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Extracellular alpha-amylase from halophilic bacteria *Marinobacter* sp. LES TG5: Isolation, optimization, and characterization

I Putu Parwata[1,*], Ketut Srie Marhaeni Julyasih[2]

[1]Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Pendidikan Ganesha, Jl. Udayana No. 11 Singaraja, Bali 81116, Indonesia

[2]Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Pendidikan Ganesha, Jl. Udayana No. 11 Singaraja, Bali 81116, Indonesia

*Corresponding author: putu.parwata@undiksha.ac.id

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ABSTRACT The growing demand for stable and effective enzymes requires the discovery of novel microbial producers. Alpha-amylase is an enzyme in high demand by various industries; however, the discovery of novel and stable alpha-amylase producers remains limited. This study aims to isolate, optimize, and characterize extracellular alpha-amylase from halophilic bacteria *Marinobacter* sp. LES TG5. Bacteria were isolated from saltwater and soil samples collected from traditional salt ponds in Les Village, Bali, Indonesia. Initial screening on starch agar yielded several amylase-producing colonies, and subsequent spectrophotometric assays identified one promising isolate (LES TG5), which demonstrated an initial activity of 0.63 U/mL. The production of amylase was significantly enhanced by a multi-stage optimization process. This involved first identifying optimal carbon and nitrogen sources, followed by a one-variable-at-a-time approach to determine the ideal nutrient levels, salt concentration, and incubation time. This optimization led to an 11-fold increase in activity, from 0.63 U/mL to 6.99 U/mL, achieved with a medium containing 2.4% (w/v) nutrient broth, 0.4% (w/v) maltose, and 3% (w/v) NaCl with an incubation time of 22 hours. Enzyme characterization revealed optimal amylase activity at pH 7, 55 °C, and 3% (w/v) NaCl. While Ca^{2+} and Mg^{2+} had no effect on amylase activity, Pb^{2+}, Fe^{2+}, Sn^{2+}, and Al^{3+} significantly reduced it. Importantly, the amylase demonstrated outstanding stability in organic solvents such as methanol, ethanol, and n-hexane, suggesting its potential as a biocatalyst for chemical synthesis in non-aqueous systems. Furthermore, its notable stability against surfactants and detergents highlights its promise as an additive in cleaning product formulations.

KEYWORDS Alpha-amylase; Halophilic bacteria; *Marinobacter* sp.; Traditional salt pond

1. Introduction

Alpha-amylase (EC 3.2.1.1) is a crucial industrial enzyme that hydrolyzes the internal α-1,4-glycosidic bonds in starch and other polysaccharides, yielding simpler compounds such as glucose and maltose (Far et al. 2020). Its broad utility is evident across diverse sectors, including brewing, paper manufacturing, textile desizing, paint formulation, wallpaper removal, sugar production, pharmaceuticals, and the development of cold-water-dispersible laundry detergents (Simair et al. 2017).

Microorganisms, particularly bacteria and fungi, are the predominant industrial producers of alpha-amylase (Mahfudz et al. 2024). The widespread adoption of microbial amylases is driven by the cost-effectiveness of large-scale production and the relative ease with which microbial strains can be engineered to produce enzymes with desired characteristics (Abo-Kamer et al. 2023).

Among microbial sources, halophilic bacteria, which thrive in high-salinity environments, have garnered significant attention in biotechnological applications. This interest is driven by their capacity to produce enzymes exhibiting remarkable activity and stability under extreme conditions (Ali et al. 2024). Research on extremozymes from various halophilic bacterial species has accelerated recently, highlighting their ability to effectively catalyze processes and maintain optimal activity across a wide range of salt concentrations (Hashem and Yousef 2024). The growing need for stable and efficient enzymes can be addressed either by identifying microorganisms that produce novel enzymes with superior activity or by enhancing the characteristics of existing enzymes (Salgaonkar et al. 2019).

Numerous halophilic bacterial genera, including *Halomonas*, *Halobacterium*, *Halothermothrix*, *Micrococcus*, *Natronococcus*, *Haloarcula*, *Nesterenkonia*, and *Haloferax*, have been reported to produce halophilic amylases, which typically exhibit high stability against varying

temperatures and salt concentrations (Yavari-Bafghi and Amoozegar 2025). However, for many industrial applications, alpha-amylase must exhibit robust stability not only at high temperatures and varying salt concentrations but also in the presence of organic solvents and detergents. The discovery of alpha-amylases that exhibit stability in the presence of both organic solvents and detergents remains limited. While previous research by Kumar and Khare (2016) reported that the amylase from *Marinobacter* sp. demonstrated good stability against nonpolar organic solvents like hexane, it showed less stability in the presence of polar organic solvents such as ethanol. This highlights the challenge in finding enzymes that are robust across a range of solvent types.

This study reports the isolation of a novel alpha-amylase from the halophilic bacterium *Marinobacter* sp. LES TG5. This bacterium was sourced from a traditional salt pond located in Les Village, Buleleng Regency, Bali Province, Indonesia. Crucially, the alpha-amylase secreted by this strain demonstrated remarkable stability in both organic solvents and detergents, a characteristic highly desirable for industrial biotechnology. The characteristics of the alpha-amylase were also compared with those produced by other Marinobacter species, demonstrating its superiority.

2. Materials and Methods

2.1. Chemicals

All chemicals, including KH_2PO_4, K_2HPO_4, NaCl, $MgSO_4$. $7H_2O$, $FeSO_4.7H_2O$, $CaCl_2$, $MnSO_4$, $CoCl_2$, $BaCl_2$, $PbSO_4$, $AlCl_3$, $CuSO_4$, EDTA, and dinitrosalicylic acid (DNS), were of pro analysis grade and were purchased from Merck (Germany). Yeast extract, tryptone, peptone, nutrient broth, agar, maltose, lactose, soluble starch, sucrose, and nutrient agar were obtained from Himedia (India). Methanol, ethanol, chloroform, n-hexane, Tween 80, and Triton-X 100 were purchased from Merck (Germany).

