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Structural and functional insights into AmyHa: a haloadapted α -amylase from *Haloarcula argentinensis* S3 optimized via response surface methodology

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25 **Abbreviations:**

26 AI: Aliphatic Index

27 ANOVA: Analysis of Variance

28 BBD: Box-Behnken Design

29 BLAST: Basic Local Alignment Search Tool

30 CFS: Cell-Free Supernatant

31 DNS: 3,5-Dinitrosalicylic Acid

32 GMQE: Global Model Quality Estimate

33 GRAVY: Grand Average of Hydropathicity

34 II: Instability Index

35 MW: Molecular Weight

36 NCBI: National Center for Biotechnology Information

37 PBD: Plackett-Burman Design

38 PCR: Polymerase Chain Reaction

39 pI: Isoelectric Point

40 QMEAN: Quantitative Model Energy Analysis

41 RSM: Response Surface Methodology

42 16S rRNA: 16S Ribosomal RNA

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44

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Abstract

The development of robust biocatalysts capable of maintaining structural integrity and catalytic efficiency under high salinity and acidic conditions remains a major challenge in enzyme engineering and industrial starch saccharification. In this study, we combined statistical bioprocess optimization with extensive in silico structural characterization to enhance the production and elucidate the haloadaptive features of an extracellular α -amylase (*AmyHa*) from the extreme haloarchaeon *Haloarcula argentinensis* strain S3. Media screening using a Plackett–Burman design identified NaCl concentration, soluble starch concentration, and medium pH as the primary factors governing enzyme yield. Subsequent optimization using a Box–Behnken response surface design identified a significant interaction between salinity and pH, indicating that high ionic strength supports enzyme synthesis under acidic conditions. The optimized culture conditions (250 g/L NaCl, 6 g/L soluble starch, and medium adjusted to pH 5.0) yielded a maximum experimental activity of 274.1 ± 1.8 U/mL, representing a 1.84-fold enhancement over the baseline. Primary sequence analysis of the *AmyHa* protein showed a highly acidic theoretical isoelectric point (pI) of 4.21, attributed to a high proportion of aspartic and glutamic acid residues (18.56%). Topological analyses indicated the absence of canonical N-terminal signal peptides and transmembrane domains, suggesting a potentially non-classical translocation pathway or an alternative initiation sequence. Homology modeling and stereochemical validation confirmed a well-defined tertiary architecture featuring a catalytic core (β/α)₈ TIM-barrel domain flanked by domains B and C. Molecular docking simulations indicated strong binding affinity towards branched starch fragments ($\Delta G = -10.0$ kcal/mol), mediated by an intricate hydrogen-bonding network surrounding the conserved catalytic triad (Asp197, Glu233, and Asp300). Overall, these findings demonstrate that *AmyHa* possesses key structural adaptations for extreme environments, offering strong potential for specialized industrial bioprocesses.

Keywords: Halophilic α -amylase; *Haloarcula argentinensis*; Protein structure; Homology modeling; Molecular docking; Haloadaptation

Introduction

Extremely halophilic archaea (haloarchaea) represent the dominant microbial populations in hypersaline ecosystems [1]. These microorganisms require salt concentrations between 20% and 30% NaCl for optimal growth [1]. To maintain cellular integrity and function under severe osmotic pressure, haloarchaea deploy specialized physiological mechanisms, including the intracellular accumulation of potassium ions ("salt-in" strategy) and the synthesis or uptake of organic osmolytes ("compatible solute" strategy) [2]. Consequently, haloarchaea, particularly members of the genus *Haloarcula*, serve as valuable sources of novel biocatalysts [3]. Enzymes derived from these extreme halophiles, termed halozymes, maintain their structural stability and catalytic activity under high ionic strength, making them robust candidates for harsh industrial applications [4]. Among these biocatalysts, α -amylase (EC 3.2.1.1; 1,4- α -D-glucan glucanohydrolase) is an endo-acting glycoside hydrolase that randomly cleaves internal α -1,4-glucosidic linkages in starch and related polysaccharides to produce maltose, maltotriose, and limit dextrins [5, 6]. Halophilic α -amylases exhibit enhanced stability at elevated salt concentrations, offering significant utility in industrial processes such as starch liquefaction, bioethanol synthesis, and detergent formulations [7]. At the molecular level, haloarchaeal α -amylases possess distinct evolutionary adaptations to hypersalinity, most notably a high proportion of acidic surface residues that coordinate a protective hydration shell [8]. Modern computational biochemistry and protein modeling tools facilitate the structural characterization of these enzymes, enabling detailed mapping of catalytic domains, surface charge distribution, and substrate-binding dynamics [9]. Although several haloarchaeal α -amylases have been characterized experimentally including those from *Haloarcula hispanica* [10], *Haloterrigena turkmenica* [11] and *Haloferax* sp. [12] exploring novel isolates from geographically isolated hypersaline environments remains essential to uncover novel structural solutions for salt-dependent stabilization. In this context, the extreme haloarchaeon *Haloarcula argentinensis* strain S3, isolated from the hypersaline ecosystem of Wadi Elmalah (Medea, Algeria), serves as a compelling model organism [3]. This strain grows optimally in media containing 25% NaCl and efficiently degrades starch as a primary carbon source. The objective of this study was to optimize the cultivation parameters for extracellular α -amylase production by *H. argentinensis* S3 using Response Surface Methodology (RSM) with Plackett–Burman and Box–Behnken experimental designs [13, 14]. Furthermore, we conducted an extensive in silico structural and functional characterization of the mature AmyHa protein. By combining secondary and tertiary structure predictions, stereochemical validation, and molecular docking simulations, this work aims to elucidate the structural

105 mechanisms driving haloadaptation and substrate specificity in glycoside hydrolases from unique North
106 African biotopes.

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108 **2 Materials and Methods**

109 **2.1 Archaeal Strain and Growth Conditions**

110 The extremely halophilic archaeon *Haloarcula argentinensis* strain S3 used in this study was previously
111 isolated from Wadi Elmalah (Medea, Algeria) [3]. The strain was revived and maintained in modified
112 Halophilic Medium (SH medium) composed of (g/L): NaCl, 160; MgCl₂ 6H₂O, 160; CaCl₂ 2H₂O, 0.1;
113 K₂SO₄, 5; soluble starch, 2, yeast extract, 1 and peptone, 1. The pH of the medium was adjusted to 7.0
114 M NaOH prior to autoclaving. Cultivation was performed in 250 mL Erlenmeyer flasks containing 50 mL
115 of medium. Flasks were inoculated with a 10% (v/v) active seed culture and incubated at 40 °C for 8 days
116 under continuous agitation at 150 rpm. Archaeal growth was monitored spectrophotometrically by
117 measuring optical density at 600 nm (OD₆₀₀) against a sterile medium blank.

118 **2.2 Phenotypic, Biochemical, and Physiological Characterization**

119 Morphological and biochemical assays were conducted according to standard protocols for halophilic
120 microorganisms [15]. Colony morphology, including shape and pigmentation, was examined after 8 days
121 of incubation at 40 °C on solid SH medium containing 2% (w/v) agar. Cell morphology and motility were
122 observed using phase contrast microscopy of exponential-phase liquid cultures. Gram staining was
123 performed using a modified protocol optimized for halophilic cell walls to prevent osmotic cell lysis [16].
124 Oxidase and catalase activities were evaluated using standard techniques. β-Galactosidase activity was
125 assessed via ortho-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis. Physiological growth
126 boundaries were determined under varying pH, temperature, and salinity parameters. The pH range was
127 evaluated from pH 5.0 to 9.0. Temperature optimization was conducted at 30 °C, 35 °C, 40 °C, 45 °C, and
128 50 °C. Salinity requirement and tolerance were investigated by supplementing liquid SH medium with
129 NaCl concentrations ranging from 10% to 30% (w/v). All physiological assays were performed in
130 triplicate, and growth was quantified at (OD₆₀₀).

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2.3 Screening for Extracellular Hydrolytic Enzyme Activities

Extracellular enzymatic screening of strain S3 was performed qualitatively on solid SH agar plates under optimal growth conditions. Substrate degradation was detected by clear zone formation surrounding colonies. For α -amylase detection, plates were supplemented with 0.2% (w/v) soluble starch. After 8 days of incubation, plates were flooded with Gram's iodine solution, identifying starch hydrolysis by distinct clear halos against a dark blue background [17]. Protease (caseinase) activity was evaluated using SH agar enriched with 1% (w/v) skim milk casein, where clear zones indicated localized substrate breakdown [18]. Cellulase, xylanase, mannanase, and pectinase activities were assessed by supplementing the medium with 1% (w/v) carboxymethylcellulose, 0.5% (w/v) xylan, 0.5% (w/v) guar gum, or 1% (w/v) pectin, respectively. Active enzyme expression was visualized by flooding plates with a 0.1% (w/v) Congo red solution for 15 min, followed by destaining with 1 M NaCl [19, 20, 21].

