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Amylase Enzyme Production in Bacteria Associated with Marine Macroalgae: Screening, Optimization and Biofilm Inhibitory Activity

Sathianeson Satheesh * and Lafi Al Solami

Department of Marine Biology, Faculty of Marine Sciences, King Abdulaziz University, Jeddah 21589, Saudi Arabia; lalsulami@kau.edu.sa

* Correspondence: ssathianeson@kau.edu.sa or satheesh_s2005@yahoo.co.in

Abstract

Bacteria associated with marine macroalgae are considered a promising source for secondary metabolites and industrially significant enzymes. Amylases, which are commercially important enzymes mainly isolated from microorganisms, exhibit antibacterial, anti-inflammatory, anti-viral and antibiofilm activities. In this study, bacteria associated with the green macroalga *Ulva fasciata* were explored for amylase enzyme production, optimization and antibiofilm activity against marine biofilm-forming bacteria. A total of 12 amylase-producing bacterial strains were obtained from the alga. Among the strains, strain MD02 showed higher amylase activity (138.2 U mL^{-1}) and strong biofilm inhibitory activity (89.5% inhibition). Molecular identification of strain MD02 showed similarity with *Bacillus* sp. The parameters influencing amylase production were initially tested using the traditional approach (one factor) followed by a two-level full factorial design and central composite design combined with response surface methodology. Results of statistical optimization showed a higher amylase yield (307.1 U mg^{-1}) at pH 7.5, 0.75% inoculum and 0.7% glucose. This study advances our knowledge of the significance of *Ulva*-associated marine bacteria as a source of amylase enzymes and an effective biofilm control agent. Overall, this study highlights the potential significance of marine-algae-associated bacteria for enzyme production and demonstrates the feasibility of cost-effective amylase enzyme production using low-cost substrates.

Keywords: marine bacteria; *Ulva fasciata*; biofilms; biofilm control; amylase enzyme; solid state fermentation

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Academic Editor: Yihan Liu

Received: 31 December 2025
Revised: 5 February 2026
Accepted: 11 February 2026
Published: 13 February 2026

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1. Introduction

Marine microorganisms are considered a promising source for the discovery of bioactive metabolites for industrial and pharmacological applications [1,2]. Due to the dynamic environmental conditions, marine organisms including microorganisms are under stress from changing salinity, pH, temperature, nutrient levels and pressure [3–5]. Microorganisms living under these conditions may produce novel enzymes to cope with environmental challenges [6–8]. Therefore, marine microorganisms are commonly used as a source for extracting many enzymes [9,10]. Microorganisms isolated from different marine habitats such as sediments, water, mangroves and invertebrates are reported to produce enzymes like amylase, chitinase, glucosidase and xylanase [9,11,12].

Marine macroalgae (seaweeds) provide habitat for many organisms including microorganisms [13,14]. Diverse bacterial communities are associated with macroalgae and

provide crucial support to algal health and growth [15,16]. The green macroalgae of the genus *Ulva* represent one of the significant niches for the microbial communities due to their surface characteristics [13]. The interactions between bacteria and marine macroalgae are a topic of interest due to the exploration of industrially and biotechnologically significant metabolites from these associated bacteria [17,18]. Bacteria associated with marine macroalgae are a source of many enzymes such as amylases, proteases, lipases, cellulases and agarases [13,14,19]. Enzymes produced by macroalgae-associated bacteria may help in the degradation of cell wall components of the algae [20]. Hence, these enzymes are considered a novel source for industrial applications due to their stability and degradation ability [20,21].

Amylases are important enzymes used in many commercial applications, hydrolysing starch [22]. They are utilized in biomedical, textile, food, detergent and chemical industries [23,24]. The interest in amylase production has increased in recent years due to its wide applications and low cost [25]. Although amylase enzymes can be isolated from plant and animal sources, microorganisms are deemed more favorable due to growth methodologies for large-scale production and the stability of the enzymes to meet commercial needs [25–27].

Among the various biotechnological applications of amylase enzymes, their antibiofilm and antibacterial activities are widely studied for potential use in environmental and clinical biofilm control [28–32]. The adhesion of microorganisms on hard substrates submerged in aquatic environments leads to the formation of biofilms [33]. The biofilm community enables the development of macrofouling communities on hard substrates in the marine environment. Biofouling causes significant technical problems for water intake systems of desalination and coastal powerplants, maritime sectors and marine aquaculture [34,35]. In the health sector, biofilms are a serious concern due to their role in infection, resistance to antibiotic treatment and tolerance to environmental stress [36,37]. Due to the increasing economic significance of biofilms on marine structures, many antibiofilm methods are used for biofilm/biofouling control. The enzyme-based biofilm control approach offers promising avenues for the use of marine microbial enzymes to fight against biofilms and antibiotic resistance [38–40].

Many factors such as pH, temperature, nutrient content in the culture medium, incubation period and inoculum size influence the production of amylase enzyme in bacteria [26]. Therefore, optimizing enzyme-producing conditions is essential for higher yields and applications. This study assessed the antibiofilm activity of alpha-amylase enzyme produced by marine bacteria associated with the green macroalgae *Ulva fasciata* against biofilm-forming bacteria. Additionally, the enzyme production conditions were optimized to understand the suitable environment for enzyme production. The results observed in this study will enhance our knowledge of the antibiofilm efficiency of alpha-amylase enzyme produced by marine-algae-associated bacteria. Although many previous studies have reported on amylase enzyme production or antibiofilm activity separately, research that integrates the production and antibiofilm efficacy of enzymes from marine sources is limited. This study bridges amylase enzyme production through optimization and biofilm inhibition efficacy, thereby enhancing the translational potential of marine microbial enzymes.