2.2. Sample collection

Soil and saltwater samples were collected from the traditional salt pond located at Les Village, Tedjakula District, Buleleng Regency, Bali Province, Indonesia (Figure 1). Specifically, soil samples were taken from each of the four ponds. Saltwater samples were collected from containers with varying drying times, which corresponded to a salinity range of 25 to 29 °Bé. All samples were transferred into sterile vials and immediately transported to the laboratory in an icebox for subsequent microbial analysis.

FIGURE 1 Traditional salt pond at Les Village, Tedjakula District, Buleleng Regency, Bali Province, Indonesia (map position: -8.130639, 115.368858).

2.3. Isolation of halophilic bacteria

Halophilic bacteria were isolated using Luria Bertani (LB) agar medium containing 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 10% (w/v) NaCl, and 1.75% (w/v) Bacto agar. The medium was incubated at 37 °C for 1 to 2 days. Growing bacterial colonies were then purified by repeatedly streaking onto fresh LB agar medium until a single, isolated colony was obtained.

2.4. Qualitative assessment for alpha-amylase-producing bacteria

The production of extracellular alpha-amylase by bacterial isolates was qualitatively assessed using starch agar (SA) medium. The SA medium comprised 2.8% (w/v) nutrient agar, 1.0% (w/v) soluble starch, and 10% (w/v) NaCl. Inoculated plates were incubated at 37 °C for 1 to 2 days. The ability of the bacteria to produce extracellular amylase was determined by observing the clear zone formed around the bacterial colonies after flooding the plates with 0.05 M iodine solution (Abo-Kamer et al. 2023).

2.5. Quantitative assessment of alpha-amylase activity

For quantitative assessment, bacterial isolates were inoculated into a liquid medium containing 2.8% (w/v) nutrient broth, 0.05% (w/v) $CaCl_2$, 0.05% (w/v) $MgSO_4$, 10% (w/v) NaCl, and 0.8% (w/v) soluble starch. This culture was subsequently incubated in a shaking incubator at 37 °C and 180 rpm. The resulting extracellular alpha-amylase was then separated from the bacterial cells by centrifugation at 8,000 × g for 10 min.

Amylase activity was determined spectrophotometrically using soluble starch as a substrate, following a modified dinitrosalicylic acid (DNS) method (Sanjaya et al. 2024). The substrate solution was prepared by mixing 1% (w/v) soluble starch and 3% (w/v) NaCl in phosphate buffer (pH 8). Reactions were initiated by adding 50 µL of crude amylase to 200 µL of substrate solution, followed by incubation at 40 °C for 30 min. The reaction was terminated by adding 250 µL of DNS reagent, and the mixture was then incubated at 100 °C for 5 min for color development. The absorbance of the mixture was subsequently measured using a UV/Vis spectrophotometer at 478 nm. As a control, crude amylase was inactivated by boiling the enzyme for 15 min before the reaction. Amylase activity was then calculated based on glucose equivalents from a standard curve and expressed in units/mL. One unit (U) was defined as the amount of enzyme that catalyzes the release of 1 µmol of glucose per minute per mL of crude enzyme.

2.6. Identification of alpha-amylase-producing bacteria

The alpha-amylase-producing bacterial isolate was identified genotypically through 16S rRNA gene analysis (Pradhan and Tamang 2019). Chromosomal DNA from the bacterium was isolated using Geneaid's Presto Mini gDNA Bacteria Kit. The 16S rRNA gene was subsequently amplified by polymerase chain reaction (PCR) using the primers Bact27F (sequence: AGAGTTTGATCATGGCTCAG) and Uni1492R (sequence: GGTTACCTTGTTACGACTT). Sequencing of the amplified gene was performed, and the resulting sequences were analyzed using DNA Baser version 3.5.4 (Heracle BioSoft). Sequence similarities with other bacterial species were determined by performing a basic local alignment search tool (BLAST) search against the National Center for Biotechnology Information (NCBI) gene database. Finally, phylogenetic analysis was conducted using MEGA version 5.0 to ascertain the genetic relatedness of the identified species to other taxa.

2.7. Optimization of alpha-amylase production

Alpha-amylase production optimization commenced with screening for the most effective carbon and nitrogen sources for amylase synthesis. The carbon sources investigated included starch, maltose, lactose, and sucrose. This selection was based on the fact that alpha-amylase primarily catalyzes the hydrolysis of starch into oligosaccharides and glucose, which are the main components of these carbohydrates. The nitrogen sources investigated included nutrient broth, tryptone, peptone, and yeast extract. These materials were selected because they are well-established as primary nitrogen sources for the growth of a wide range of microorganisms. Following this, the optimal concentrations of selected carbon and nitrogen sources, as well as salt, were determined. Finally, the incubation time for optimal amylase production was established. The optimization of nutrient composition, including carbon sources, nitrogen sources, and salt levels, as well as incubation time, was performed using a one-variable-at-a-time (OVAT) approach. This method assumes no significant interaction between the variables.

For each optimization experiment, the bacterium was inoculated into the respective medium and incubated in a shaking incubator at 37 °C and 180 rpm. The resulting extracellular amylase was then separated from the bacterial cells by centrifugation at 8,000 × g for 10 min. Amylase activity was subsequently assayed using starch as the substrate.

2.8. Characterization of alpha-amylase

The optimum pH for alpha-amylase activity was determined by assaying enzyme activity across a pH range of 4 to 10. The buffer systems utilized for this purpose were acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–8.0), and glycine-NaOH buffer (pH 9.0–10.0).

To determine the optimum temperature, amylase activity was measured at various temperatures ranging from 25 °C to 80 °C.