2.4 Quantitative Determination of α -Amylase Activity

Submerged fermentation was conducted in modified SH medium supplemented with 1.0% (w/v) soluble starch. Following 8 days of cultivation at 40 °C and 150 rpm, cultures were centrifuged at 10,000 x g for 20 min at 4 °C. The resulting cell-free supernatant (CFS) served as the crude enzyme extract. The quantitative reaction mixture contained 500 μ L of 1.0% (w/v) soluble starch dissolved in 50 mM sodium phosphate buffer (pH 7.0, supplemented with 200 g/L NaCl) and 500 μ L of appropriately diluted crude enzyme extract. The mixture was incubated at 45 °C for exactly 10 min. The reaction was stopped immediately by adding 1.0 mL of 3,5-dinitrosalicylic acid (DNS) reagent [22]. The tubes were incubated in a boiling water bath for 5 min, rapidly cooled on ice, and the absorbance was measured at 540 nm against a substrate free blank. The concentration of released reducing sugars was calculated using a D-glucose standard curve. Enzyme Unit Definition: One unit (U) of α -amylase activity was defined as the amount of enzyme required to liberate 1 μ mol of reducing sugar equivalents (expressed as D-glucose) per minute under the specified assay conditions.

2.5 Molecular identification and phylogenetic analysis

Genomic DNA extraction and purification from strain S3 were performed using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) using the archaeal-specific forward primer 21F (5'-TTCCGGTTGATCCTGCCGGA-3') and the universal prokaryotic reverse primer RP2

(5'-ACGGCTACCTTGTTACGACTT-3') [23]. The PCR mixture (50 μ L) contained 300 ng of template genomic DNA, 10 pmol of each primer, and 2 U of GoTaq DNA Polymerase with 10 X Taq buffer and 10 mM dNTP mix (Promega, USA). Amplification was executed in a PTC-200 thermocycler (MJ Research, Waltham, MA, USA) under the following conditions: an initial denaturation step at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 10 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension step at 72 °C for 10 min. The resulting PCR products were resolved on a 1% (w/v) agarose gel stained with ethidium bromide, visualized under ultraviolet light, and subsequently purified and sequenced on an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained 16S rRNA gene sequence was subjected to a similarity search against the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLASTn) to identify closely related taxonomic neighbors. Multiple sequence alignments were performed using the ClustalW algorithm in MEGA 12 software. Phylogenetic trees were reconstructed using both the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods with bootstrap analysis based on 1,000 replicates to evaluate the structural robustness of the tree topology. *Methanospirillum hungatei* served as the outgroup.

2.6 PCR Amplification and Sequencing of The α -Amylase Gene (*AmyHa*)

The α -amylase gene (*AmyHa*) from *Haloarcula argentinensis* strain S3 was amplified by PCR using specific oligonucleotide primers designed against conserved homologous loci of haloarchaeal glycoside hydrolases (GenBank accession no. AJ890146) [3]: forward primer 5'-CTTCCACCCGAAAGACGACA-3' and reverse primer 5'-AACGGTGATCTGGACCCAAC-3'. Thermal cycling conditions were set as follows: an initial denaturation at 95 °C for 3 min; 25 cycles of denaturation at 95 °C for 45 s, primer annealing at 51 °C for 45 s, and extension at 72 °C for 1 min; followed by a final extension step at 72 °C for 10 min. The specific PCR amplicon was resolved by 1% (w/v) agarose gel electrophoresis, purified using the Geneaid Gel Extraction Kit, and sequenced in both forward and reverse directions on an ABI PRISM 3100-Avant Genetic Analyzer [3]. Sequence assembly, contig alignment, and consensus generation were executed using BioEdit software (version 7.2.5), and the finalized nucleotide sequence was deposited in the NCBI GenBank database

2.7 *In silico* Protein Characterization and Sequence Analysis

The nucleotide sequence of *AmyHa* was translated using the ExPASy Translate tool, and homology searches were executed via BLASTp. Multiple sequence alignments with reference archaeal α -amylases were conducted using ClustalW, and an evolutionary tree was built in MEGA 12 using the Maximum Likelihood method based on the JTT matrix model with 1,000 bootstrap replicates. Physicochemical properties including molecular weight (M_w), theoretical isoelectric point (pI), amino acid composition, instability index (II), aliphatic index (AI), molar extinction coefficient, and grand average of hydropathicity (GRAVY) were computed *via* the ExPASy ProtParam server [28, 29]. Secondary structure elements were predicted using SOPMA and PSIPRED v4.0. The presence of N-terminal signal peptides and transmembrane domains was evaluated using SignalP 6.0, DeepTMHMM-1.0, and TOPCONS web servers.

2.8 Homology Modeling, Quality Validation, and Molecular Docking

Three-dimensional homology modeling of the mature *AmyHa* tertiary structure was reconstructed using the ExPASy SWISS-MODEL workspace [57], selecting the crystallographic coordinates of α -amylase from *Haloarcula japonica* (PDB ID: 7V53; 98.4% sequence identity) as the template [32]. Model quality was evaluated based on Global Model Quality Estimate (GMQE) scores [57]. Geometric properties, atomic energy parameters, and structural reliability were assessed using the ExPASy QMEAN scoring server [35]. Backbone dihedral angles (ϕ, ψ) were validated through Ramachandran plot analysis using the PROCHECK suite on the SAVES v6.1 online server. Active-site mapping and functional domain annotations were performed using I-TASSER [9], NCBI CD-search against the Conserved Domain Database [31], and InterPro web utilities [30]. Three-dimensional structural visualizations and molecular electrostatic surface displays were generated using PyMOL software. Molecular docking simulations were executed using the AutoDock Vina v1.5.7 software package [24]. Polar hydrogen atoms were added to the macromolecular receptor model, and Gasteiger charges were assigned [24]. Ligand structures representing malto-oligosaccharide models were retrieved from the PubChem database, comprising a branched amylopectin fragment (CID: 439207), linear α -maltotetraose (CID: 446495), an amylopectin core fragment (CID: 439341), a branched glycogen core model (CID: 439177), and a linear amylose fragment (CID: 21239054). The

docking grid box dimensions were set to 126 X 126 X 126 Å, centered at spatial x = 2.8, y = 7.6, and z = 0.5, fully encompassing the predicted $(\beta/\alpha)_8$ TIM-barrel catalytic cleft [24, 32]. Bound ligand conformations were ranked based on the lowest calculated binding free energy (ΔG , kcal/mol) [24].

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2.9 Experimental Design and Statistical Optimization

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Screening of critical culture variables was executed using a 12-run Plackett–Burman Design (PBD) evaluating eleven operational factors at two levels (-1 and +1) [14]. Variables showing statistically significant effects ($p < 0.05$) were selected for optimization via a Box–Behnken Design (BBD) under Response Surface Methodology (RSM) across 17 experimental runs at three levels (-1, 0, +1) [13]. Experimental data were fitted to a second-order polynomial equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

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where Y is the predicted enzyme activity, β_0 is the model intercept, β_i linear coefficients, β_{ii} quadratic coefficients, β_{ij} interaction coefficients, and X_i , X_j coded independent variables. Design-Expert version 13 software was used for experimental layout, Analysis of Variance (ANOVA), and 3D response surface generation. Model validity was evaluated via determination coefficients (R^2 , adjusted R^2 , predicted R^2) and Fisher's F -test.

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3 Results

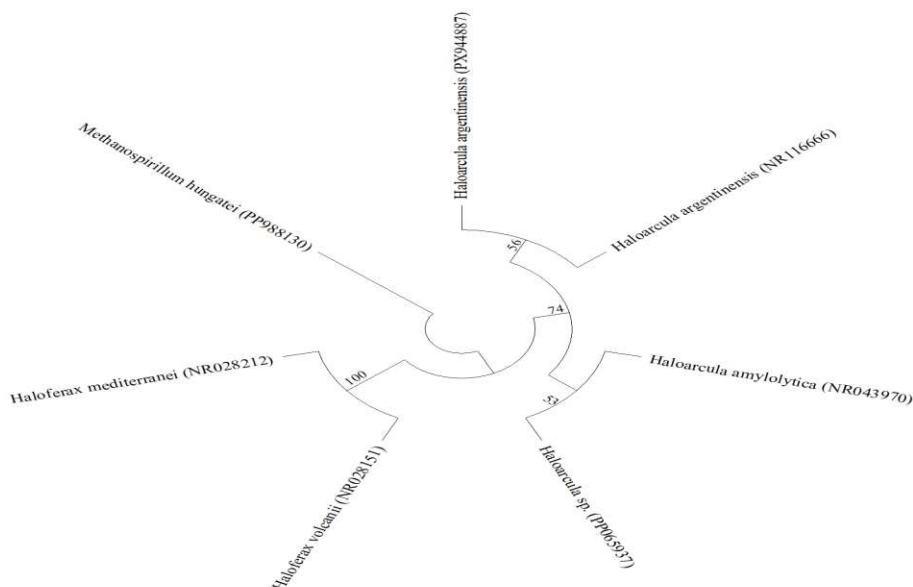
3.1 Phenotypic, Biochemical, and Physiological Profiling of Strain S3