2. Materials and Methods

2.1. Isolation of Marine Bacteria

The green macroalga *Ulva fasciata* was collected from the Obhur coastal area of Jeddah. The collected algal samples were rinsed with filtered and sterilized seawater. Each algal sample (1 g) was cut into small pieces using sterile scissors and placed in a tube containing 9 mL of sterile seawater. The test tubes were then vigorously vortexed to release the

microbiota associated with the algae. The resulting suspension was serially diluted and plated on marine agar plates (Zobell Marine Agar, HiMedia, Mumbai, India). The plates were placed in an incubator at 30 °C for 24–48 h. After incubation, the bacterial colonies that grew on the plates were isolated and purified using the streak plate method. The purified bacteria colonies were stored on marine agar slants at 4 °C for further studies.

2.2. Screening of Amylase-Enzyme-Producing Bacteria

The bacterial isolates were screened for amylase enzyme production in order to select the best amylase-enzyme-producing strains. The method described by Shaw et al. [41] was used with slight modifications. The screening was conducted using starch agar medium (Himedia, Mumbai, India). The composition of the culture medium was as follows: soluble starch (20 g/L), peptone (20 g/L) and agar (20 g/L). The culture plates were kept in an incubator at 30 °C for 48 h. Following the incubation period, Gram's iodine solution was added to the plates to observe clear zone formation resulting from starch hydrolysis [42]. A total of 12 amylase-producing bacterial strains were isolated with clear zone sizes ranging from 9 to 21 mm. Strains exhibiting a zone of hydrolysis greater than 10 mm were selected for submerged fermentation and enzyme assay.

2.3. Culture of Bacteria and Amylase Production

Initially, the bacterial strains were activated on LB medium containing yeast extract (5 g/L), NaCl (10 g/L) and tryptone (10 g/L) in conical flasks. The flasks were placed in an incubator shaker (37 °C) for 24 h at 150 rpm. Thereafter, the bacterial culture was transferred to a culture medium consisting of 20 g/L of glucose, 10 g/L of sodium chloride and 10 g/L of yeast extract. The other components of the culture medium included $MgSO_4$ (1 g/L), KH_2PO_4 (2 g/L) and $CaCl_2$ (2 g/L). Following that, the culture was kept in a shaker incubator at 37 °C for 18 h at 120 rpm. After the initial growth and activation of the bacteria using nutrient medium, the bacterial culture was used as an inoculum for amylase production under submerged fermentation conditions.

A fermentation medium was prepared for enzyme production (using the submerged fermentation method). The medium consisted of peptone (0.6%), potassium chloride (0.05%), sodium chloride (0.3%), magnesium sulphate (0.05%) and starch (1%). The pH of the fermentation medium was adjusted to 7. To begin the process, 50 mL of the fermentation medium was placed in a conical flask and 0.5 mL of bacterial culture was added. The flasks were then kept in an incubator shaker set at 37 °C and 130 rpm agitation. The cultures were incubated for 24 h and centrifuged at 8000× *g* at 4 °C for 15 min. After centrifugation, the supernatant was collected and used for the amylase enzyme assay.

2.4. Amylase Assay

Amylase enzyme activity was determined using soluble starch as a substrate according to the method reported previously [43]. Briefly, the crude enzyme (1 mL) extracted from the bacteria was mixed with an equal volume of soluble starch (1% *w*/*v*) in a test tube. The samples were mixed thoroughly and placed in an incubator at 37 °C in a water bath for 15 min. Thereafter, 2 mL of 3,5 dinitrosalicylic acid (DNS) was added to the test tubes and they were kept for 5 min in a boiling water bath. The tubes were cooled immediately in running water to halt the enzymatic reaction. The absorbance of the sample was determined using a spectrophotometer at 540 nm. Substrate (starch) and enzyme were used as a blank for the measurement. The enzyme activity (U/mL) was calculated based on the change in sugar concentration using the standard curve. The enzyme activity is expressed as U/mL for submerged fermentation, whereas the activity is reported as U mg^{-1} after solid-state fermentation.

2.5. Determination of Biofilm Inhibitory Activity of Crude Enzyme

The biofilm-forming bacterium *Vibrio alginolyticus* IMB11 (GenBank accession number: ON003958) was used as the target organism for the antibiofilm assay. This bacterium was obtained from the stock culture from our laboratory at King Abdulaziz University. The bacterial strain was inoculated in marine broth (Himedia, Mumbai, India) and kept in an incubator at 28 °C for 24 h. The biofilm inhibitory activity of the α-amylase enzyme was determined by microtiter plate assay as outlined previously [44]. In brief, crude α-amylase enzyme from the selected bacterial strain MD02 (100 µL/mL) was added to the wells containing the target bacterial culture (in marine broth, 10^7 CFU/mL). The controls were maintained without the addition of the enzyme, and marine broth with enzyme was used as a blank. The plates were then incubated at 37 °C for 24 h in an incubator. After incubation, the contents of the wells were decanted and washed three times with distilled water to remove unattached bacterial cells. The wells were air-dried, and 200 µL of crystal violet (1%) was added. The plates were left for 15 min at room temperature (28 °C) for staining of the cells. Following this, the excess stain was washed away gently with distilled water, and the wells were dried again. The cells bound to the stain were solubilized by adding 200 µL of ethanol (95%) and the absorbance of the wells was read using a microplate reader (Bio Tek, Elx 800, Winooski, VT, USA) at 570 nm. The biofilm inhibition percentage was calculated using the following formula:

$$\text{Percentage Biofilm inhibition activity } (\%) = 100 - \left(\frac{(B \times 100)}{C}\right)$$

where B is the OD_{570} of the wells treated with the enzyme and C is OD_{570} of the wells without the addition of the enzyme.

2.6. Identification and Molecular Characterization of Bacteria

DNA was isolated from the potent bacterial strain using InstaGene matrix according to the procedure outlined by the manufacturer. Genomic DNA was amplified using universal primers as previously described [32,45]. The amplicon was purified and sequenced using an automated sequencer (Applied Biosystems, Foster city, CA, USA). The identification of the bacterium was carried out by NCBI BLAST search (BLASTN 2.17.0+), and the sequence was submitted to GenBank (NCBI) (accession number: PX760387). The phylogenetic analysis was conducted using the MEGA program (version 4).