The effect of divalent cations on amylase activity was assessed by incubating the enzyme solution for 1 hour in 10 mM solutions of various cations, including Mg^{2+}, Mn^{2+}, Ba^{2+}, Sn^{2+}, Al^{3+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Pb^{2+}, and Ca^{2+}. Following incubation, the residual amylase activity was then measured.

Tolerance to salt concentrations (NaCl) was determined by assaying extracellular amylase activity at varying NaCl concentrations, ranging from 0 to 21% (w/v).

Finally, tolerance to organic solvents, surfactants, and detergents was assessed by measuring the residual amylase activity after incubating the enzyme for 1 h in 25% and 50% (v/v) concentrations of these agents. The organic solvents tested included methanol, ethanol, chloroform, and n-hexane. Surfactants utilized were Tween 80 and Triton X-100, while commercial detergents tested included dishwashing liquid and liquid laundry detergent.

2.9. Statistical Analysis

Data for both amylase activity and cell dry weight of the bacteria are presented as the mean ± standard deviation. All statistical analyses were conducted using Microsoft Excel (version 16).

3. Results and Discussion

3.1. Isolation of halophilic bacteria producing extracellular alpha-amylase

Initial isolation efforts yielded over 50 bacterial colonies from saltwater and soil samples collected from the salt ponds in Les Village, Tedjakula District, Buleleng Regency, Bali. From this collection, a total of 24 colonies were screened for their potential to produce extracellular alpha-amylase using starch agar medium. These included isolates from saltwater samples (AG1, AG2, AG3, AG5, AG6, AG7, AG8) and soil samples (TG1, TG2, TG3, TG5, TG6, TG7, TG8, TG12, TG13, TG14, TG15, TG16, TG17, TG18, TG21, TG22, TG24). The results, depicted in Figure 2, showed the formation of a clear zone around bacterial colonies, indicative of extracellular alpha-amylase production. Nine bacterial colonies exhibited this clear zone (Figure 2a): eight isolates from soil samples (TG1, TG5, TG6, TG7, TG8, TG13, TG15, and TG16) and one isolate from saltwater samples (AG6). These data suggest that bacterial colonies originating from soil samples (TG) demonstrated a higher potential for extracellular amylase production compared to those from saltwater samples (AG).

Bacterial colonies exhibiting potential for extracellular alpha-amylase production on starch agar medium were subsequently subjected to quantitative amylase activity assays; the results are presented in Figure 2b. Of the nine colonies tested, two isolates, TG5 and TG6, demonstrated high amylase activity, with respective activities of 0.63 U/mL and 0.44 U/mL. Isolate TG7 also produced amy-

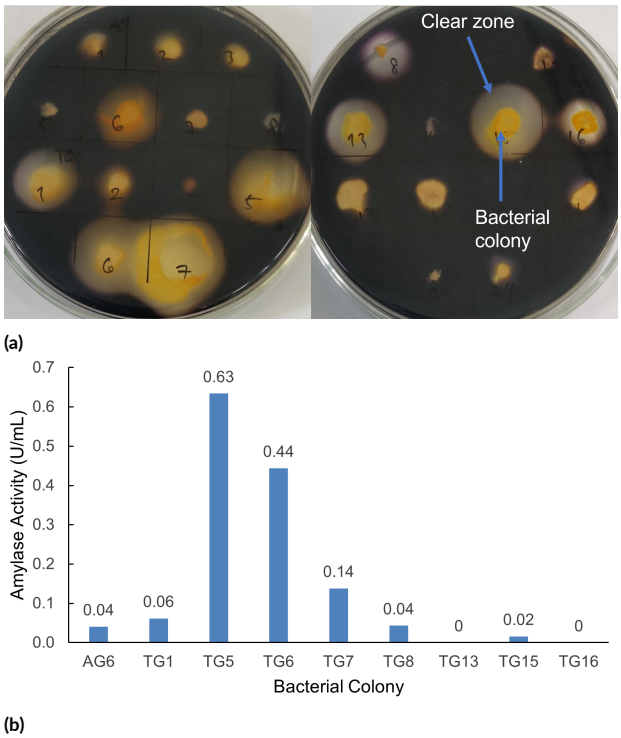

FIGURE 2 Extracellular alpha-amylase activity of halophilic bacteria. (a) amylase activity on starch agar medium, (b) quantitative assay of amylase activity.

lase, albeit with a lower activity of 0.14 U/mL, while the amylase activity for TG1, TG8, TG15, and AG6 was below 0.1 U/mL. Interestingly, no amylase activity was detected for isolates TG13 and TG15 in the quantitative assay, despite positive qualitative results on starch agar medium. These findings confirm the significant potential of halophilic bacteria isolated from the Les Village salt pond for extracellular alpha-amylase production. The most promising isolate, TG5, was selected for further optimization of its amylase production.

3.2. Identification of the best alpha-amylase-producing bacterium (TG5)

The most promising alpha-amylase-producing halophilic bacterium, isolate TG5, was identified through phylogenetic analysis of its 16S rRNA gene sequence. As depicted in Figure 3, the phylogenetic tree constructed using MEGA 11 software shows that isolate TG5 clustered within the *Marinobacter* sp. branch, indicating a close

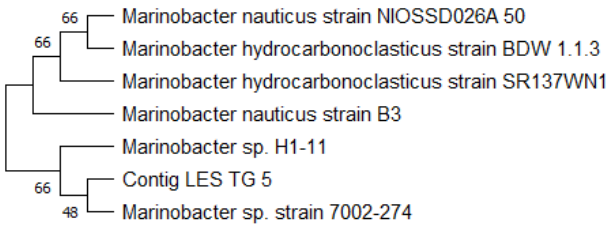

FIGURE 3 Phylogenetic analysis of halophilic bacteria isolate TG5 based on 16S rRNA gene sequence.

evolutionary relationship and high sequence homology with this genus.

