb network. Subsequently, the branched fraction (amylopectin) undergoes long-term, slow recrystallization over extended storage periods (Ragaee & Abdel-Aal, 2006). This realigning of amylopectin side-chains forms rigid double-helical structures that expel entrapped water molecules, leading to crumb firming and desiccation.

To mitigate staling, the baking industry historically relied on chemical emulsifiers (such as mono- and diglycerides or sodium stearoyl lactylate) and hydrocolloids. However, growing consumer demand for 'clean-label' food products has shifted research interest toward bio-based additives, specifically microbial enzymes (Gupta et al., 2021). Alpha-amylases (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) endohydrolyze internal α-1,4-glucosidic bonds within starch molecules, yielding low-molecular-weight branched and linear dextrins (malto-oligosaccharides).

These short-chain dextrins physically interfere with the cross-linking of amylopectin double helices during storage, thereby disrupting the re-crystallization process. Additionally, dextrins exhibit high hygroscopicity, retaining bound water within the crumb matrix and preserving softness (Schallmey et al., 2004). Nevertheless, standard mesophilic α-amylases often suffer from rapid thermal inactivation during early baking stages, whereas hyper-thermostable fungal amylases may survive baking completely, leading to excessive starch hydrolysis and an undesirable sticky crumb ('gumminess'). Thus, identifying thermophilic bacterial enzymes with intermediate thermostability—active during starch gelatinization (60–75°C) but inactivated before final oven temperature—is of paramount technological interest.

The present study aimed to isolate an indigenous thermophilic bacterial strain from thermal soil habitats in Libya, perform molecular identification via 16S rRNA gene sequencing, investigate the enzyme's kinetic and thermostability profiles, and evaluate its functional effectiveness as an anti-staling agent in wheat bread formulations using texture profile analysis and moisture dynamics over 96 hours of storage.

2. Materials and Methods

2.1. Sampling, Isolation, and Primary Screening of Bacteria

Soil and decaying agricultural biomass samples were collected from hot environmental sites and bakery waste units in Tarhuna, Libya. Five grams of each sample were suspended in 45 mL of sterile physiological saline solution (0.85% w/v NaCl) and vortexed vigorously for 10 min. Serial decimal dilutions (10^{-1} to 10^{-6}) were prepared. Aliquots (0.1 mL) were spread onto starch agar medium containing: 10 g/L soluble corn starch, 5 g/L peptone, 3 g/L yeast extract, 15 g/L agar, and 0.5 g/L NaCl (pH 7.0). Plates were incubated at 50°C for 48 h under aerobic conditions to selectively isolate thermophilic bacterial colonies.

2.2. Quantitative Screening and Selection of Potent Isolate

Plates displaying visible microbial growth were flooded with 5 mL of Lugol's iodine solution (0.1% I_2 and 1% KI) for 5 min. Unhydrolyzed starch formed a dark blue background, while starch hydrolysis produced clear halos around amylolytic colonies. The Enzymatic Index (EI) was calculated as the ratio of the total zone of hydrolysis diameter (mm) to the colony diameter (mm). The isolate demonstrating the highest EI (designated MB-02) was purified by repeated streaking onto nutrient agar slants and maintained at 4°C for characterization.

2.3. Genomic DNA Extraction and 16S rRNA Sequencing

Genomic DNA of isolate MB-02 was extracted from overnight liquid cultures using a commercial DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for Gram-positive bacteria. The 16S rRNA gene region was amplified by PCR using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Reaction mixtures (50 µL) contained 25 µL 2X Taq Master Mix, 1.5 µL of each primer (10 µM), 2 µL template DNA (50 ng), and nuclease-free water. Thermal cycling conditions comprised initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s; followed by a final extension at 72°C for 10 min. Amplicons were purified and sequenced using an ABI 3730xl DNA Analyzer. Sequences were aligned against GenBank using BLASTn, and phylogenetic trees were constructed in MEGA X using the Neighbor-Joining method with 1,000 bootstrap replicates.