The extremely halophilic archaeon strain S3, isolated from the hypersaline biotope of Wadi Elmalah in Medea, Algeria, displayed classical phenotypic and biochemical traits characteristic of the family Halobacteriaceae [3]. Colonies grown on solid SH agar plates at 40 °C for 8 days were small, circular, and smooth, showing a prominent pink-to-red pigmentation indicating active C50 carotenoid biosynthesis. Microscopic examination showed pleomorphic, non-motile, Gram-negative cells. Biochemical testing confirmed positive activities for both oxidase and catalase, along with active β -galactosidase expression

252 verified through o-nitrophenyl- β -D-galactopyranoside (ONPG) hydrolysis. Physiological profiling
 253 demonstrated an obligate requirement for high ionic strength: strain S3 failed to grow at salinities below
 254 15% (w/v) NaCl. Optimal growth occurred between 20% and 25% (w/v) NaCl. Temperature experiments
 255 characterized strain S3 as a mesophilic-to-moderately thermophilic isolate, capable of growing between
 256 30 °C and 50 °C with peak growth at 40 °C [43]. Additionally, the strain tolerated a wide pH span (5.0 to
 257 9.0) with an optimum at pH 6.0

258 3.2 Taxonomic Identification and Phylogenetic Analysis

259 Sequencing of the nearly full-length 16S rRNA gene (approximately 1,300 bp) definitively resolved the
 260 taxonomic identity of strain S3. Comparative BLASTn searches supported the isolate belongs to the
 261 domain Archaea, clustering strictly within the family Halobacteriaceae and the genus *Haloarcula*. The
 262 sequence shared 99.25% identity with the validated type strain *Haloarcula argentinensis* HST01-
 263 2R^T(NR_116666). We officially deposited the sequence in the NCBI GenBank database under accession
 264 number PX944887. Phylogenetic trees reconstructed using both Neighbor-Joining and Maximum
 265 Likelihood algorithms yielded congruent topologies. Strain S3 consistently fell within a strongly
 266 supported monophyletic clade alongside recognized *Haloarcula argentinensis* species, supported by high
 267 bootstrap values across 1,000 pseudoreplicates. This topological arrangement distinguishes strain S3 from
 268 related haloarchaeal taxa, including *Haloarcula amylolytica* and *Haloferax mediterranei*, with
 269 *Methanospirillum hungatei* anchoring the tree as the outgroup (Fig. 1).



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Fig. 1 Phylogenetic tree of the archaeal strain S3 constructed using MEGA 12 depending on 16S rRNA gene sequences. Strain code and the accession number are showed on the tree.

3.3 Hydrolytic Screening and Baseline Enzyme Production

Qualitative plate assays demonstrated that *Haloarcula argentinensis* S3 possesses a versatile extracellular enzymatic repertoire. Amylolytic activity was predominant (+++), producing wide starch hydrolysis halos on agar. The strain also exhibited moderate caseinase, pectinase, and xylanase activities (++) , alongside low levels (+) of cellulase and mannanase (Table 1). Under unoptimized quantitative cultivation (160 g/L NaCl, 2 g/L soluble starch, pH 7.0 at 45 °C), strain S3 reached an extracellular α -amylase yield of 150.02 U/mL after 5 days. However, kinetic monitoring revealed early substrate depletion at 2 g/L starch, leading to a premature plateau in enzyme activity.

Table 1 Qualitative screening of extracellular hydrolytic enzyme production by *Haloarcula argentinensis* strain S3.

Strain	α -Amylase	Cellulase	Caseinase	Pectinase	Xylanase	Mannanase
S3	+++	+	++	++	++	+

(–, no activity; +, low activity; ++, moderate activity; +++, high activity)

3.4 Screening Critical Variables via Plackett–Burman Design

We used a 12-run Plackett–Burman Design (PBD) to evaluate eleven operational parameters and isolate the primary factors governing enzyme synthesis. Extracellular α -amylase activity varied widely across runs, ranging from 139.00 U/mL (*Run 11*) to 274.00 U/mL (*Run 6*) (Table 2). Linear regression demonstrated that the screening model was markedly significant ($p = 0.0094$) with an acceptable determination coefficient ($R^2 = 0.74$). Analysis of Variance (ANOVA) demonstrated that out of eleven

296 factors, only medium pH (*C*), NaCl concentration (*D*), and soluble starch concentration (*G*) significantly
 297 affected enzyme yield ($p < 0.05$) (Table 3). The Pareto diagram designated medium pH as the single most
 298 influential variable, followed directly by salinity and starch concentration (Fig. 2).

299 **Table 2** Plackett–Burman experimental design matrix for screening medium factors

Runs	A	B	C	D	E	F	G	H	I	J	K	Enzymatic Activity (U/ml)*
1	1	-1	1	1	1	-1	-1	-1	1	-1	1	187.29
2	1	-1	-1	-1	1	-1	1	1	-1	1	1	229.24
3	1	1	1	-1	-1	-1	1	-1	1	1	-1	196.11
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	168.84
5	1	-1	1	1	-1	1	1	1	-1	-1	-1	238.063
6	-1	-1	-1	1	-1	1	1	-1	1	1	1	274.00
7	-1	1	-1	1	1	-1	1	1	1	-1	-1	266.82
8	-1	1	1	-1	1	1	1	-1	-1	-1	1	149.59
9	1	1	-1	1	1	1	-1	-1	-1	1	-1	250.21
10	1	1	-1	-1	-1	1	-1	1	1	-1	1	193.753
11	-1	1	1	1	-1	-1	-1	1	-1	1	1	139.00
12	-1	-1	1	-1	1	1	-1	1	1	1	-1	179.42

300 * Values represent means of three replicates.

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302 **Table 3** ANOVA evaluation for the Plackett–Burman screening model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	16524.78	3	5508.26	7.76	0.0094	(sig.)
C-pH	7173.14	1	7173.14	10.11	0.0130	
D-NaCl	4737.41	1	4737.41	6.68	0.0324	
G-Starch	4614.23	1	4614.23	6.50	0.0342	
Residual	5677.26	8	709.66			
Cor Total	22202.04	11				

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Enzymatic activity

A: Temperature
B: Inoculum size
C: pH
D: NaCl
E: CaCl₂
F: MgCl₂
G: Starch
H: Corn starch
J: Yeast extract
K: Tryptone
L: Time

Positive Effects
Negative Effects

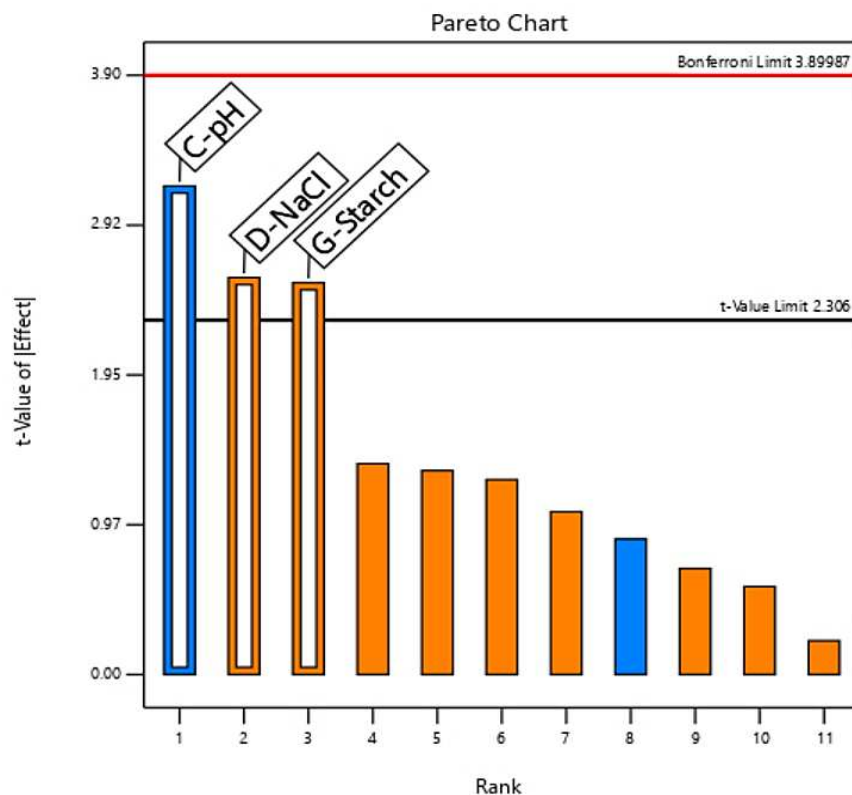


Fig. 2 Pareto Diagram presenting significant factors influencing α -amylase production based on PBD results.

3.5 Response Surface Optimization and Empirical Validation

To map parameter interactions and locate the optimum, we deployed a 17-run Box–Behnken Design (BBD) incorporating five center points. Extracellular α -amylase activity varied from 126.83 U/mL (*Run* 9: 300 g/L NaCl, 6 g/L starch, pH 6.0) to 270.88 U/mL (*Run* 2: 300 g/L NaCl, 6 g/L starch, pH 4.0) (Table 4). Multiple regression analysis yielded the following second-order polynomial equation for predicted α -amylase activity (Y):

$$Y = 251.36 + 3.87A + 10.94B - 9.35C - 3.64AB + 62.66AC - 6.93BC - 25.57A^2 - 13.68B^2 - 30.72C^2$$

where A , B , and C correspond to coded values for NaCl concentration, starch concentration, and medium pH, respectively.

