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Amylase Production from Thermophilic *Bacillus* sp. BCC 021-50 Isolated from a Marine Environment

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Abstract: The high cost of fermentation media is one of the technical barriers in amylase production from microbial sources. Amylase is used in several industrial processes or industries, for example, in the food industry, the saccharification of starchy materials, and in the detergent and textile industry. In this study, marine microorganisms were isolated to identify unique amylase-producing microbes in starch agar medium. More than 50 bacterial strains with positive amylase activity, isolated from marine water and soil, were screened for amylase production in starch agar medium. *Bacillus* sp. BCC 021-50 was found to be the best amylase-producing strain in starch agar medium and under submerged fermentation conditions. Next, fermentation conditions were optimized for bacterial growth and enzyme production. The highest amylase concentration of 5211 U/mL was obtained after 36 h of incubation at 50 °C, pH 8.0, using 20 g/L molasses as an energy source and 10 g/L peptone as a nitrogen source. From an application perspective, crude amylase was characterized in terms of temperature and pH. Maximum amylase activity was noted at 70 °C and pH 7.50. However, our results show clear advantages for enzyme stability in alkaline pH, high-temperature, and stability in the presence of surfactant, oxidizing, and bleaching agents. This research contributes towards the development of an economical amylase production process using agro-industrial residues.

Keywords: *Bacillus* sp. BCC 021-50; amylase production; characterization and marine organisms

1. Introduction

Amyloglucosidase enzymes are involved in the hydrolysis of starchy materials into oligosaccharides and, finally, into simple glucose units [1]. Amylases are of three types, including α-amylase that hydrolyzes α-1,4 bonds and bypasses branched linkages, β-amylase that breaks down α-1,4 and cannot bypass α-1,6 branch linkages and produces maltose as a product, and γ-amylase (glucoamylase) attacks the substrate from the non-reducing end, and hydrolyzes α-1,4 and α-1,6 linkages, consequently releasing monosaccharides as the end product [2]. Amylases are applied in several industrial processes, including saccharification of starchy materials, pharmaceuticals, food, and detergent and textile industries [3,4]. Amylases are produced from all sources of life (plants, animals, and microorganisms) and the demand of production is continuously increasing due to the wide range of industrial applications. Therefore, microbial sources are exploited for several industrially-important bioproducts.

Among microorganisms, bacterial strains are preferred over fungal strains for production of enzymes and other value-added products [5].

Several terrestrial microorganisms have been used for enzyme production, for example, protease, xylanase, amylase, chitinase, cellulase, lipase, and inulinase, [6–9]. However, limited studies are reported on α-amylase production from marine microbial strains [3]. Moreover, marine microorganisms are reported to produce enzymes with industrially-important properties, such as stability at elevated temperature and alkaline pH conditions [10]. These bacterial characteristics are essentially important for amylase production using cost-effective materials as energy sources.

High fermentation medium cost is one of the major concerns in amylase production from microbial sources. Amylase demand is continuously increasing and researchers are attempting to establish economical fermentation processes. Several agro-industrial residues have been utilized for amylase production and many other value-added products. Researchers are looking for novel microbial strains to produce amylase enzymes with industrially-important properties, for example, alkaline pH-stable, thermo-stable, and surfactant-stable amylase. In addition, those microorganisms should utilize agricultural waste material as cost effective carbon and nitrogen sources. Therefore, in this study, marine microorganisms were isolated and screened for amylase production.

Current research was carried out to optimize cultivation conditions for the growth of *Bacillus* sp. BCC 021-50 and the production of high amylase concentration from locally-isolated marine bacterial strains, and could play an effective role in the commercialization of amylase production.

2. Materials and Methods

2.1. Isolation and Screening of Amylase-Producing Microbes

Bacterial strains were isolated from marine soil and water near local seashores for amylase production. These cultures were screened to obtain hyper amylase-producing strains by growing in starch agar medium. The medium contained soluble starch (20 g/L), peptone (20 g/L), agar (20 g/L), and plates were incubated for 48 h. After 48 h incubation, plates were flooded with gram iodine solution to observe clear zone formation due to starch hydrolysis. Several bacterial strains were screened and the size of clear zones were 12 to 40 mm. Strains showing 32, 35, and 40 mm zone sizes were selected for fermentation experiments. *Bacillus* sp. BCC 021-50 was identified and used for subsequent experiments as the best strain based on morphological (simple and gram staining) and biochemical (catalase and Voges-Proskauer) tests (data provided as supplementary material Table S1).