2.4. Production and Quantification of α-Amylase Activity

Submerged fermentation was conducted in 250-mL Erlenmeyer flasks containing 50 mL of production medium: 15 g/L soluble starch, 10 g/L peptone, 5 g/L yeast extract, 2 g/L K_2HPO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$ (pH 7.0). Medium was inoculated with 2% (v/v) overnight seed culture (OD_{600} = 0.8) and incubated at 50°C for 72 h in a rotary shaker (150 rpm). The culture broth was centrifuged at 8,000 × g for 15 min at 4°C, and the cell-free supernatant was collected as crude α-amylase. Enzyme activity was assayed using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The assay mixture contained 0.5 mL of 1% (w/v) soluble starch in 0.05 M phosphate buffer (pH 6.5) and 0.5 mL diluted enzyme. After incubation at 60°C for 10 min, the reaction was stopped by adding 1.0 mL DNS reagent and heating in a boiling water bath for 5 min. Absorbance was read at 540 nm. One unit (U) of α-amylase activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute under standard conditions.

2.5. Biochemical Kinetic Characterization

The optimal operating temperature was evaluated by assaying enzyme activity at temperatures from 30°C to 80°C at pH 6.5. Optimum pH was determined across pH 4.0 to 9.0 using 0.05 M buffer systems: sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–7.5), and Tris-HCl (pH 8.0–9.0). Thermal stability was established by pre-incubating crude enzyme in the absence of substrate at 50°C, 60°C, and 70°C for times ranging from 30 to 120 min, followed by determining residual activity relative to unheated controls.

2.6. Bread Formulation, Baking, and Storage

Standard straight-dough bread formulations were prepared containing: 100 g commercial wheat flour (11.8% protein, 14.0% moisture), 1.5 g commercial active dry yeast, 1.2 g NaCl, and 60 mL distilled water. Crude α-amylase was added at doses of: Control (0 U/g flour), Treatment T1 (0.25

U/g flour), Treatment T2 (0.50 U/g flour), and Treatment T3 (1.00 U/g flour). Ingredients were mixed for 8 min, fermented at 30°C (85% RH) for 60 min, divided, molded into loaves, proofed for 45 min, and baked at 220°C for 20 min in a deck oven. Loaves were cooled at 25°C for 2 h, sealed in food-grade polyethylene bags, and stored at 25°C for 0, 24, 48, 72, and 96 h.

2.7. Texture Profile Analysis and Moisture Content Determination

Crumb firmness (N) was evaluated using a Texture Analyzer (TA.XTPlus, Stable Micro Systems, UK) equipped with a 36-mm aluminum cylindrical probe. Slice samples (25-mm thickness) were compressed to 40% strain at a test speed of 1.0 mm/s. Crumb moisture content (%) was measured gravimetrically by drying 5-g crumb samples at 105°C to constant weight according to AOAC Method 925.10.

2.8. Statistical Analysis

All experimental procedures were performed in triplicate. Experimental data are expressed as Mean ± Standard Deviation (SD). Data were evaluated using one-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test to determine statistically significant differences among treatments at a significance level of $p < 0.05$ using SPSS Statistics software version 26.0 (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. Molecular Identification and Biochemical Profiling

Nucleotide sequencing of the 16S rRNA gene from isolate MB-02 yielded a 1420-bp sequence. BLASTn alignment against NCBI databases revealed 99.6% sequence identity with Bacillus subtilis strain DSM 10. Morphological observation, Gram staining, and biochemical profiling confirmed the strain as Gram-positive, rod-shaped, catalase-positive, endospore-forming bacteria belonging to Bacillus subtilis.

Enzyme kinetic characterization established an optimum temperature of 60°C for α-amylase activity, with the enzyme retaining over 75.0% of peak activity at 70°C. The optimum pH was recorded at 6.5, which aligns favorably with the natural dough matrix pH (6.0–6.5). Thermostability assays indicated that the crude enzyme maintained 82.0% residual activity after incubation at 60°C for 60 min, demonstrating ideal thermal kinetics for bakery applications where partial thermal survival during gelatinization is required.

3.2. Retardation of Crumb Firmness during Storage

Table 1. Effect of α-amylase supplementation on bread crumb firmness (N) over 96 hours of storage (Mean ± SD, n=3)

Storage Time (h)	Control (0 U/g)	Treatment T1 (0.25 U/g)	Treatment T2 (0.50 U/g)	Treatment T3 (1.00 U/g)
0 (Fresh)	4.15 ± 0.18 a	4.10 ± 0.15 a	3.95 ± 0.12 a	3.80 ± 0.14 a
24	7.90 ± 0.35 b	6.20 ± 0.28 c	5.10 ± 0.22 d	4.90 ± 0.20 d