3.3. Optimization of alpha-amylase production by *Marinobacter* sp. LES TG5

Optimization of extracellular alpha-amylase production from *Marinobacter* sp. LES TG5 commenced with screening for the most effective carbon and nitrogen sources for bacterial productivity; the results are presented in Figure 4. Among the four carbon sources evaluated, maltose yielded the highest amylase activity at 2.8 U/mL, followed by starch (1.78 U/mL), sucrose (1.11 U/mL), and lactose (1.05 U/mL). Amylase activity generally correlated with bacterial cell density, as expressed by cell dry weight. Maltose also supported the highest bacterial density at 3.55 mg/mL, followed by starch (3.35 mg/mL), sucrose (2.20 mg/mL), and lactose (1.80 mg/mL) (Figure 4a).

For nitrogen sources, nutrient broth resulted in slightly higher amylase activity (1.95 U/mL) compared to yeast extract (1.93 U/mL). Tryptone provided an amylase activity of 0.93 U/mL, while peptone was a poor nitrogen source, yielding only 0.29 U/mL. Bacterial cell density generally aligned with the amylase activity produced, except for yeast extract. Although yeast extract produced the highest cell density at 6.2 mg/mL, followed by nutrient broth (4.0 mg/mL), tryptone (2.6 mg/mL), and peptone (1.9 mg/mL) (Figure 4b), this high cell density did not cor-

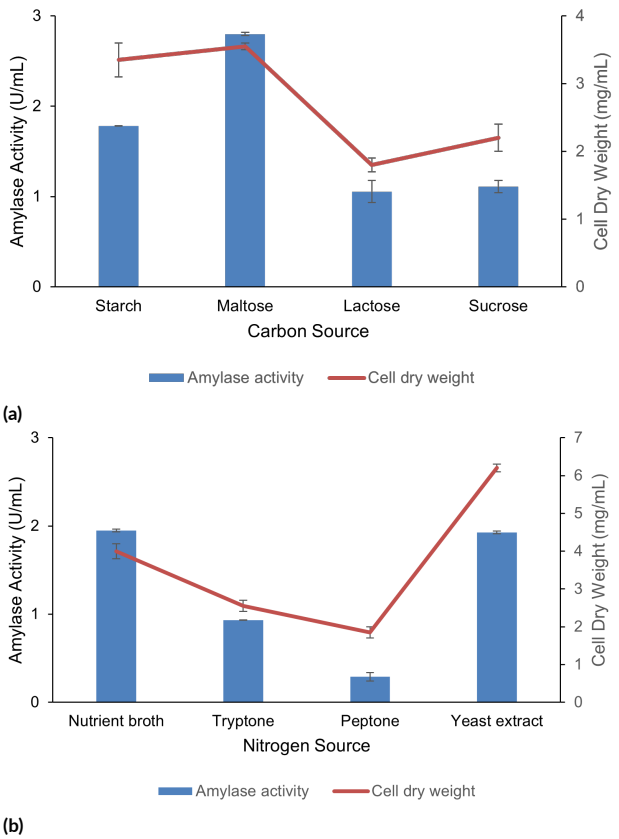

FIGURE 4 The activity of extracellular alpha-amylase produced by *Marinobacter* sp. LES TG5 on various carbon sources (a) and nitrogen sources (b).

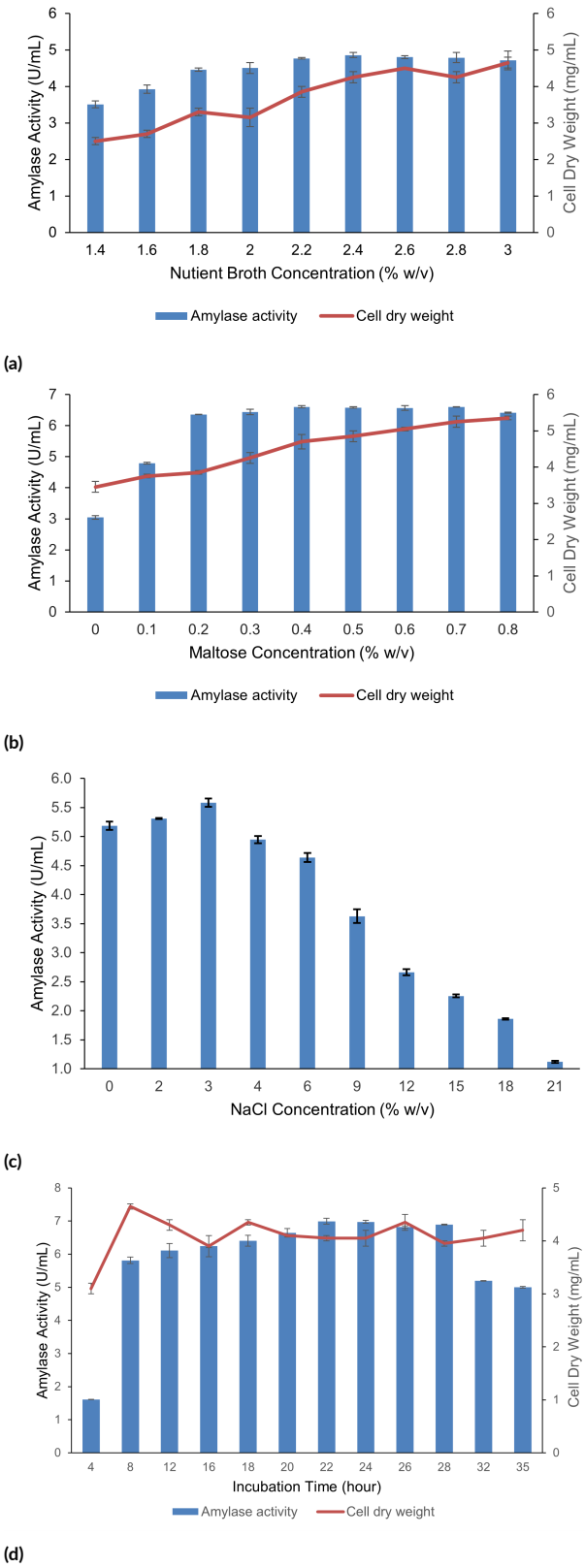

FIGURE 5 The activity of extracellular alpha-amylase produced by *Marinobacter* sp. LES TG5 on various concentrations of nutrient broth (a), maltose (b), NaCl (c), and variation of incubation time (d)

respond to a proportional increase in amylase activity.