2.2. Cultivation Conditions of Marine Bacteria

In the first step, the strain was activated on LB medium containing 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl. Then bacterial cells were incubated at 37 °C in a shaking incubator for 24 h at 150 rpm. 10% v/v culture was transferred in the seed culture medium (glucose (20 g/L), yeast extract (10 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), NaCl (10 g/L), $CaCl_2$ (2 g/L), and KH_2PO_4 (2 g/L)), and incubated at 120 rpm, at 37 °C for 18 h. Finally, a 2 % v/v inoculum was used in the enzyme production medium (glucose (20 g/L), yeast extract (10 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), NaCl (10 g/L), $CaCl_2$ (2 g/L), and KH_2PO_4 (2 g/L)) and was incubated at 37 °C for 120 h in a shaking incubator. Samples were collected at regular intervals of 12 h and the growth (OD) was measured at 600 nm using a spectrophotometer. The culture broth was centrifuged at 11,300× g for 10 min to collect cell-free supernatant for α-amylase activity. The chemicals used for this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Merck and Co., Inc., (Kenilworth, NJ, USA) and commercial sources. All solutions were prepared in double-distilled water. All media were sterilized at 115 °C for 20 min prior to inoculation.

2.3. Amylase Assay

Amylase activity was checked based on the determination of reducing sugars according to method reported elsewhere [11]. Briefly, 1.0 mL of sample and an equal amount of substrate (1.0% w/v soluble starch) were mixed thoroughly and test tubes were incubated at 37 °C for 15 min in a water bath. After 10 min, the reaction was stopped by addition of 2.0 mL of DNS reagent and tubes were kept in a boiling water bath for 5 min. Tubes were cooled to room temperature and absorbance was measured at 540 nm against substrate and enzyme blanks. The blanks were prepared by replacing the sample and substrate with the same amount of water. All other reagents were added in the same concentrations. One unit of amylase activity is defined as the dosage of the enzyme applied for releasing 1 µmol of glucose under optimized assay conditions.

2.4. Effect of Temperature

Amylase production was checked at different temperatures. 250-mL flask containing 50 mL of fermentation medium was inoculated with 2% v/v inoculum from 24 h-old seed culture and flasks were incubated in a shaking incubator at different temperatures ranging from 30–60 °C, for 36 h. The initial pH was adjusted to 7.0. Amylase activity was determined from culture broth obtained after centrifugation (11,300× g for 10 min at 10 °C).

2.5. Effect of the Carbon Source

Bacillus sp. BCC 021-50 culture was grown in 250 mL conical flaks containing 50 mL of fermentation medium, supplemented with 20 g/L of carbon sources including glucose, date syrup, molasses, fructose, starch, maltose, and galactose. Then, 2% v/v of seeds culture was inoculated in each flask and the culture was incubated at 50 °C for 36 h. At the end, the fermentation medium was centrifuged (11,300× g for 10 min at 10 °C) to collect samples for further analysis and stored at 4 °C.

2.6. Effect of the Nitrogen Source and the Influence of the Initial pH

Organic and inorganic nitrogen substances were evaluated as nitrogen sources for amylase production. The fermentation medium was supplemented with ammonium nitrate, ammonium chloride, ammonium sulfate, urea, yeast extract, casein, tryptone, meat extract, and peptone at a concentration of 10 g/L. Additionally, biosynthesis of amylase was determined at different initial pH values ranging 5.0 to 11.0. Fermentation and biochemical analysis was done as described above.

2.7. Amylase Activity and Stability at Different pH

A pH range of 6.0–11.0 was assessed for maximal amylase activity by changing the pH using different buffer solutions: phosphate buffer pH 6.0–7.5; Tris–HCl buffer pH 8.0–8.5, and glycine–NaOH buffer pH 9.0–11.0. Stability was determined by incubating enzymes in the above-mentioned buffers for 1 h at 40 °C and then amylase activity was checked under assay conditions.

2.8. Amylase Activity and the Stability at Different Temperatures

The effect of temperature on crude amylase was studied by incubating reactions at different temperature ranges (30–85 °C) in glycine-NaOH buffer (pH 7.5) for 1 h. Thermostability was determined by pre-incubating enzyme for 1 h at different temperature ranges 30–85 °C.

2.9. Enzyme Stability in Surfactant, Oxidizing, and Bleaching Agents

For applicability of crude amylase in biotechnological processes "especially in detergent industries" stability was determined with surfactants and bleaching agents. Stability of amylase was assayed in the presence of Triton X-100, Tween 20 and 40, Sodium dodecyl sulfate (surfactants), and sodium hypochlorite (bleaching agent) under optimized assay conditions (pH 7.5 and 40 °C for

1 h). Amylase activity with the addition of different compounds was measured as the percent of relative activity compared to control as 100% (without additives).