Table 4 Box–Behnken experimental matrix with actual and predicted α -amylase activities.

Run	A (NaCl, g/L)	B (Starch, g/L)	C (pH)	Experiment (U/mL)	Predicted (U/ml)
1	200	4	5	199.01	193.65
2	300	6	4	270.88	270.94
3	250	4	6	188.17	193.59
4	250	4	4	192.97	198.43
5	300	8	5	217.91	223.27
6	250	8	6	207.07	201.61
7	250	8	4	239.60	234.18
8	300	4	5	214.19	208.67
9	300	6	6	126.83	126.93
10	200	8	5	217.29	222.81
11	200	6	6	244.55	244.49
12	200	6	4	137.98	137.88
13	250	6	5	253.18	251.36
14	250	6	5	230.91	251.36
15	250	6	5	260.04	251.36
16	250	6	5	260.66	251.36
17	250	6	5	251.99	251.36

ANOVA statistical testing indicated the robustness of the quadratic model (Table 5). An F -value of 24.66 ($p = 0.0002$) verified high statistical significance. The determination coefficient ($R^2 = 0.97$) indicated that the model accounts for 97.0% of the total variability in enzyme yield. Furthermore, the adjusted R^2 (0.93) agreed closely with the predicted R^2 (0.82) ($\Delta < 0.2$), while a low coefficient of variation ($CV = 4.96\%$) and an adequate precision ratio of 17.34 underscored high precision and signal clarity. The non-significant lack of fit ($F = 0.54$, $p = 0.6794$) showed that experimental noise alone accounts for minor residual variances. Linear effects B and C, the cross-over interaction AC ($p < 0.0001$), and all quadratic terms (A^2 , B^2 , C^2) were statistically significant ($p < 0.05$). Three-dimensional response surface plots illustrated parameter interdependencies (Figs. 3–5). Confirmation fermentations performed under optimal conditions

331 (250 g/L NaCl, 6 g/L soluble starch, pH 5.0) yielded 274.1 ± 1.8 U/mL extracellular α -amylase activity,
 332 closely matching the predicted maximum of 279.4 U/mL and achieving a 1.84-fold improvement over the
 333 unoptimized baseline (149.03 ± 2.62 U/mL).

334 **Table 5** ANOVA summary for the second-order response surface model.

335

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Model	26017.50	9	2890.83	24.66	0.0002	Highly Sig.
<i>A-NaCl</i>	119.97	1	119.97	1.02	0.3454	Not Sig.
<i>B-Starch</i>	957.69	1	957.69	8.17	0.0244	Sig.
<i>C-pH</i>	699.57	1	699.57	5.97	0.0446	Sig.
<i>AB</i>	53.00	1	53.00	0.45	0.5229	Not Sig.
<i>AC</i>	15702.60	1	15702.60	133.94	< 0.0001	Extremely Sig.
<i>BC</i>	192.24	1	192.24	1.64	0.2412	Not Sig.
<i>A</i> ²	2753.86	1	2753.86	23.49	0.0019	Sig.
<i>B</i> ²	788.17	1	788.17	6.72	0.0358	Sig.
<i>C</i> ²	3974.00	1	3974.00	33.90	0.0006	Sig.
Residual	820.65	7	117.24			

<i>Lack of Fit</i>	236.91	3	78.97	0.54	0.6794	Not Sig.
<i>Pure Error</i>	583.74	4	145.94			
Cor Total	26838.15	16				

336

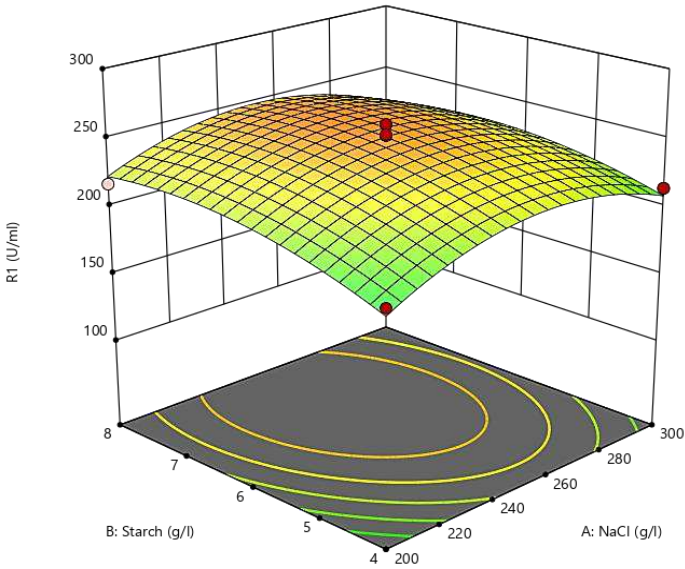
Factor Coding: Actual

R1 (U/ml)
Design Points:
● Above Surface
○ Below Surface
126.83 270.88

X1 = A
X2 = B

Actual Factor
C = 5

3D Surface

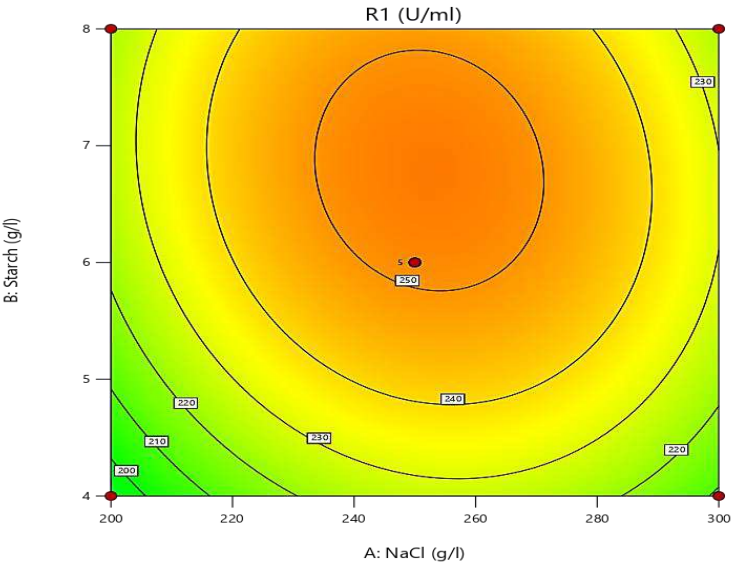


337

Factor Coding: Actual

R1 (U/ml)
● Design Points
126.83 270.88
X1 = A
X2 = B

Actual Factor
C = 5



338

339

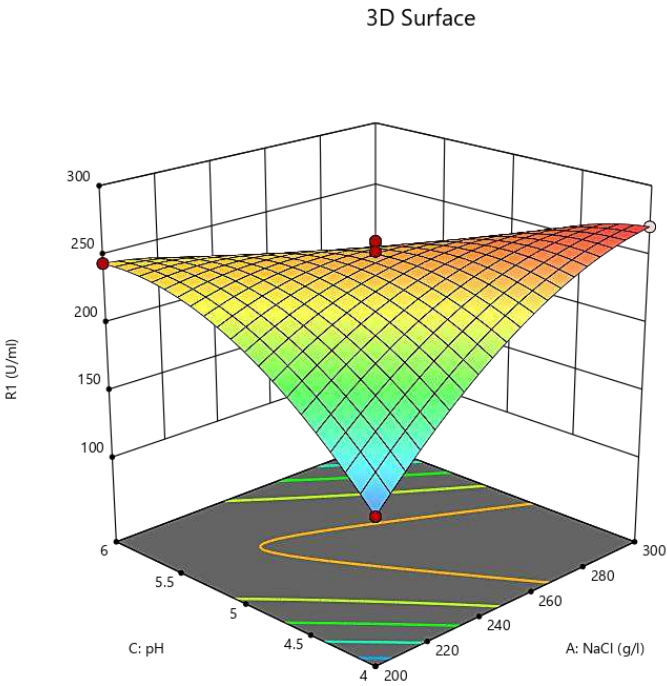
340 **Fig. 3** 3D surface graph and contour plot illustrating the interaction between A: NaCl (g/l) and
341 B: Starch (g/l), and their effect on α -amylase production by S3.