2.7. Screening of Different Substrates for Amylase Production in Solid-State Fermentation

Substrates such as potato peel, sweet potato peel, wheat bran and tapioca peel were screened for higher enzyme production. The raw materials for these substrates were collected from the local market. The substrates were dried, ground into a powder and sieved through a 2 mm mesh. The substrates (about 5 g) were taken in conical flasks, then hydrated with 4 mL of distilled water and autoclaved. A freshly prepared algal-associated bacterial culture (1×10^6 CFU/mL) was then inoculated (0.5 mL) into the flasks. The flasks were incubated in a static incubator at 37 °C for 48 h. The screening assay revealed higher enzyme activity in tapioca peel (see Supplementary Table S1). Due to the observed higher enzyme activity, tapioca peel was chosen as the substrate for further optimization studies.

2.8. Optimization of Amylase Production by One-Factor-at-a-Time Approach

This experiment was conducted in 100 mL flasks containing 5 g of the substrate (which showed the best amylase enzyme activity) to test the influence of factors such as moisture content, inoculum size and pH on enzyme production. The effect of moisture content on enzyme production was checked by adjusting the moisture content of the substrate

between 60% and 80%. Furthermore, the optimum inoculum size for higher amylase enzyme production was determined by testing inoculum sizes between 0.25% and 1.25%. The inoculum size range was selected based on preliminary trials. The pH of the substrate was also adjusted between 6.0 and 9.0 using buffer solutions to know the effects on enzyme production. In addition to the factors mentioned, amylase production was estimated using carbon sources such as starch, sucrose, maltose, xylose, glucose and lactose. The bacterial isolate was grown in basal media and selected carbon sources were added to the substrate at a concentration of 1% (*w*/*w*). Finally, the influence of nitrogen sources on amylase enzyme production was tested by adding 1% (*w*/*w*) of ammonium sulphate, sodium nitrate, potassium nitrate, beef extract, peptone and yeast extract to the substrate. The flasks were placed in an incubator at 37 °C for 72 h. Thereafter, the cell-free supernatant obtained was used for the measurement of amylase enzyme activity.

2.9. Statistical Optimization of Amylase Production Using Tapioca Peel in Solid-State Fermentation

2.9.1. Screening of Factors Influencing Amylase Production via a Statistical Approach

The factors identified through the traditional one-factor approach were used for secondary screening using a 2^5 full factorial design. The two-level full factorial design is an important statistical method for screening a minimal number of variables to enhance the product yield. The design consisted of 32 experiments with the five selected factors being moisture content (60–80%), pH (6.0–8.0), inoculum (0.5–1.0%), starch (carbon source) and ammonium sulphate (nitrogen source). Two levels (low and high, represented by − and +) were chosen for screening the variables (Table 1). To conduct the experiment, 5 g of tapioca peel powder was combined with an appropriate buffer at a specified pH. The culture medium was then sterilized at 121 °C for 30 min and cooled. The bacterial culture was added to the mixture and placed in an incubator for 72 h at 37 °C. After 72 h of fermentation, amylase was extracted, and the enzyme activity was assessed. One-way analysis of variance (ANOVA) was used to test the model's significance. The statistical analysis was conducted using "Design Expert" software (version 8.0.1).

Table 1. Levels of factors analyzed in a two-level full factorial design for the optimization of amylase biosynthesis.

Factor	Name	Units	−1	1
A	Moisture	%	60	80
B	pH		6	8
C	Inoculum	%	0.5	1
D	Glucose	%	0.1	1
E	Ammonium sulphate	%	0.1	0.5

2.9.2. Optimization of α-Amylase Enzyme Production Using Central Composite Design Combined with Response Surface Methodology

Amylase production was analyzed using central composite design (CCD) and response surface methodology (RSM). A total of 20 experimental runs were conducted based on a designed matrix for amylase production. The inoculum, pH and glucose contents ranged from 0.5 to 1%, 6.5 to 8.5, and 0.2 to 1.2%, respectively (Table 2). All five variables were tested at five different levels. Enzyme activity was measured, and the amount of amylase produced was calculated. The significance of the model was assessed statistically, and the significant factors and concentrations were identified. The statistical software Design Expert (version 8.0.1) was used to test the second-order polynomial coefficients. The predicted amylase production was experimentally validated in triplicate. The predicted

variable concentrations were chosen, amylase-producing bacteria were inoculated, and the experiment was conducted in replicates (n = 3). The results are expressed as mean ± SD.

Table 2. Central composite design model for the analysis of factors influencing amylase production.

Factor	Name	Units	Low Actual	High Actual
A	pH		6.5	8.5
B	Inoculum	%	0.5	1
C	Glucose	%	0.2	1.2

3. Results and Discussion

3.1. Screening of Marine Bacteria for Amylase Production

In this study, a total of 12 amylase-producing bacterial strains were isolated from the green alga *U. fasciata*. The zone of hydrolysis varied from 9 to 21 mm. The size of the clear zones produced by the bacteria on starch agar plates is presented in Table 3. Among the 12 strains, six showed a zone diameter greater than 15 mm. Results indicated that all the isolated bacterial strains from the alga were capable of producing amylase enzymes. Bacteria associated with algae are reported to produce many hydrolytic enzymes [21]. These enzymes are crucial for the degradation and mineralization of algal wastes in marine ecosystems [46]. In a previous study, 59.7% of bacteria isolates from different macroalgae exhibited amylase activity [18]. Further, Comba González, Hoyos, Kleine and Castaño [14] reported amylase enzyme activity in about 50% of the epiphytic bacteria associated with the green alga *Ulva lactuca*. The observed results in this study further agree with previous studies which showed the production of enzymes by the majority of bacteria collected from marine sources [9,26,47,48]. Overall, the screening assay proved that bacteria associated with marine macroalgae are a source of amylase enzymes that can be utilized for industrial and biotechnological applications.