Following the screening results, maltose and nutrient broth were selected as the optimal carbon and nitrogen sources for subsequent amylase production. Further optimization of the production medium then focused on the concentrations of nutrient broth, maltose, and salt. The study optimized nutrient broth concentration within a range of 1.4% to 3.0% (w/v), with the results presented in Figure 5a. Amylase activity increased from 3.5 U/mL to 4.9 U/mL as the nutrient broth concentration rose from 1.4% to 2.4% (w/v). However, at nutrient broth concentrations exceeding 2.4% (w/v), the amylase activity remained relatively stable. Bacterial cell density generally mirrored the trend observed for amylase activity. These findings indicate that 2.4% (w/v) is the optimum nutrient broth concentration for amylase production by *Marinobacter* sp. LES TG5.

Maltose optimization was conducted across concentrations ranging from 0% to 0.8% (w/v), with the results presented in Figure 5b. The bacterium exhibited extracellular amylase activity of 3.05 U/mL even without maltose supplementation. However, the presence of maltose significantly enhanced amylase production, reaching 4.79 U/mL and 6.36 U/mL with the addition of 0.1% (w/v) and 0.2% (w/v) maltose, respectively. Further increases in maltose levels did not substantially boost amylase production, which achieved an optimal value of 6.61 U/mL at 0.4% (w/v) maltose. Figure 4b also illustrates that increasing maltose content in the medium gradually increased bacterial cell density from 3.45 mg/mL (at 0% w/v maltose) to 5.35 mg/mL (at 0.8% w/v maltose).

Subsequent optimization focused on the salt content in the medium. Figure 5c shows that the bacterium could grow and produce extracellular amylase with an activity of 5.64 U/mL and a cell density of 2.5 mg/mL even without added salt. Increasing the salt content continuously enhanced cell density, though not extracellular amylase activity proportionally. Amylase activity increased from 5.64 U/mL to 6.62 U/mL with an increase in NaCl content from 0% to 3% (w/v). At NaCl concentrations above 3% (w/v), amylase activity slightly decreased and then remained relatively stable up to 10% (w/v) NaCl. These data indicate that 3% (w/v) is the optimal NaCl concentration for amylase production by the bacterium.

The final optimization involved the incubation time; the results are presented in Figure 5d. After 4 hours of incubation at 37 °C and 180 rpm, extracellular amylase activity reached 1.61 U/mL. Extending the incubation time to 8 hours more than tripled amylase production to 5.81 U/mL. Subsequent increases in incubation time resulted in a slower rise in amylase production, reaching an optimum activity of 6.99 U/mL at 22 hours. Amylase activity remained relatively stable from 22 to 28 hours of incubation but significantly decreased thereafter. Bacterial cell density exhibited a similar trend to amylase activity. Incubation for 8 hours led to a very significant increase in bacterial density, reaching 4.65 mg/mL. Following this, bacterial density tended to stabilize up to 35 hours of incubation.

3.4. Characterization of alpha-amylase

3.4.1 Effect of NaCl, pH, and temperature

The initial characterization of the extracellular alpha-amylase produced by *Marinobacter* sp. LES TG5 focused on its activity across varying salt concentrations, pH levels, and temperatures. Figure 6a illustrates the effect of salt on amylase activity. Optimal activity was observed at NaCl concentrations below 9% (w/v), reaching a maximum of 5.58 U/mL at 3% (w/v) NaCl. Conversely, NaCl concentrations exceeding 9% (w/v) led to a drastic decrease in amylase activity, with a reduction of over 50%. Notably, despite being produced by a halophilic bacterium, the amylase maintained good activity at 0% (w/v) NaCl, measuring 5.18 U/mL.