Factor Coding: Actual

R1 (U/ml)
Design Points:
● Above Surface
○ Below Surface
126.83 270.88

X1 = A
X2 = C

Actual Factor
B = 6



342

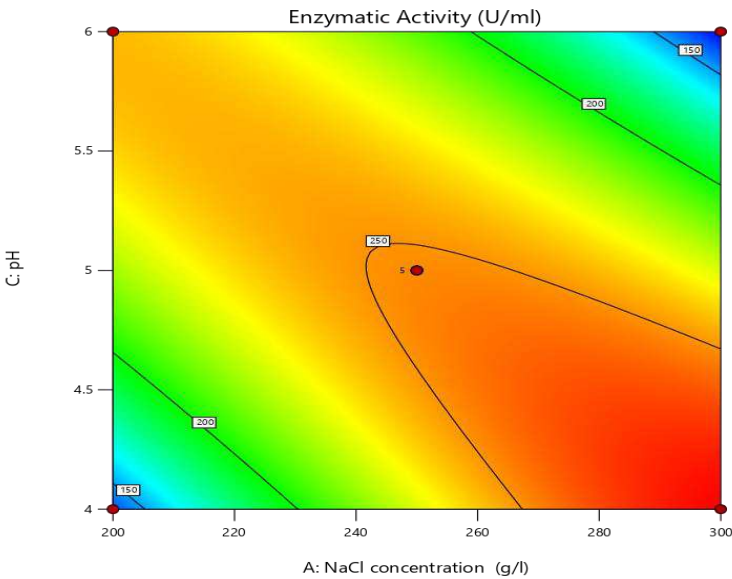
343

344

Factor Coding: Actual

Enzymatic Activity (U/ml)
● Design Points
126.83 270.88
X1 = A
X2 = C

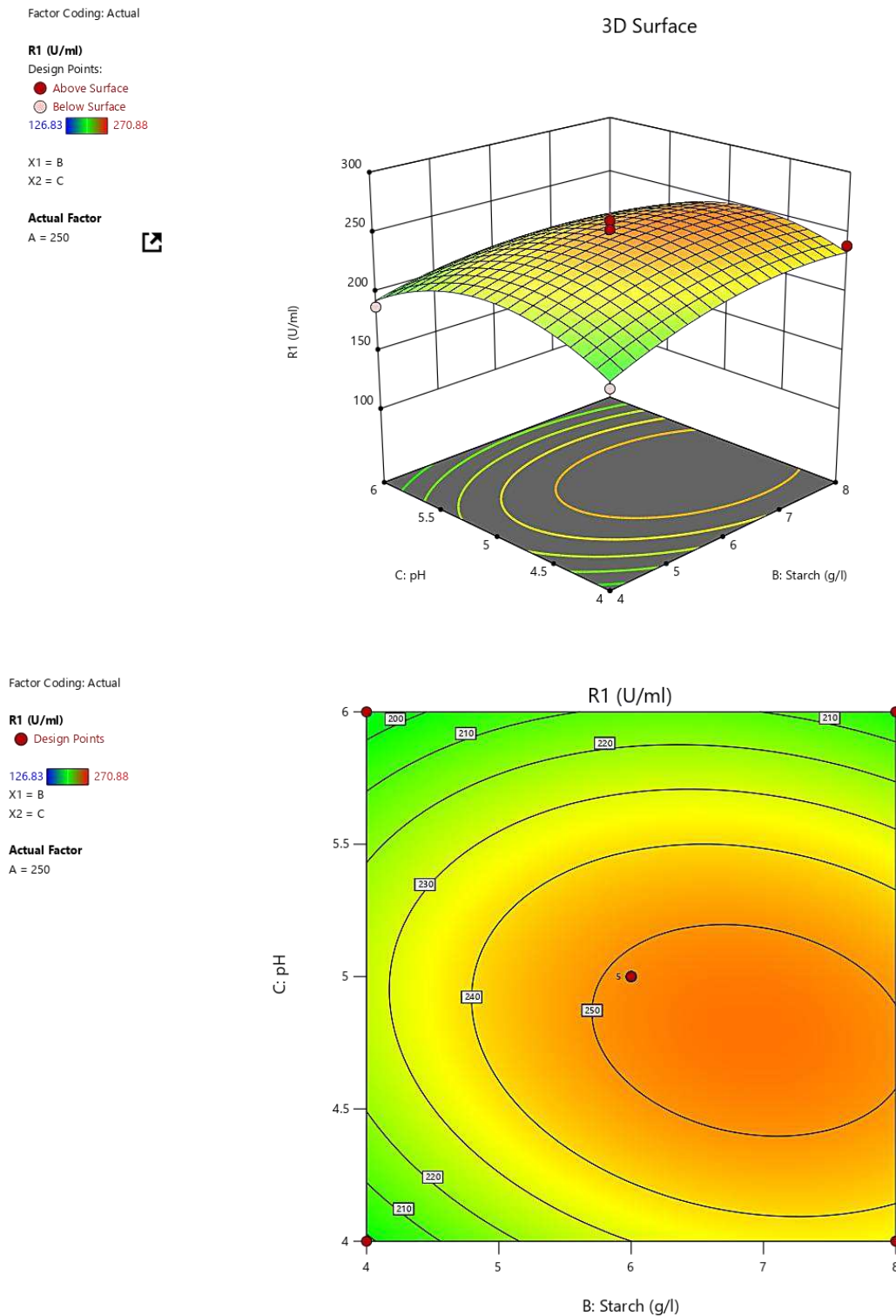
Actual Factor
B = 6



345 **Fig. 4** 3D surface graph and contour plot illustrating the interaction A: NaCl (g/l) and C: pH,
 346 and their effect on α -amylase production by S3.

347

348



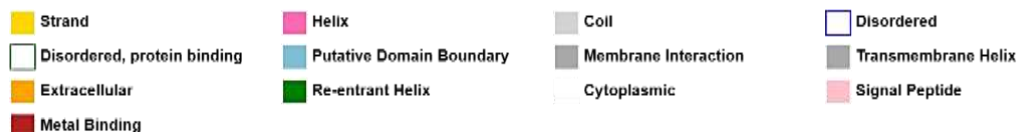
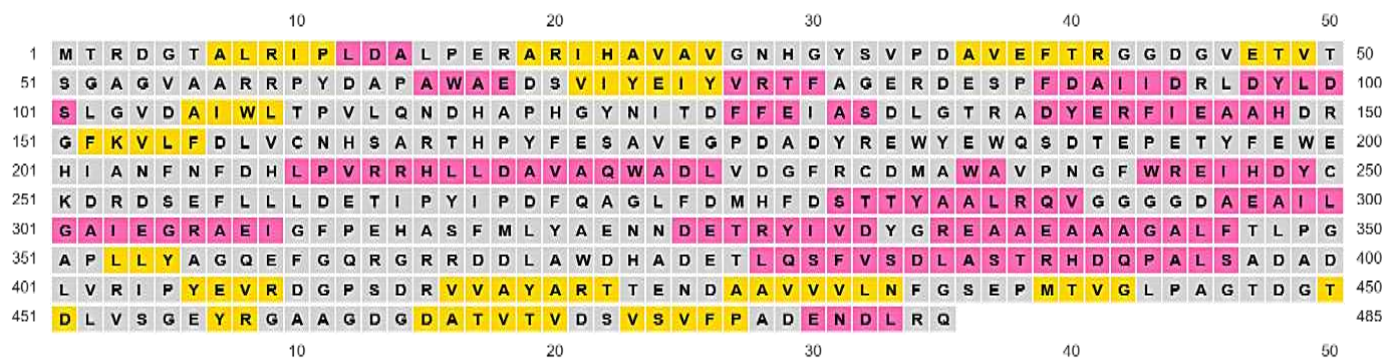
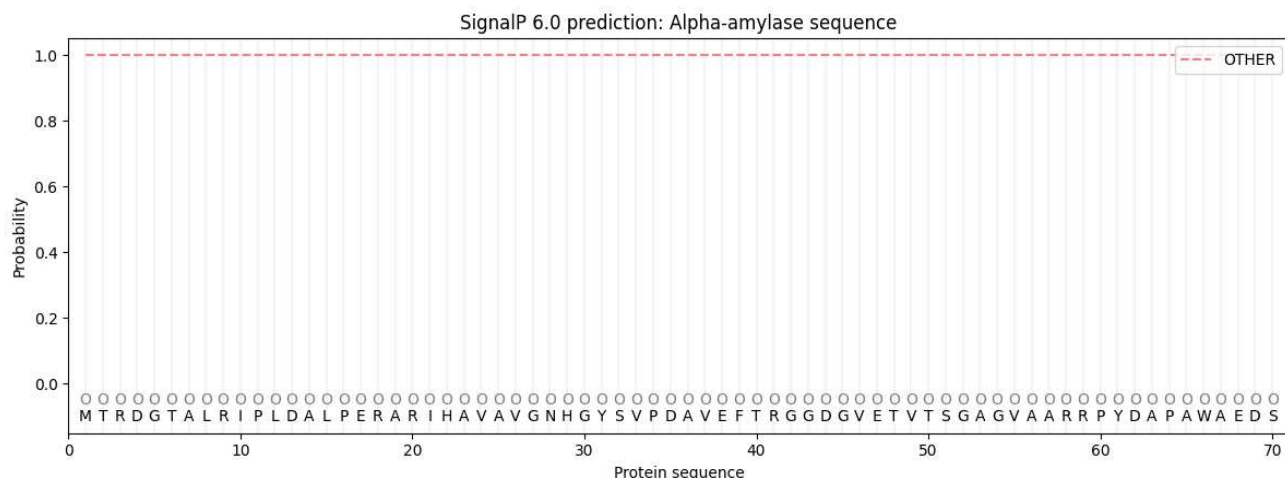
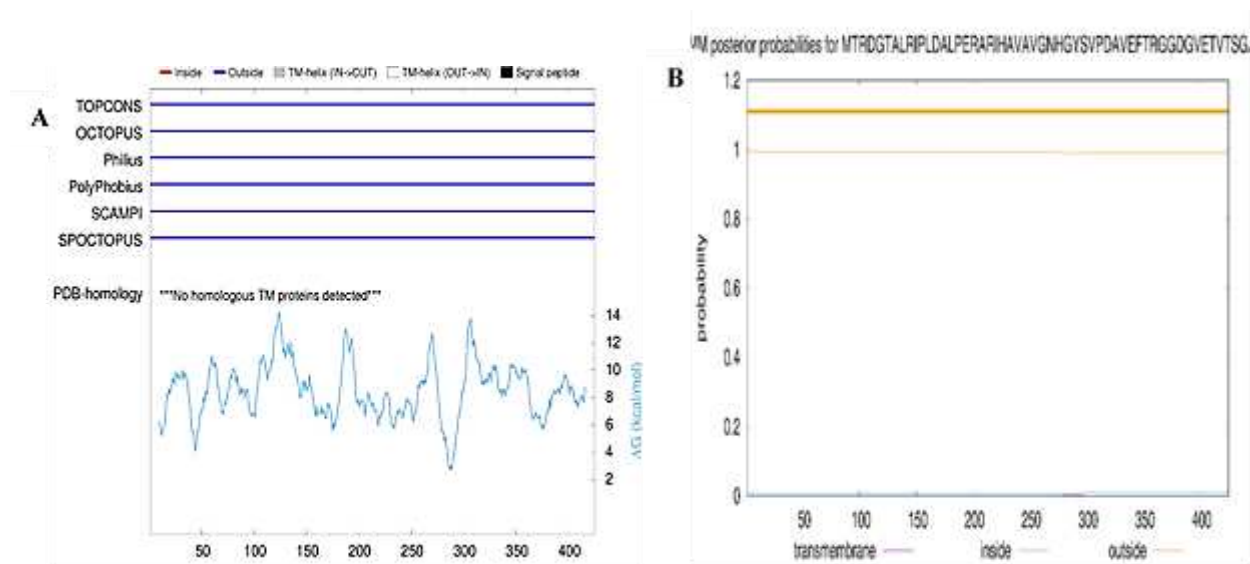