Table 3. The zone of hydrolysis produced by potential alpha-amylase-producing bacterial strains isolated from the marine alga in the starch iodine test.

Strain	Zone of Clearance (mm)
MA01	9 ± 1
MA02	10 ± 0
MA03	18 ± 1
MD01	16 ± 1
MD02	21 ± 2
MK03	19 ± 1
MU02	14 ± 2
MU04	17 ± 1
MU09	10 ± 0
MU10	9 ± 1
MV04	12 ± 0
MU06	17 ± 2

3.2. Production of Amylase in Submerged Fermentation

The amylase enzyme activity of the crude enzyme extracted from bacterial strains using the submerged fermentation process is presented in Table 4. Among the 12 strains, strain MD02 exhibited the highest amylase activity of 138.2 U/mL followed by MK03. The strains MA03 and MU04 also showed good amylase enzyme activity. The observed higher amylase enzyme activity in some of the strains indicated the capability of amylase enzyme production by alga-associated bacteria. Previously, Alonazi et al. [49] reported a maximum

amylase enzyme activity of 16 U/mL in *Bacillus pacificus* associated with the brown alga *Turbinaria ornata*. Furthermore, a *Halomonas* sp. isolated from a sponge showed a maximum enzyme activity of 75.27 U/mg [50].

Table 4. Amylase enzyme activity of marine bacteria associated with the green alga *U. fasciata*.

Strain	Amylase Activity (U/mL)
MA02	38.1 ± 5.6
MA03	104.1 ± 3.3
MD01	40.5 ± 4.4
MD02	138.2 ± 2.1
MK03	117.1 ± 1.4
MU02	29.8 ± 2.2
MU04	97.4 ± 1.1
MU09	3.9 ± 0.52
MV04	2.5 ± 0.3
MU06	89.04 ± 4.2

3.3. Biofilm Inhibitory Activity of Crude Enzyme

The crude enzyme extracted from strain MD02 showed the highest biofilm inhibition of 89.5% followed by strain MU06 with 72.5% inhibition (Figure 1). Furthermore, the amylase enzyme of all bacterial strains exhibited comparatively higher biofilm inhibition than the control. The biofilm inhibitory activity of the amylase enzyme may be due to its action on key polysaccharides used by the bacteria for attachment. The mature biofilm consists of a microbial community enclosed by extracellular polymeric substances (EPS) produced by microorganisms [51,52]. The degradation of polysaccharides associated with the biofilm is the major antibiofilm mechanism of amylase enzymes [53]. Previous studies have reported that targeting biofilm EPS with amylase enzymes can reduce the adhesion and growth of biofilms on substrates and pave the way for alternative biofilm control strategies [24,54,55]. Enzymes obtained from bacteria associated with macroalgae may possess thermostability and withstand environmental stress [56], and these characteristics are important for biofilm inhibitory activity. Further, the antibiofilm activities of amylase enzymes isolated from bacteria have been extensively documented in the literature [30,31,57,58]. Although the biofilm inhibition rate varied between bacterial strains and collection sites, the observed biofilm inhibition percentage of the amylase enzyme extracted from strain MD02 indicated the effectiveness of this bacterium for antibiofilm applications. Generally, inhibition above 50% is considered good antibiofilm activity [59], and the observed biofilm inhibition of above 80% aligns with many previous studies which reported the very strong antibiofilm activity of amylase enzyme [30,31]. Moreover, this biofilm inhibition assay has limitations. Only one biofilm-forming bacterium was used in this study to know the inhibitory activity. Also, the degradation ability of amylase enzyme on established biofilms was not tested in this study. The crude enzyme used in this study may contain other extracellular metabolites produced by the bacteria. Hence, further studies including more bacterial strains and biofilm degradation assays are necessary to understand the biofilm inhibition efficiency of the amylase enzyme. Based on amylase enzyme activity and biofilm inhibitory activity, strain MD02 was selected for further optimization studies.

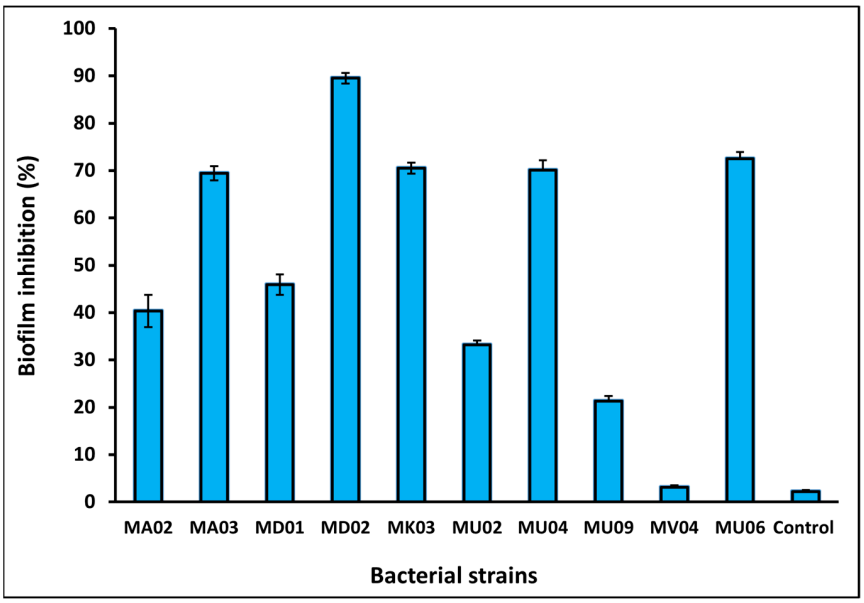


Figure 1. Biofilm inhibitory activity of bacteria associated with the green alga *U. fasciata*. Values are expressed as mean ± SD (n = 3). Error bars represent the SD. Control without enzyme treatment.