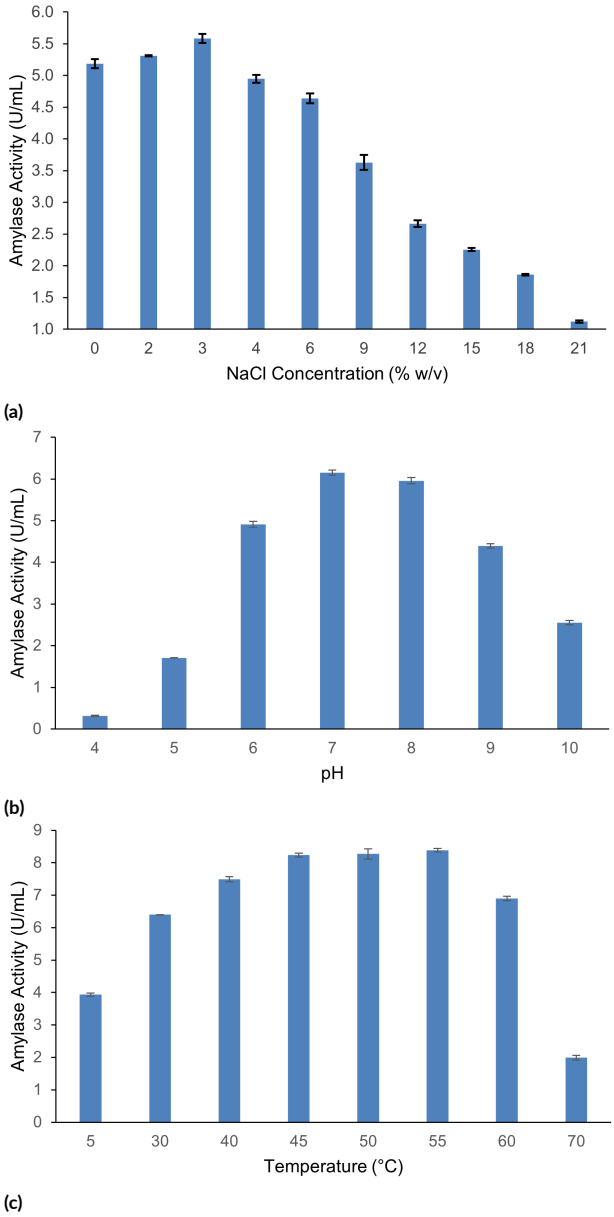

FIGURE 6 Effect of NaCl (a), pH (b), and temperature (c) on the activity of extracellular alpha-amylase produced by *Marinobacter* sp. LES TG5.

The amylase demonstrated robust activity within the pH range of 6 to 9, achieving an optimum of 6.15 U/mL at pH 7 (Figure 6b). Acidic conditions below pH 6 significantly impaired amylase activity, causing a reduction of over 70%. Similarly, under alkaline conditions above pH 9, amylase activity decreased substantially by more than 50%.

Regarding temperature, the amylase exhibited good tolerance from 30 °C to 60 °C, with maximum activity of 8.38 U/mL occurring at 55 °C (Figure 6c). However, temperatures above 60 °C resulted in a sharp decline in activity, with only 24% of its catalytic activity remaining at 70 °C. Interestingly, the amylase retained over 45% of its catalytic activity even at 5 °C.

3.4.2 Effect of cations, organic solvents, surfactants, and commercial detergents

Next, we characterized the alpha-amylase to determine the effect of cations, organic solvents, and surfactants/detergents on its activity. Figure 7a illustrates the impact of various cations and EDTA on amylase activity. Cations like Ca^{2+} and Mg^{2+} did not significantly affect amylase activity, while Mn^{2+}, Co^{2+}, and Ba^{2+} caused a slight decrease. Conversely, Pb^{2+}, Al^{3+}, Fe^{2+}, and Sn^{2+} drastically reduced amylase activity by over 50%. Notably, Cu^{2+} led to a complete loss of catalytic activity. The addition of EDTA caused the amylase to lose more than half of its activity, suggesting its classification as a metalloenzyme.

Amylase activity in the presence of various organic solvents is presented in Figure 7b. The enzyme's activity was unaffected by n-hexane at either 25% or 50% (v/v) solvent concentrations. Amylase also demonstrated good stability in methanol and ethanol at 25% (v/v), retaining over 80% of its catalytic activity. However, at 50% (v/v) concentrations of methanol and ethanol, activity dropped drastically to approximately 10% of the control. Chloroform had a pronounced negative effect on amylase activity at both 25% and 50% (v/v), reducing activity to approximately 20% of the control.

The influence of surfactants and detergents on amylase activity is shown in Figure 7c. Surfactants, specifically Triton X-100 and Tween 80, resulted in a significant decrease in amylase activity by over 50%. In contrast, the amylase maintained more than 50% of its activity after 1 hour of incubation in 25% (v/v) dishwashing liquid and liquid laundry detergent. However, at 50% (v/v) concentrations, amylase activity in both commercial detergents decreased by over 50%.

3.5. Discussion

This study successfully isolated halophilic bacteria capable of producing extracellular alpha-amylase from traditional salt ponds in Les Village, Tedjakula District, Buleleng Regency, Bali Province, Indonesia. Interestingly, we obtained more amylase-producing bacterial isolates from soil samples compared to seawater samples. The

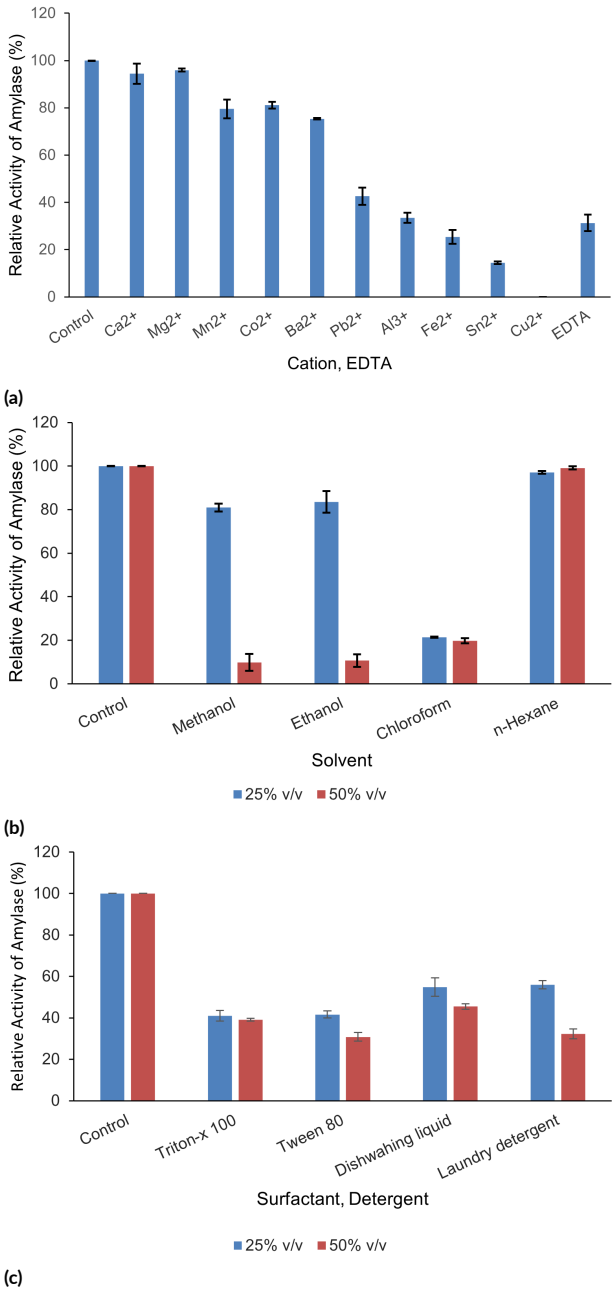

FIGURE 7 Effect of cation (a), solvent (b), and surfactant/detergent (c) on the activity of extracellular alpha-amylase produced by *Marinobacter* sp. LES TG5.