Fig. 7 Computational prediction of non-classical secretion pathways for the AmyHa protein sequence executed via the SignalP 6.0 server



394 **Fig. 8** Transmembrane topology and hydrophobic profile predictions for the mature AmyHa
 395 sequence compiled via consensus web servers.

396 An instability index (II) of 32.41 classifies *AmyHa* as a stable protein (threshold < 40). An aliphatic index
 397 (AI) of 88.65 indicates good thermal stability, while a negative grand average of hydropathicity (GRAVY
 398 = -0.482) reflects a hydrophilic surface architecture. Secondary structure predictions executed via SOPMA
 399 and PSIPRED v4.0 showed a composition dominated by random coils (42.15%), followed by α -helices
 400 (34.20%), extended strands (16.45%), and β -turns (7.20%), with helical regions predominantly
 401 concentrated in the central and C-terminal domains (Fig. 9). mol).



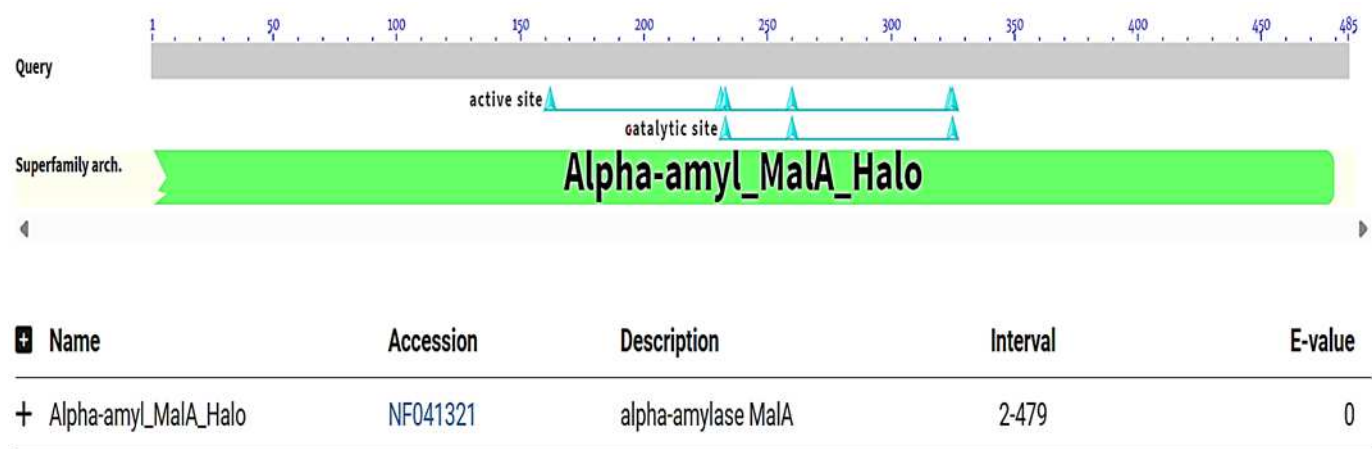
402
 403
 404 **Fig. 9** Secondary structure architectural layout and topology analysis of the mature AmyHa protein
 405 predicted via the PSIPRED v4.0 server.

406 3.7 Domain Architecture, Homology Modeling, and Validation

407 Functional domain annotation using the NCBI CD-search utility mapped a substantially significant match
 408 spanning residues 2 to 479 (E -value = 0), assigning the primary sequence to the Alpha-amyl_MalA_Halo
 409 superfamily (accession NF041321) (Fig. 10). InterPro database searches cross-validated this domain
 410 assignment by identifying convergent signatures for the core GH13 catalytic domain (Pfam PF00128).

411

412 Using ExPASy SWISS-MODEL, we generated a three-dimensional homology model based on the
 413 crystallographic structure of α -amylase from *Haloarcula japonica* (PDB ID: 7V53; 98.4% sequence
 414 identity) as the template (Fig. 11). The resulting tertiary structure displays the typical three-domain
 415 organization of GH13 glycoside hydrolases: an N-terminal catalytic domain A organized around a $(\beta/\alpha)_8$
 416 TIM-barrel fold, an asymmetrical loop insertion domain B protruding between strand $\beta 3$ and helix $\alpha 3$, and
 417 a C-terminal β -sheet domain C
 418 PROCHECK Ramachandran plot analysis established high stereochemical quality, demonstrating that
 419 93.3% of all amino acid residues were positioned in the most favored energy regions, 6.5% in additionally
 420 allowed regions, 0.3% in generously allowed regions, and 0.0% in disallowed regions (Fig. 12). Finally,
 421 model reliability was corroborated by the ExPASy QMEAN server, which generated native-like local
 422 quality distribution scores and an excellent overall QMEAN Z-score of 0.04, confirming native-like
 423 geometry (Fig. 13) .



425 **Fig. 10** Conserved domain architecture and functional superfamily annotations mapped for the
 426 mature AmyHa protein using the NCBI CD-search utility. The alignment confirms the
 427 classification of AmyHa within the core α -amylase catalytic superfamily (Alpha-
 428 amyl_MalA_Halo, accession NF041321) based on conserved active-site sequence motifs.

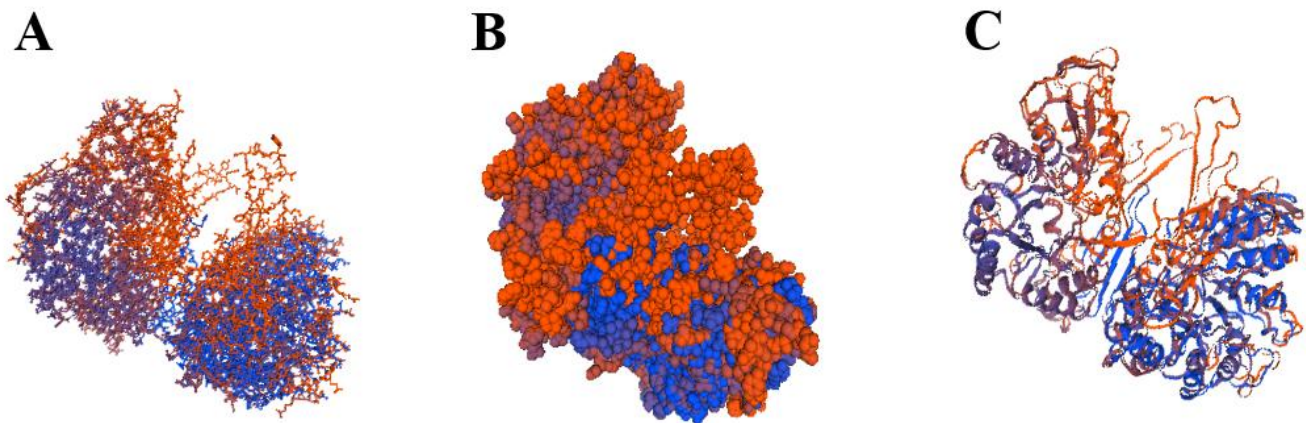


Fig. 11 Three-dimensional tertiary structure model of the mature *AmyHa* protein from *Haloarcula argentinensis* strain S3 predicted via sequence homology modeling using SWISS-MODEL. (A) Wire-frame structural framework, (B) molecular electrostatic surface layout, and (C) ribbon diagram representation showing the overall fold architecture.