3.4. Identification of Bacterium MD02

The bacterial strain MD02 that exhibited higher amylase activity and biofilm inhibitory activity was identified as *Bacillus* sp. Figure 2 shows the phylogenetic tree of the strain MD02 based on 16S rRNA gene sequences. The tree explains the relationship between the MD02 isolate and closely related *Bacillus* sequences retrieved from NCBI GenBank. The phylogenetic analysis demonstrated a close similarity of the 16S rRNA gene sequence of this strain with existing gene sequences of *Bacillus* (in Firmicutes) in the NCBI database. Generally, *Bacillus* spp. are considered as a biotechnologically important group of bacteria due to their capability to produce several industrially significant enzymes including amylase enzyme [60]. Further, many studies indicated that *Bacillus* is a key taxon associated with different macroalgal groups [61–63]. The association of *Bacillus* sp. with the macroalga *U. lactuca* observed in this study indicated the significance of marine macroalgae for the exploration of novel marine microbiota.

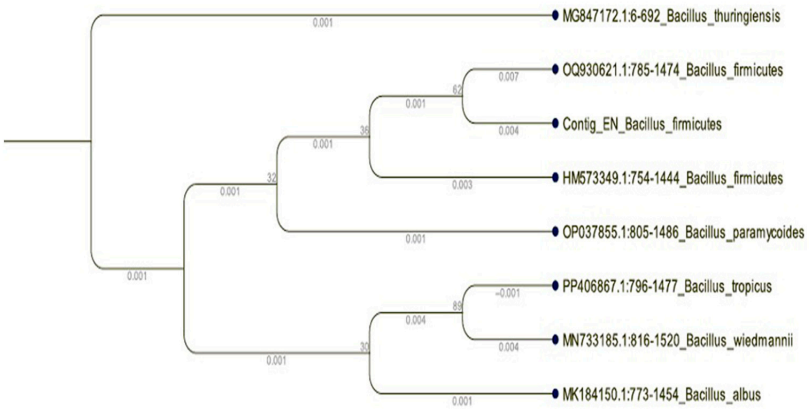


Figure 2. Phylogenetic analysis of bacterial strain MD02 using 16S rRNA gene. The strain was identified as *Bacillus* sp.

3.5. Optimization of Factors Influencing Amylase Enzyme Production Through One-Factor-at-a Time Approach

The medium composition and fermentation conditions for higher enzyme yield may vary between bacterial strains. Hence, an initial optimization study was conducted to

find the parameters for higher amylase production. Figures 3 and 4 show the enzyme production by the bacterium under different conditions. In this traditional method, the amylase enzyme production by the strain MD02 (identified as *Bacillus* sp.) was found to be higher at 70% moisture and pH 8. Moisture content of the substrate used for the fermentation is one of the significant factors which influence the growth of bacteria [64]. Generally, bacteria require 70% moisture content substrate for better growth and enzyme production [64–66].

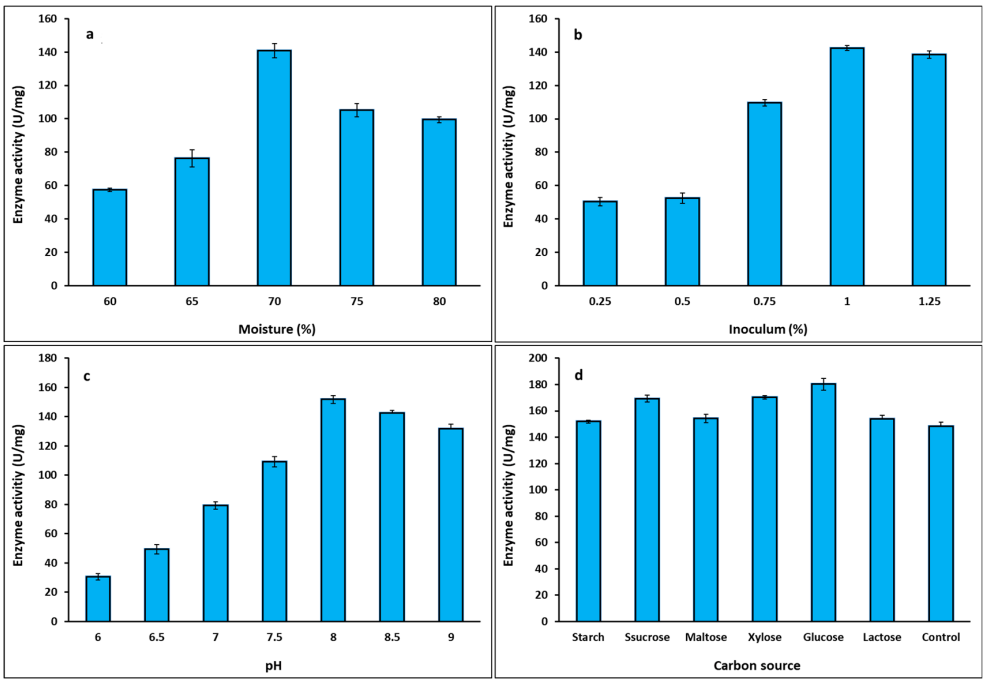


Figure 3. Influence of moisture (**a**), inoculum (**b**), pH (**c**) and carbon sources (**d**) on amylase enzyme production in marine-algae-associated bacterial strain MD02. Values are mean ± SD (n = 3). Error bars represent the SD.