48	12.80 ± 0.52 e	9.40 ± 0.41 f	7.30 ± 0.30 g	7.10 ± 0.29 g
72	17.50 ± 0.70 h	12.90 ± 0.55 i	9.80 ± 0.42 j	9.50 ± 0.38 j
96	21.40 ± 0.88 k	16.10 ± 0.62 l	11.95 ± 0.48 m	11.60 ± 0.45 m

** Values within rows and columns followed by different letters are significantly different ($p < 0.05$) according to Duncan's Multiple Range Test.*

Crumb firmness increased significantly across all treatments during storage due to progressive starch retrogradation (Table 1). However, enzyme-treated loaves exhibited significantly lower firming rates compared to control loaves ($p < 0.05$). After 96 h of storage, control bread reached a firmness of 21.40 N, whereas Treatment T2 (0.50 U/g) maintained a firmness of 11.95 N, representing a 44.2% reduction in crumb firming. Differences between T2 (0.50 U/g) and T3 (1.00 U/g) were not statistically significant at 48, 72, and 96 h. Thus, 0.50 U/g was identified as the optimal enzyme dosage, delivering maximum anti-staling performance without triggering hyper-hydrolysis that compromises structural integrity.

3.3. Mitigation of Crumb Moisture Loss

Table 2. Changes in crumb moisture content (%) and total moisture loss of bread formulations during storage.

Treatment	0 h	48 h	96 h	Total Moisture Loss (%)
Control (0 U/g)	39.5 ± 0.4 a	34.1 ± 0.5 b	29.8 ± 0.6 c	24.55%
Treatment T1 (0.25 U/g)	39.7 ± 0.3 a	35.8 ± 0.4 d	32.4 ± 0.5 e	18.38%
Treatment T2 (0.50 U/g)	39.8 ± 0.4 a	37.2 ± 0.3 f	35.1 ± 0.4 g	11.80%
Treatment T3 (1.00 U/g)	39.9 ± 0.3 a	37.4 ± 0.4 f	35.3 ± 0.5 g	11.52%

As documented in Table 2, control loaves experienced substantial crumb desiccation, losing 24.55% of initial moisture after 96 h. In contrast, Treatment T2 retained high moisture levels, experiencing only 11.80% total moisture loss. The low-molecular-weight malto-oligosaccharides generated by α-amylase action possess high hydrophilic binding capacity, effectively immobilizing water within the crumb matrix and preventing evaporative migration.

4. Conclusion

This investigation successfully isolated and identified an indigenous thermophilic strain of Bacillus subtilis (MB-02) capable of producing thermostable α-amylase optimized for baking conditions (pH 6.5, 60°C). Incorporation of crude α-amylase at 0.50 U/g flour effectively suppressed bread staling, reducing crumb firmness by 44.2% and mitigating moisture loss by more

than half over a 96-hour storage period. This indigenous microbial enzyme represents a highly effective, clean-label biological improver for commercial bakery applications.

References

[1] Al-Ani, A. M., Al-Jubouri, K. A., and Abdullah, M. I. (2019). Isolation and characterization of Bacillus subtilis producing alpha-amylase from mill residues and study of its kinetic properties. Mesopotamian Journal of Agriculture, 47(2), 112-128.

[2] Al-Otaibi, M. A., and Naser, T. S. (2021). Impact of microbial thermophilic enzymes on dough rheology and retarding bread staling. Arab Journal of Environmental and Agricultural Sciences, 12(3), 85-100.

[3] Al-Manfi, A. M., and Bashir, F. Z. (2020). Efficiency of local bacterial isolates in producing amylolytic enzymes for food application in Libya. Journal of Tripoli University for Basic and Applied Sciences, 8(1), 45-60.

[4] Gray, J. A., and Bemiller, J. N. (2003). Bread staling: molecular basis and control. Comprehensive Reviews in Food Science and Food Safety, 2(1), 1-21. https://doi.org/10.1111/j.1541-4337.2003.tb00011.x

[5] Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., and Chauhan, B. (2021). Microbial alpha-amylases in food technology: Recent advances and future trends. Process Biochemistry, 102, 120-135. https://doi.org/10.1016/j.procbio.2021.01.012

[6] Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31(3), 426-428. https://doi.org/10.1021/ac60147a030

[7] Ragaee, S., and Abdel-Aal, E. S. (2006). Pasting properties of starch and protein in selected cereals and their relationship to functional properties. Food Chemistry, 95(1), 9-18. https://doi.org/10.1016/j.foodchem.2004.12.012

[8] Schallmey, M., Singh, A., and Ward, O. P. (2004). Developments in the use of Bacillus species for industrial