2.10. Statistical Analysis

Three replicates of each sample were used for statistical analysis. Data were reported as means and least significant difference tests were conducted to identify differences among the means. For the determination of significant difference, a *t*-test was performed at $p < 0.05$.

3. Results and Discussion

3.1. Isolation, Screening, and Identification of Amylase-Producing Marine Bacteria

Fermentation medium cost is one of the important factors in microbial enzyme production and utilization of agro-industrial waste can play a vital role in the reduction. Locally-isolated strains and cheap substrates can produce inexpensive amylase and reduce the enzyme's production cost. In this study, we attempted to establish economical fermentation processes with the locally-isolated strain *Bacillus* sp. BCC 021-50. Previously, amylase production has been carried out from several bacterial and fungal strains, including *Bacillus megaterium* [12], *Bacillus amyloliquefaciens* 04BBA15 [13], *Bacillus cereus* strain BRSC-S-A26MB [14], *Bacillus* sp. RKY3 [15], *Penicillium expansum* MT-1 [16], and *Aspergillus fumigatus* [17]. Due to increasing amylase use in various research fields, many research groups are interested in the isolating and screening of hyper amylase-producing strains from different sources. In the present study, more than 50 bacterial strains were isolated from a local seashore. These strains were screened for starch hydrolyzing activity using starch agar medium. Several strains showed positive results for amylase activity with clear zone formation (data not shown) and few of them were selected for further confirmation of extracellular amylase activity in fermentation medium. The best one was identified as *Bacillus* sp. BCC 021-50 per Bergey's manual of bacterial determination. BCC 021-50 can produce extracellular enzymes and cultural conditions were optimized for the highest possible amylase titer.

3.2. Optimization of Fermentation Conditions for Amylase Production

Fermentation conditions of *Bacillus* sp. BCC 021-50 were optimized in terms of incubation time, temperature, carbon source, nitrogen source and influence of initial pH to enhanced growth and the highest α-amylase concentration. The time profile of amylase production and bacterial growth is shown in Figure 1. The amylase titer in the culture broth increased with time, up to 1634 U/mL, after 36 h and decreased thereafter. Thus, our results suggested that amylase production was growth-dependent. A decrease in amylase concentration on further incubation might be due to the decrease in cell growth, a deficiency of nutrients, and a change in the final pH [18]. In previous studies, the highest amylase production from *Bacillus licheniformis* after 36 h under solid-state fermentation was observed, when rice husk was used as a carbon source [19], but 20% v/v inoculum was used in comparison to 2.0% v/v used in our study. No doubt higher inoculum can be cost effective if the processing time is considerably reduced for large-scale amylase production. Another study [20] achieved the highest amylase concentration from *Bacillus amyloliquefaciens* after 42 h when wheat bran and groundnut oil cake were supplemented as the carbon source. Elhalem et al. [21] obtained maximum amylase production from *Bacillus amyloliquefaciens* after 48 h incubation in submerged fermentation conditions. By contrast, Bozic et al. [22] reported maximal amylase yield from *Bacillus licheniformis* after 72 h in shaker flask conditions. Ackan et al. [23] found a maximum amylase titer from *Bacillus subtilis* RSKK96 after 72 h of incubation. Amylase activity was stimulated by calcium ions (Ca^{2+}) [24], and most of the α-amylases are metalloenzymes, thus, $CaCl_2$ was the best nitrogen source for the fermentation medium.