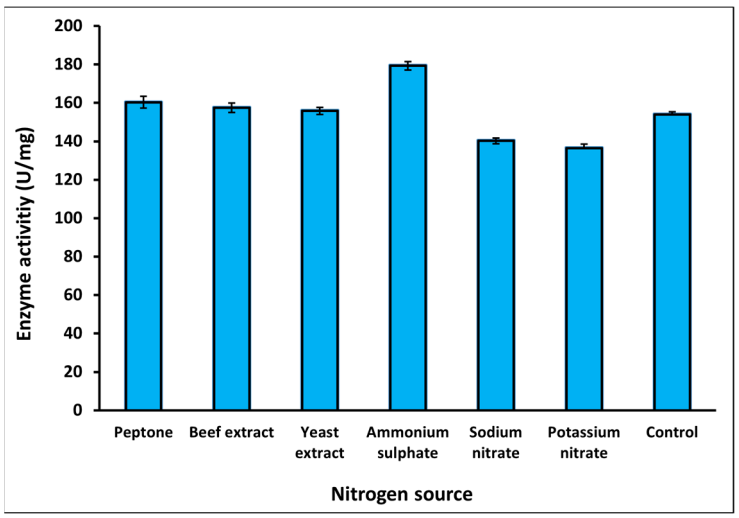


Figure 4. Effects of different nitrogen sources on amylase enzyme activity in marine bacterium *Bacillus* sp. associated with the green alga *U. fasciata*. Values are mean ± SD (n = 3). Error bars represent the SD.

The higher amylase enzyme activity observed at pH 8 indicated alkaline preference of the bacterial strain. The optimum pH for amylase enzyme production may vary depending

on culture medium composition and bacterial species. For example, a study conducted by Alonazi et al. [49] showed pH 9 for optimum enzyme production in bacteria isolated from the brown alga *Turbinaria ornata*. Additionally, an optimum pH 6 for higher amylase enzyme activity in a marine bacterium was reported by Elmansy et al. [26]. To sum up, the literature shows the optimum pH values between 6 and 10 for marine bacteria for higher amylase enzyme activity [67–69]. Enzyme activity showed an increase with the increase in bacterial inoculum of up to 1%. However, no further increase was observed at 1.25%. The results of different inoculum sizes on amylase enzyme activity indicated a dose-dependent effect, although lower and higher inoculum sizes produced low enzyme activity. While there is no unanimity on optimum inoculum size for higher amylase activity in the literature, many studies have reported the inoculum range between 0.5% and 8% for different *Bacillus* species [26,70,71].

Among the different carbon sources tested, the highest enzyme activity was observed in glucose followed by sucrose and xylose. Further, the fermentation medium prepared using ammonium sulphate as nitrogen source showed higher amylase enzyme activity than other nitrogen sources (Figure 4). Many previous studies indicated starch as a preferred carbon source for higher enzyme activity in *Bacillus* species [49,66,72]. However, a previous study by Adetiloye et al. [73] showed higher yield of amylase enzyme in *Bacillus* sp. using glucose as carbon source indicating its suitability in fermentation. While carbon source for amylase enzyme production varies greatly depending on bacterial taxa [74], using glucose as carbon source may inhibit amylase synthesis due to the accumulation of reducing sugars. Hence, further studies are required to optimize the concentration of glucose suitable for amylase enzyme production. The traditional one-factor approach is generally useful for selecting the factors that induce enzyme activity. However, a one-factor-at-a-time approach may not provide the interaction between factors [75,76]. Hence, the factors and nutrient sources which showed high amylase activity were further evaluated by the statistical approach.

3.6. Optimization of Amylase Production by Screening of Selected Variables Affecting Enzyme Production with a Two-Level Full Factorial Design

A two-level full factorial design was used to screen the five significant factors that influence amylase production in SSF. Thirty-two experiments were performed to analyze the influence of five factors on amylase biosynthesis (Table 5). Fisher's test was performed to test the significance of each variable on amylase biosynthesis. According to the designed model, four factors (moisture, pH, inoculum and glucose) strongly affect the biosynthesis of amylase from bacteria. However, ammonium sulphate had little or no effect on amylase production ($p > 0.05$). The F value of 5.43 indicated that the designed model was significant ($p < 0.05$) (Supplementary Table S1). In this experiment, B (pH), C (inoculum), D (glucose), CD and BCD were significant model terms. The adequate precision value was 11.57, indicating an adequate signal of the model. The R^2 value of this two-level full factorial model was 0.908, and the final equation was in terms of coded factors.

$$\begin{aligned}\text{Enzyme activity} = {} & +58.45 + 7.87\text{A} + 20.50\text{B} + 13.11\text{C} + 16.47\text{D} + 1.83\text{E} + 4.64\text{AB} + 7.52\text{AC} + 7.33\text{AD} \\ & +6.46AE + 3.21B + 5.47BD + 7.29BE + 8.78CD + 3.00DE + 4.74ABC + 3.06ABD + 4.78ACD + 3.83ADE + \\ & 16.95BCD + 6.23BDE\end{aligned} \quad (1)$$

Table 5. Screening of variables via a two-level full factorial design. Amylase activity is expressed as U/mg.

Run	Moisture	pH	Inoculum	Glucose	Ammonium Sulphate	Enzyme Activity (U/mg)
1	60	6	0.5	0.1	0.5	10.2
2	80	8	1	0.1	0.1	40.2
3	60	6	0.5	1	0.1	79.4
4	60	8	1	0.1	0.5	40.4
5	80	8	1	1	0.1	190.4
6	80	6	1	1	0.5	76.2
7	60	8	1	1	0.1	70.2
8	60	8	0.5	1	0.1	50.2
9	80	8	1	1	0.5	180.2
10	60	6	1	1	0.5	15.2
11	80	8	0.5	1	0.5	105.2
12	80	6	1	0.1	0.1	50.3
13	60	6	0.5	1	0.5	10.3
14	60	6	1	1	0.1	60.9
15	80	8	1	0.1	0.5	69.3
16	60	6	1	0.1	0.5	40.6
17	60	8	0.5	1	0.5	60.6
18	60	6	0.5	0.1	0.1	10.5
19	80	8	0.5	0.1	0.1	68.5
20	60	8	1	0.1	0.1	40.5
21	60	8	0.5	0.1	0.5	59.4
22	80	6	0.5	1	0.1	39.9
23	80	6	1	0.1	0.5	38.5
24	80	6	0.5	0.1	0.5	9.6
25	80	6	0.5	1	0.5	59.3
26	80	6	0.5	0.1	0.1	5.2
27	80	6	1	1	0.1	50.4
28	60	8	0.5	0.1	0.1	79.3
29	60	6	1	0.1	0.1	50.7
30	80	8	0.5	1	0.1	19.3
31	60	8	1	1	0.5	130.9
32	80	8	0.5	0.1	0.5	58.5