most potent amylase producer identified was Marinobacter sp. This finding aligns with research by Raddadi et al. (2017), who successfully isolated *Marinobacter* species from marine sediments, further supporting the notion that soil or sediment environments are common habitats for these halophilic bacteria. While various *Marinobacter* species have been extensively studied for their biotechnological potential, reports on their amylolytic activity are less common. To date, one previous study has documented amylase production by another *Marinobacter* sp., also isolated from water and soil samples sourced from a salt lake (Kumar and Khare 2015). A comparison of the optimal conditions for amylase production and enzyme characteristics for both bacterial isolates is summarized in Table 1.

The optimization experiments demonstrated that the improved media composition and incubation time significantly enhanced extracellular alpha-amylase production by *Marinobacter* sp. LES TG5. Among the nitrogen sources tested, nutrient broth proved to be the most effective for amylase synthesis by this bacterium. Given that nutrient broth is composed of 0.5% (w/v) peptone and 0.15% (w/v) yeast extract, and considering that peptone alone was a poor nitrogen source for amylase production. In contrast, yeast extract yielded only slightly lower activity than nutrient broth; it can be inferred that yeast extract is a primary contributor to amylase production by *Marinobacter* sp. LES TG5 within the nutrient broth formulation.

This finding contrasts with a previous study by Kumar and Khare (2015) on *Marinobacter* sp EMB8, which reported that tryptone resulted in higher amylase activity compared to yeast extract or a combination of peptone and yeast extract. In our current study, however, tryptone showed a lesser contribution to amylase production than both yeast extract and nutrient broth. This discrepancy suggests that different strains within the same Marinobacter species may exhibit distinct preferences for nutrient sources when it comes to optimizing amylase production.

In this study, maltose was identified as the optimal carbon source for extracellular alpha-amylase production by *Marinobacter* sp. LES TG5. This finding differs from previous research on *Marinobacter* sp. EMB8, where starch was identified as the optimal carbon source for amylase production (Kumar and Khare 2015). However, our results align with other studies, such as those on the thermohalophilic bacterium *Bacillus* sp. NRC22017 (Elmansy et al. 2018) and the halophilic bacterium *Cytobacillus oceanisediminis* isolate AHB6 (Hashem and Yousef 2024). Both of which found maltose to be superior to starch for amylase synthesis. It's known that maltose and glucose, being products of starch catabolism by alpha-amylase, can act as catabolite repressors for alpha-amylase production. For instance, maltose concentrations between 0.5–1.5% (w/v) were shown to reduce amylase production from *Streptomyces* sp. MSC702 by over 20%, with even more drastic drops (over 70%) at concentrations exceeding 1.5% (w/v) (Singh et al. 2012). Interestingly, in contrast to this typical catabolite repression, our study observed an increase in alpha-amylase production by *Marinobacter* sp. LES TG5 with maltose concentrations ranging from 0.2–0.8% (w/v), reaching a maximum at 0.4% (w/v) maltose. This suggests a unique regulatory mechanism in *Marinobacter* sp. LES TG5, where maltose might initially stimulate or not repress amylase production within this concentration range.

Marinobacter sp. LES TG5 demonstrated the ability to produce extracellular alpha-amylase across a broad NaCl concentration range of 0–10% (w/v), with optimal activity achieved at 3% (w/v) NaCl. Notably, the bacterium even produced amylase with high activity in the

TABLE 1 Properties of 33 isolates analyzed for various plant growth-promoting characteristics.

Parameter	*Marinobacter* sp. LES TG5 (this study)	*Marinobacter* sp. EMB8 Kumar and Khare (2015, 2012)
Production of alpha-amylase		
Optimum nitrogen source	nutrient broth, yeast extract	Casein enzyme hydrolysate, tryptone
Optimum carbon source	maltose	starch
NaCl concentration range	0–18% w/v	1–20% w/v
Optimum NaCl concentration	3% w/v	5% w/v
Optimum incubation time	22 hours	54 hours
Characterization of alpha-amylase		
Optimum pH	7	7
Optimum temperature	55 °C	45 °C
Optimum NaCl concentration	3% w/v	1% w/v
Cations (Ca^{2+} and Mg^{2+})	unaffective	unaffective
Absolute inhibitor	Cu^{2+}	Hg^{2+}
Residual activity after incubation in 25% v/v n-hexane	97% (*)	108% (**)
Residual activity after incubation in 25% v/v ethanol	84% (*)	36% (**)

(*) Incubation at 37 °C for 1 hour; (**) Incubation at 37 °C for 24 hours

complete absence of salt. This finding suggests that the bacterial isolate exhibits characteristics of both halophilic and halotolerant organisms. This characteristic is highly advantageous for enzyme production from halophilic bacteria, offering flexibility to conduct processes with or without salt. In contrast, amylase production from *Marinobacter* sp. EMB8 was reported to be very low without NaCl (Kumar and Khare 2015), highlighting a key difference between the strains.

Regarding incubation time, *Marinobacter* sp. LES TG5 produced high amylase activity after just 8 hours, reaching its optimum at 22 hours. This contrasts sharply with *Marinobacter* sp. EMB8, whose amylase production showed a significant increase only after 30 hours of incubation, reaching maximum activity at 54 hours (Kumar and Khare 2015). This rapid production profile demonstrates the superior efficiency of *Marinobacter* sp. LES TG5 in synthesizing amylase compared to previously studied strains, making it a promising candidate for industrial applications requiring faster enzyme yields.