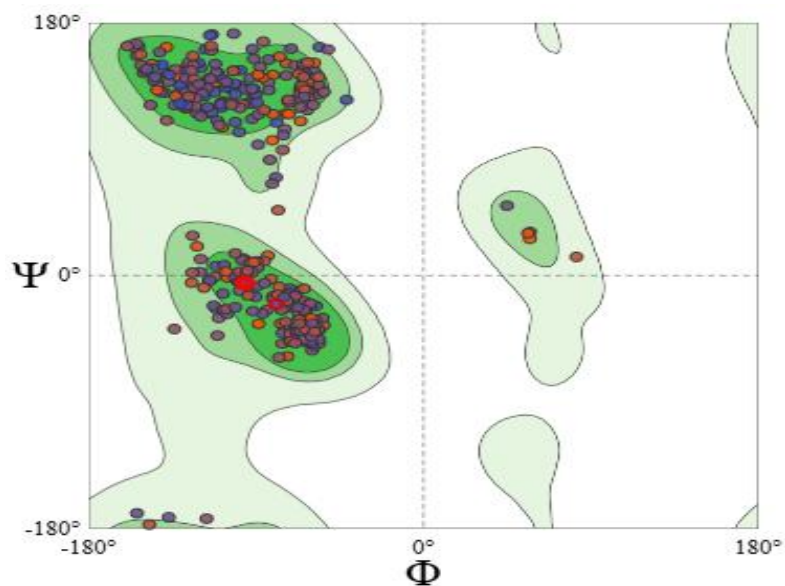
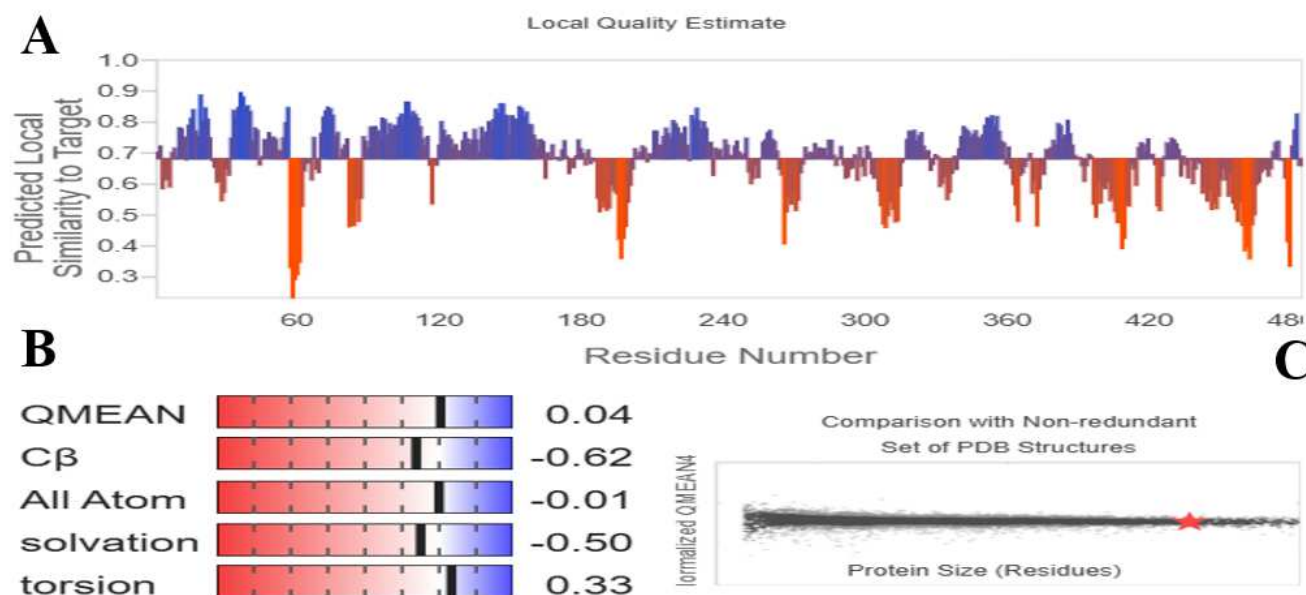


Fig. 12 Ramachandran plots for AmyHa model: Ramachandran plot mapping the backbone dihedral angles (Φ , Ψ) for every residue. The phi (Φ) angle is plotted on the x-axis, and the psi (Ψ) angle on the y-axis, each spanning -180° to 180° . Individual residues appear as colored dots,

452 while green shading marks the regions where Φ/Ψ combinations are sterically allowed or
 453 RABLERed for protein backbones. Clusters of points within the dark green regions represent
 454 residues adopting energetically favorable conformations, whereas points outside these regions
 455 indicate less common or strained backbone geometries.
 456



457
 458 **Fig. 13** Local quality estimation and QMEAN-based structural validation matrix calculated for
 459 the calculated AmyHa model coordinates. Panel (A) charts the continuous QMEANDisCo local
 460 quality scores across the individual amino acid residues, panel (B) summarizes the specific
 461 QMEAN Z-scores comprising overall quality (0.04), β -carbon geometry (-0.62), all-atom
 462 interactions (-0.01), solvation forces (-0.50), and torsion profiles (0.33) where the color gradient
 463 indicates structural agreement with native folds, and panel (C) compares the overall target score
 464 distribution against a reference dataset of high-resolution experimentally determined structures.
 465
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472 **3.8 Molecular Docking Simulations and Substrate Preferences**

473 We conducted molecular docking simulations using AutoDock Vina, targeting the predicted (β/α)₈ TIM-
474 barrel catalytic cleft. Docking short-chain oligosaccharide models established a distinct binding
475 preference in descending order: branched amylopectin fragment > linear α -maltotetraose > amylopectin
476 fragment = glycogen fragment > starch fraction > linear amylose. The branched amylopectin model
477 yielded the lowest binding free energy ($\Delta G = -10.0$ kcal/mol). Ligand positioning within the pocket was
478 stabilized by hydrogen bonding and hydrophobic interactions with conserved residues, including Glu304,
479 Ile303, Arg306, Phe311, Pro312, Glu313, Ala315, Ala397, Asp398, Ala399, Asp400, and Leu401.
480 Crucially, docking validated close contact with the conserved catalytic triad: nucleophile Asp197,
481 acid/base donor Glu233, and structural stabilizer Asp300. Conversely, linear amylose showed the weakest
482 affinity ($\Delta G = -5.9$ kcal/mol) (Table 6).

483

484 **Table 6** Binding affinity of α -amylase with docked carbohydrate complexes via AutoDock Vina

Substrates / Oligomeric Models	PubChem CID	Degree of Polymerization (DP) / Structural Features	Affinity (kcal/mol)
Branched amylopectin fragment	CID: 439207	Branched oligosaccharide (α -1,4 and α -1,6 linkages)	-10.0
α -maltotetraose	CID: 446495	Linear tetrasaccharide (4 glucose units)	-7.1
Amylodextrin core fragment	CID: 439341	Linear malto-oligosaccharide	-6.8
Branched glycogen core fragment	CID: 439177	Short branched chain model	-6.8
Soluble starch model fraction	—	Short-chain glucan model	-6.0
Linear amylose fragment	CID: 21239054	Linear malto-oligosaccharide (α -1,4 linkages)	-5.9