The improvement of amylase production in marine bacteria has been the main focus of various studies owing to their ability to synthesize several valuable biochemicals with significant bioactivity [26,48,77,78]. The wide application of amylases in several industrial and environmental processes has attracted the attention of industrialists and researchers for environmental and industrial uses. Moreover, each bacterial strain has specific nutrient demands and environmental conditions to increase amylase production. Two-level factorial design experiments have been used for screening variables for enzyme production [79–81]. The two-level factorial design has several advantages over other screening methods because this method is efficient and provides valid information through the analysis of various factors at two different levels. The Plackett–Burman design is a widely used screening method for determining significant variables, and this method is likely based on screening the number of variables [82–84]. The two-level factorial design is best suited for screening a minimum number of independent variables. The significant interactions observed between inoculum and glucose (CD), and pH, inoculum and glucose (BCD) in the two-level factorial design of this study indicated the importance of these parameters in enhanced amylase

enzyme production. Understanding these interactions can lead to more efficient and cost-effective enzyme production for industrial use.

3.7. Optimization of Selected Variables for Amylase Production via Central Composite Design and Response Surface Methodology

After initial screening to identify the significant factors via a two-level full factorial design, a CCD experiment was performed to optimize the biosynthesis of bacterial amylase. The relationships between amylase production and three independent variables (pH, inoculum and glucose) were studied, and the response Y (amylase yield) was calculated (Table 6). Design Expert software was used to analyze quadratic polynomial regression; fitness was tested and the regression equation was as follows:

Table 6. Amylase enzyme activity observed with the central composite design used in this study.

Runs	pH	Inoculum (%)	Glucose (%)	Enzyme Activity (U/mg)
1	6.5	0.5	1.2	150.2
2	6.5	0.5	0.2	12.9
3	7.5	0.75	0.7	240.5
4	6.5	1	1.2	69.2
5	7.5	0.32	0.7	155.9
6	7.5	0.75	−0.14	70.4
7	8.5	1	0.2	49.8
8	7.5	0.75	0.7	269.2
9	7.5	0.75	1.54	286.3
10	5.81	0.75	0.7	2.4
11	7.5	1.17	0.7	110.6
12	8.5	0.5	1.2	87.5
13	7.5	0.75	0.7	289.5
14	7.5	0.75	0.7	304.8
15	7.5	0.75	0.7	297.4
16	8.5	1	1.2	228.4
17	8.5	0.5	0.2	69.6
18	7.5	0.75	0.7	285.4
19	6.5	1	0.2	30.5
20	9.181793	0.75	0.7	5.9

Final Equation in Terms of Coded Factors:

$$\text{Enzyme activity} = +281.37 + 13.06A - 1.35B + 53.86C + 23.06AB + 2.56AC + 7.76BC - 99.47A2 - 53.83B2 - 37.88C2 \quad (2)$$

The ANOVA results revealed that the designed CCD model explained 93.73% of the variation in the results (R^2 = 0.937), revealing that the designed model had an acceptable degree of fit and that an R^2 value > 0.75 was preferable. The *p*-value and the lack-of-fit value were 0.93 and 4.59, respectively. In this study, C, A2, B2 and C2 were significant model terms. A summary of the ANOVA results for the quadratic model is depicted in Supplementary Table S2. The 2D contour plot and 3D response surface were used to study the interaction of all three variables and the effect of each variable change on amylase production. The designed CCD model was best fitted to a quadratic polynomial regression equation, and response surface graphs (2D contour and 3D surface plots) were obtained (Figure 5). The elliptical contour plot obtained in this study revealed the significance of the tested factors. Figure 5 depicts the relationship between pH and inoculum. Amylase production reached a maximum (304.8 U/mg) when the culture medium pH was adjusted to 7.5 and inoculated

with 0.75% inoculum and 0.7% glucose. The culture conditions varied on the basis of the substrate and bacterial strain used for amylase production. The interactions observed in this study have been previously reported by various research groups [85–87]. Sharif, Shah, Fariq, Jannat, Rasheed and Yasmin [72] reported improved amylase production via RSM using bacteria at pH 7.0, 1.25% substrate, 70 °C and 300 µL inoculum. In *Bacillus aryabhattai*, improved production of amylase was achieved in culture medium containing 5.0% peptone, 10.25% starch and 5.18% yeast extract at pH 7.3. This condition was increased 1.39-fold compared with that of the unoptimized medium [88]. Similarly, the bacterium *Bacillus cereus*, which is isolated from water, produces amylase. The statistical optimization approach increased amylase production, and the suitable culture conditions were 2% inoculum, 5% starch, 48 h fermentation, pH 8.0 and 45 °C. These culture conditions improved amylase production by 1.2-fold compared with that in unoptimized media [73]. The 3D surface plot observed in this study revealed that amylase production was improved by increasing the initial pH of the medium and inoculum concentration. Compared with the other two variables (pH and inoculum), increasing the glucose concentration increased amylase activity, revealing that the effect of glucose on amylase activity was more significant than that of pH and inoculum. The model *F* value was 16.60, which revealed that the model was significant ($p < 0.0001$). The R^2 value of the model was >0.75, indicating that the model was best fitted ($R^2 = -0.93$).