The optimization of media nutrition and incubation time in this study successfully increased alpha-amylase production from *Marinobacter* sp. LES TG5 by over 11-fold, rising from an initial 0.63 U/mL to 6.99 U/mL. For comparison, a similar optimization effort for *Marinobacter* sp. EMB8 by Kumar and Khare (2015) resulted in a 12-fold increase in amylase production. While the relative improvement in *Marinobacter* sp. EMB8 was slightly higher, the optimized activity achieved by *Marinobacter* sp. LES TG5 still represents a substantial enhancement, making it a promising candidate for industrial amylase production.

The optimum pH for alpha-amylase activity from *Marinobacter* sp. LES TG5 in this study was observed to be pH 7, aligning with findings for a similar bacterium, *Marinobacter* sp. EMB8. However, the optimum temperature for amylase activity in our study was slightly higher, at 55 °C, compared to 45 °C for *Marinobacter* sp. EMB8 (Kumar and Khare 2012). Interestingly, the amylase produced by *Marinobacter* sp. LES TG5 demonstrated notable stability at low temperatures, retaining over 45% of its activity at 5 °C. This characteristic is particularly advantageous given the increasing focus on low-temperature processes in the food and beverage industry. Such processes offer several benefits, including enhanced control over cold-active enzymes, minimization of undesirable chemical reactions, effective prevention of contamination and spoilage, and better preservation of labile and complex flavor profiles (Pulicherla et al. 2011).

The optimum salt content for alpha-amylase activity in this study was 3% (w/v) NaCl, which is slightly higher than the 1% (w/v) NaCl reported for amylase from *Marinobacter* sp. EMB8 (Kumar and Khare 2012). Furthermore, while amylase from *Marinobacter* sp. EMB8 reportedly lost its catalytic activity in the absence of NaCl, the amylase from *Marinobacter* sp. LES TG5 maintained high activity even without salt. This characteristic is particularly advantageous for industrial applications that do not utilize salt in their production processes, such as the alcohol, textile, and paper industries.

Several studies have reported an increase in alpha-amylase activity in the presence of Ca^{2+} and Mg^{2+}, including amylase produced by *Haloarcula* sp. HS (Gómez-Villegas et al. 2021) and by *Pseudomonas balearica* VITPS19 (Kizhakedathil and C 2021). However, the amylase from *Marinobacter* sp. LES TG5 in this study was not significantly affected by either of these cations. Another study reported that the amylase activity produced by *Bacil-*

lus licheniformis RA31 was also not affected by Mg^{2+} but was drastically reduced by Ca^{2+} (Sharma et al. 2022). Our results are consistent with other research showing that cations like Fe^{2+} and Cu^{2+} negatively impact amylase activity, as observed in *Pseudomonas balearica* VITPS19 and *Bacillus licheniformis* RA31. Notably, Cu^{2+} caused a complete loss of catalytic activity for the amylase from *Marinobacter* sp. LES TG5, an extreme inhibitory effect also reported for Bacillus licheniformis RA31 amylase.

The alpha-amylase produced by *Marinobacter* sp. LES TG5 in this study exhibited excellent stability in n-hexane solvent, a characteristic similar to the amylase from *Marinobacter* sp. EMB8 (Kumar and Khare 2012). Furthermore, the amylase from *Marinobacter* sp. LES TG5 demonstrated superior stability to ethanol and methanol compared to amylase from *Bacillus licheniformis* RA31. Our enzyme maintained over 80% of its catalytic activity after 1 hour of incubation in 25% (v/v) ethanol or methanol. In contrast, the amylase activity from Bacillus licheniformis RA31 decreased by more than 50% after just 10 minutes of incubation in 20% ethanol or methanol (Sharma et al. 2022). The notable tolerance of amylase to organic solvents is highly advantageous for its application in catalytic processes, particularly in the synthesis of maltooligosaccharides (MOS) (Kumar and Khare 2012). This is significant because MOS is an important compound widely utilized in both the food and pharmaceutical industries (Yavari-Bafghi and Amoozegar 2025).

The alpha-amylase from *Marinobacter* sp. LES TG5 also displayed good stability against surfactants and commercial detergents. The enzyme retained over 50% of its activity after 1 hour of incubation in 25% (v/v) liquid detergent or dishwashing liquid. Even in a 50% (v/v) detergent solution, the enzyme maintained more than 30% of its activity. This stability against commercial detergents is also a characteristic reported for amylase produced by *Bacillus licheniformis* RA31 (Sharma et al. 2022). Such resilience provides significant advantages for the application of these enzymes as detergent additives, enhancing cleaning efficiency.

4. Conclusions

This study successfully isolated *Marinobacter* sp. LES TG5, a halophilic bacterium producing extracellular alpha-amylase, from traditional salt ponds in Les Village, Bali, Indonesia. Optimization of the production medium and incubation time significantly enhanced amylase yield, increasing it by 11-fold (from 0.63 U/mL to 6.99 U/mL). These findings present significant opportunities for the development of industrial-scale amylase production. Characterization revealed that the amylase exhibits optimal activity at pH 7, 55 °C, and 3% (w/v) NaCl. These findings demonstrate the enzyme's potential as a biocatalyst for production processes conducted under moderate temperatures and in the presence of low salt concentrations. Notably, the amylase demonstrated excellent stability in organic solvents such as methanol, ethanol, and n-hexane, highlighting its potential as a biocatalyst for chemical synthesis in non-aqueous environments. Furthermore, its considerable stability against surfactants and detergents suggests its promise as an effective additive in various cleaning products.

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Authors' contributions

PP designed the study. PP, KSMJ carried out the laboratory work and analyzed the data. PP wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that there is no conflict of interest.

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