485

486 **4 Discussion**

487 The physiological and biochemical profiling of *Haloarcula argentinensis* strain S3 highlights a
488 specialized evolutionary adaptation to hypersaline environments [3, 40]. The obligate salinity requirement
489 for growth (minimum 15% w/v NaCl) and optimal proliferation between 20% and 25% (w/v) NaCl reflect
490 the classic "salt-in" osmoadaptation strategy characteristic of the family Halobacteriaceae [1, 2, 41]. To
491 counterbalance severe external osmotic pressure, haloarchaea accumulate high intracellular
492 concentrations of potassium ions (K^+), forcing cellular proteins to evolve unique structural modifications
493 that maintain solubility and catalytic activity under extreme ionic strength [39, 41]. A similar strict
494 halophilic requirement was described for a family IV esterase from *Haloarcula japonica*, which required
495 elevated salt concentrations for structural stability and maximal activity [42]. Although belonging to a
496 distinct glycoside hydrolase family, *AmyHa* shares these haloadaptive trends, reinforcing the status of
497 haloarchaea as a rich bio-resource for robust, salt-tolerant biocatalysts [4, 48]. An intriguing physiological
498 trait of strain S3 is its growth optimum at pH 6.0 [3, 43]. While most characterized haloarchaea prefer
499 neutral or mildly alkaline habitats [44], the slightly acidic preference of strain S3 mirrors the natural soil
500 chemistry of the Wadi Elmalah ecosystem from which it was isolated [3]. Parallel adaptations to mildly
501 acidic niches have been documented in *Haladaptatus paucihalophilus*, *Halococcus qingdaonensis*, and
502 *Natrinema gari*, indicating that acid tolerance is more widespread among haloarchaea than previously
503 recognized [45, 46, 47]. Furthermore, strain S3 exhibited a versatile extracellular hydrolytic repertoire,
504 secreting α -amylase alongside protease, xylanase, pectinase, cellulase, and mannanase [3, 25]. This multi-
505 enzyme secretion profile reflects a flexible ecological strategy allowing haloarchaea to decompose
506 heterogeneous organic polymers in hypersaline matrices [4, 25]. From a biotechnological perspective,
507 such multi-hydrolase capacity enhances the industrial utility of strain S3 for consolidated bioprocesses
508 operating under harsh saline regimes [48]. Statistical optimization using Response Surface Methodology
509 (RSM) successfully overcome early kinetic limitations and maximized extracellular enzyme yield [13, 14,
510 50]. The Plackett–Burman design demonstrated that salinity, medium pH, and substrate availability act as
511 primary regulatory triggers for amylolytic expression [12, 14, 27, 49]. Subsequent Box–Behnken
512 modeling demonstrated excellent predictive accuracy ($R^2 = 0.97$), validating the second-order polynomial
513 equation [13, 50]. Interestingly, the highly significant interaction between salinity and pH (AC term, $p <$
514 0.0001) suggests that elevated NaCl concentrations provide electrostatic shielding that stabilizes the cell
515 envelope and extracellular proteins against acid-induced charge disruptions [3]. Under optimized
516 parameters (250 g/L NaCl, 6 g/L soluble starch, pH 5.0), extracellular α -amylase production reached 274.1
517 ± 1.8 U/mL, representing a 1.84-fold enhancement over the unoptimized baseline [3, 50]. Similar

518 statistical optimization strategies have been effectively applied to maximize amyolytic yields in diverse
519 microbial systems [49, 50]. At the molecular level, primary sequence analysis of *AmyHa* revealed classical
520 signatures of haloadaptation [3, 8, 41]. Its acidic theoretical isoelectric point (pI 4.21) and the massive
521 overrepresentation of acidic amino acids (18.56% Asp and Glu) constitute key structural features [3, 8].
522 Surface-exposed negative charges coordinate a tight, protective hydration shell that prevents protein
523 aggregation and salting-out under high ionic stress [8, 39, 41]. Identical structural adaptations occur across
524 haloarchaeal glycoside hydrolases, including α -amylases from *Haloarcu* *hispanica*, *Haloarcu* *marismortui*, and *Halalkalicoccus jeotgali* [8]. As highlighted in recent structural studies on salt-tolerant
525 biocatalysts, an abundance of acidic surface residues acts as the primary driving force for maintaining
526 catalytic solubility and preventing denaturation under hyper-saline regimes [55]. Topological predictions
527 via SignalP 6.0, TOPCONS, and TMHMM demonstrated the absence of a canonical N-terminal signal
528 peptide or transmembrane helices, pointing to a soluble extracellular protein [3, 51, 52]. Rather than
529 prematurely asserting a non-classical secretion mechanism, these negative predictions suggest that *AmyHa*
530 may utilize an alternative translocation route or an unconventional signal motif [51, 52]. In haloarchaea,
531 folded extracellular enzymes often bypass classical Sec pathways and exploit unconventional
532 translocation systems [51, 52]. Furthermore, the high proportion of random coils (42.15%) in *AmyHa*
533 likely provides the native backbone with the structural flexibility required to maintain catalytic
534 conformation under fluctuating environmental salinities [3]. Finally, molecular docking simulations
535 provided structural insights into active-site organization and substrate coordination within the $(\beta/\alpha)_8$ TIM-
536 barrel catalytic cleft [24, 32, 36, 37]. *AmyHa* exhibited a marked binding preference for branched
537 oligosaccharide fragments ($\Delta G = -10.0$ kcal/mol for amylopectin) over linear amylose ($\Delta G = -5.9$ kcal/mol)
538 [36, 37]. Ligand positioning within the catalytic pocket confirmed close contact with conserved residues
539 surrounding the catalytic triad (*Asp197*, *Glu233*, *Asp300*), which drives α -(1,4)-glucosidic bond cleavage
540 [38]. Similar structural architectures and catalytic mechanisms have been established for microbial
541 glycoside hydrolases [32, 33, 38]. In agreement with recent catalytic domain investigations, active-site
542 stability and precise hydrogen-bonding networks surrounding the catalytic pocket are critical for
543 governing substrate affinity and enzymatic cleavage efficiency in α -amylases [56]. Together with its
544 stability under combined hypersalinity and acidic conditions, these structural features highlight *AmyHa* as
545 a promising biocatalyst for industrial starch processing under multi-extreme conditions [5, 6, 7].
546

547 4.1 Limitations of the Study

548 It is important to acknowledge certain limitations inherent to the experimental design of this work. First,
549 while the three-dimensional structural architecture, active-site dynamics, and substrate binding
550 preferences of AmyHa were comprehensively characterized, these insights rely primarily on homology
551 modeling and molecular docking simulations. Although supported by high stereochemical validation
552 scores, crystal structure determination via X-ray crystallography or cryo-EM will be essential to
553 definitively resolve the atomic coordinates and map the exact spatial orientation of surface-exposed acidic
554 residues driving molecular haloadaptation. Second, the non-classical translocation route predicted for
555 AmyHa reflects computational topology analyses; thus, experimental validation through native protein
556 purification, N-terminal Edman sequencing, and cellular localization assays will be required to confirm
557 the mature 5' processing and secretion machinery. Finally, evaluating enzyme performance under pilot-
558 scale bioreactor conditions and in the presence of industrial organic solvents or chemical detergents will
559 provide a more comprehensive assessment of the technological feasibility of AmyHa for multi-extreme
560 bioprocesses

561 **Conclusion**

562

563 The current investigation successfully established an integrated optimization and in silico
564 characterization framework for the extracellular α -amylase (*AmyHa*) expressed by the extreme
565 halophile *Haloarcula argentinensis* strain S3. The sequential application of a Plackett-Burman
566 screening matrix and a Box-Behnken response surface design efficiently isolated the critical
567 parameters and determined that an acidic medium formulation paired with elevated salinity
568 levels triggers a substantial increase in enzymatic expression. The mathematical models showed
569 high consistency with the experimental data, leading to a measured production of 274.1 ± 1.8
570 U/mL under the optimized conditions, which corresponds to a notable 1.84-fold enhancement
571 compared to the initial unoptimized cultural baseline. The structural and functional
572 characteristics of the mature enzyme were further supported by comprehensive bioinformatic
573 modeling, stereochemical validation, and molecular docking simulations. The primary sequence
574 analysis revealed a distinctively acidic surface profile that explains the structural solubility of
575 the molecule under high ionic stress, while the molecular docking models suggested a high
576 binding affinity toward branched starch fractions like amylopectin within the classical β/α_8 TIM-
577 barrel catalytic core. These preliminary findings suggest that *Haloarcula argentinensis* strain S3

could serve as a promising candidate for specialized industrial bioprocesses that require catalytic stability under concurrent acidic and hypersaline conditions. However, because these structural models and kinetic specificities rely heavily on computational predictions and controlled laboratory fermentations, several future investigations are necessary to fully evaluate the industrial feasibility of this system. Future perspectives should focus on the purification of the native enzyme to clarify its physical properties and verify the calculated substrate affinities through classical kinetic assays. Furthermore, crystallographic analysis is required to confirm the calculated tertiary coordinates, clarify the dynamic role of the clustered acidic residues, and verify the structural mechanisms driving its molecular haloadaptation. Finally, scaling up the fermentation process in automated bioreactors and assessing enzyme performance in the presence of chemical detergents or organic solvents would provide useful insights into the applicability of AmyHa in starch processing, textile, and biotechnological operations.

Authors contributions

M.S. contributed to conceptualization, study design, methodology, investigation, data curation, laboratory experimentation, formal analysis, and writing the original draft. S.K.-G. contributed to conceptualization, supervision, validation, project administration, funding acquisition, and writing, review and editing. S.A.-T. contributed to methodology, visualization, validation, and writing, review and editing. A.N. contributed to phylogenetic analysis, bioinformatics, and methodology. R.N. contributed to resources and investigation. N.S.-S. contributed to methodology and investigation. S.K. contributed to investigation. A.M. contributed to investigation and formal analysis. All authors read and approved the final version of the manuscript.

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608

609 **Declarations**

610 Conflict of interests the authors declare no competing interests.

611

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