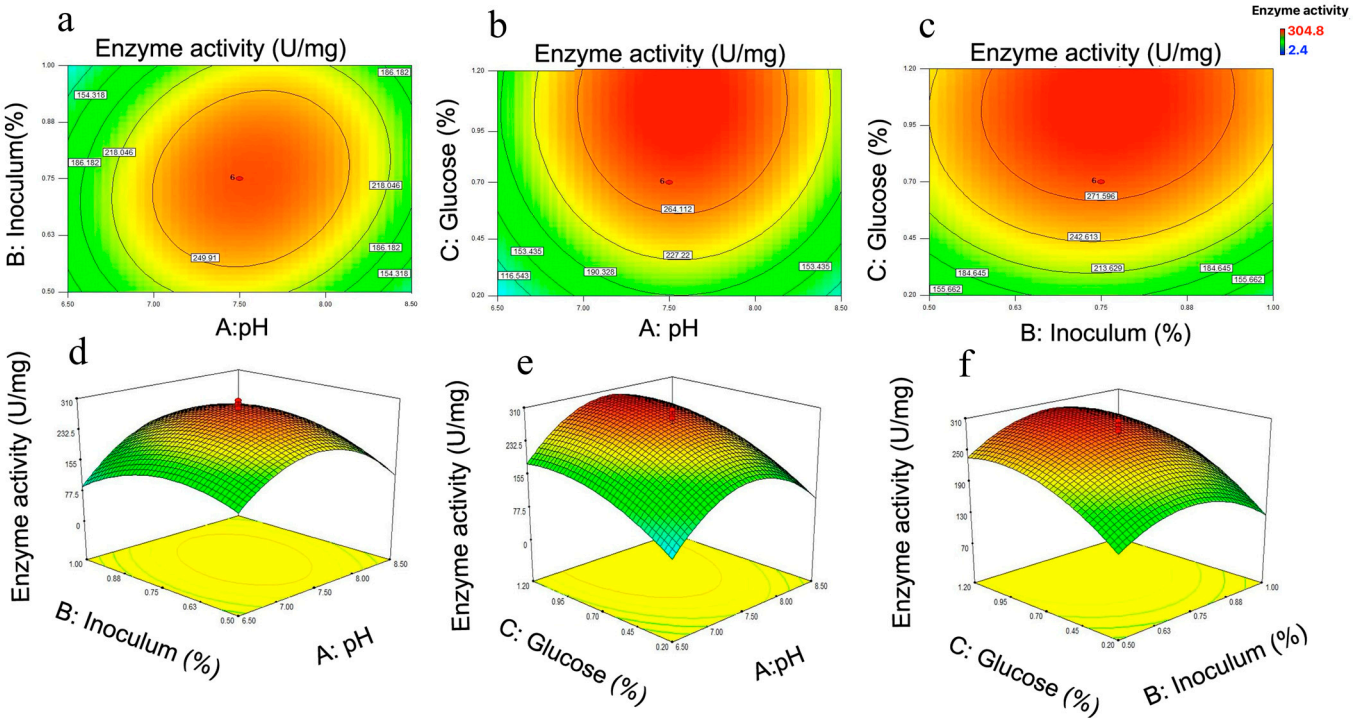


Figure 5. 2D contour plots and 3D response surface plots between inoculation and pH (**a**,**d**), glucose and pH (**b**,**e**), and glucose and inoculum (**c**,**f**).

3.8. Validation of the Model

The maximum amylase activity predicted by the designed CCD model was 315.2 U/mg. To analyze the reliability of the predicted model, the potential of the final predicted optimum medium was validated via the SSF. The observed amylase production was 307.1 U/mg, and this value was very close to the predicted value, indicating the reliability of the model. The CCD model has been widely recognized to determine the interactions among and between the selected variables to help determine their optimal variable concentrations to achieve the maximum enzyme yield. *F*-test analysis of the quadratic model

revealed statistical significance, and it was used to test the significance of the model to achieve the highest possible enzyme yield. Generally, a high *F* value for a designed model indicates statistical significance. In this study, amylase production increased twofold compared with that in an unoptimized culture medium. Previously, many studies have used response surface methodology for optimizing bioprocess conditions for amylase production [76,89,90]. Overall, the amylase activity observed in the statistical optimization method was greater than that of the unoptimized single-factor approach. The high amylase enzyme yield proved the validity of the model used in this study.

4. Conclusions

This study demonstrated that marine bacteria associated with macroalgae are an efficient source of biologically active α-amylase enzyme with antibiofilm potential. Among the screened strains, *Bacillus* sp. showed higher enzyme activity and effectively inhibited the biofilm formation by *V. alginolyticus*. The use of tapioca peel as a substrate for solid-state fermentation indicated a cost-effective and sustainable approach for amylase enzyme production from marine bacteria. The optimization studies resulted in a twofold increase in enzyme yield compared to traditional methods indicating the robustness of the predicted model. The strong biofilm inhibitory activity of the amylase enzyme observed in the study further confirmed the role of enzymes in biofilm control strategies. However, further studies using different biofilm-forming bacterial strains and degradation effects of amylase enzyme on mature biofilms are necessary to understand antibiofilm efficiency. Further, the results observed in this study were based on crude enzymes extracted from bacteria. Hence, testing the activity of purified enzyme and its stability under different environmental conditions is necessary for its applications. Overall, this study underscores the biotechnological significance of marine-algae-associated bacteria in enzyme production and biofilm control.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation12020112/s1, Figure S1: Effects of different substrates on amylase enzyme activity in marine-algae-associated bacterium; Table S1: Analysis of variance for the determination of significant factors via two-level full factorial design; Table S2: Central composite design and analysis of variance.

Author Contributions: Conceptualization, S.S.; methodology, S.S.; validation, S.S. and L.A.S.; formal analysis, S.S.; investigation, S.S. and L.A.S.; resources, L.A.S.; data curation, S.S.; writing—original draft preparation, S.S.; writing—review and editing, S.S. and L.A.S.; project administration, L.A.S.; funding acquisition, L.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, Saudi Arabia, under the grant no. (IPP: 667-150-2025). The authors, therefore, acknowledge with thanks DSR for technical and financial support.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data generated/analyzed in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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