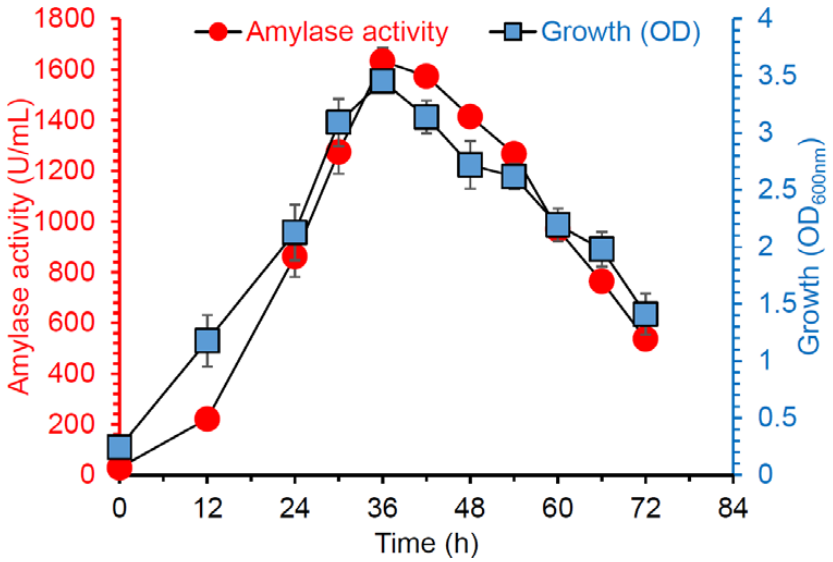

Figure 1. Batch profile of amylase production and *Bacillus* sp. BCC 021-50 growth in synthetic medium containing (g/L) glucose (20), yeast extract (10), $MgSO_4 \cdot 7H_2O$ (1.0), NaCl (10), $CaCl_2$ (2.0), and KH_2PO_4 (2.0), incubated in a shaking incubator at 37 °C with an initial pH 7.0. The experiments were performed in triplicate and data presented in the figure are the average of three parallel experiments. Error bars are shown for standard deviation.

Figure 2 shows the effect of fermentation temperature on synthesis of amylase from *Bacillus* sp. BCC 021-50 (30–60°C), when grown in fermentation medium containing 20 g/L of glucose and 10 g/L of yeast extract, and incubated for 36 h at an initial pH of 7.0. Enzyme concentration increased with the increase of temperature, and maximum amylase yield was noted at 50 °C (3314 U/mL). A further increase of the temperature reduced the amylase titer, probably due to the decreasing microbial growth and denaturation of the enzyme at higher temperature. As previously reported [25], the highest amylase concentration was obtained from the *Bacillus* strain at 45 °C on starch agar medium, and Bozic et al. [22] obtained maximum amylase production from *Bacillus licheniformis* at 37 °C [23], finding a maximum amylase titer from *Bacillus subtilis* RSKK96 at 37 °C after 72 h of incubation under submerged fermentation conditions. Thermophilic microorganisms are required for commercial amylase production to reduce the contamination chances and obtaining thermophilic enzymes. Our results show the thermophilic nature of the newly-isolated strain and growth was achieved in shaker flask fermentation conditions for amylase production. Further experiments are designed based on the obtained results to perform open amylase production that could possibly save sterilization costs and energy. In addition, crude amylase will be applied to hydrolyze starchy materials, and fermentable sugars could be converted into biochemical end products, (ethanol, lactic acid, amino acids, etc.).

In the next step, the effect of different carbon sources, including glucose, date syrup, molasses, fructose, starch, maltose, and galactose, were checked for amylase concentration; the results are shown in Figure 3. The amylase titer was greatly influenced by the carbon sources and the highest enzyme yield of 4827 U/mL was obtained when 20 g/L of molasses was supplemented as the carbon source in comparison to other sugars. In our previous studies, molasses and date syrup were evaluated as cost effective carbon sources for protease, pectinase, and alkaline phosphate production [5,18,26,27]. However, agro-industrial residues, molasses, and date syrup were used in the fermentation medium for amylase production from *Bacillus* sp. BCC 021-50.

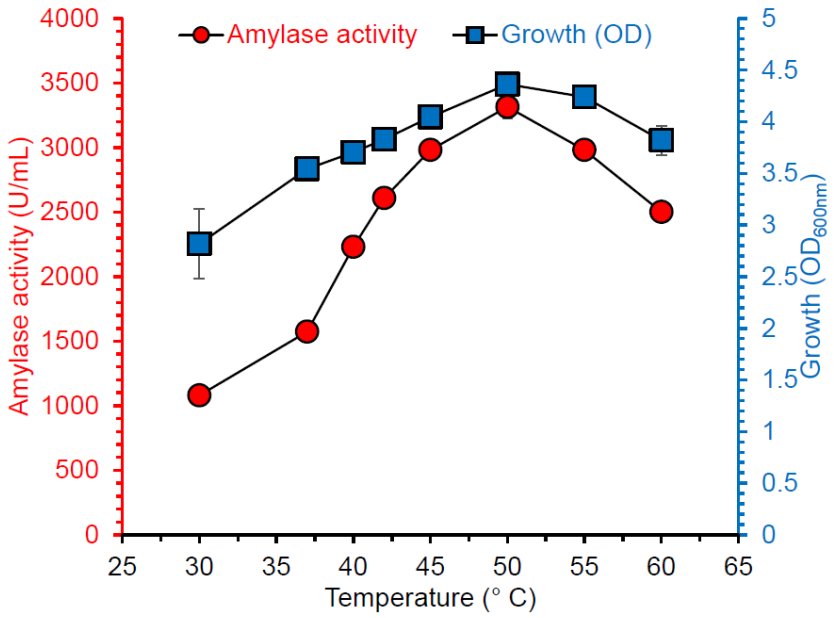

Figure 2. The effect of fermentation temperature on amylase production and cell growth (*Bacillus* sp. BCC 021-50) for 36 h. The medium initially contained glucose (20 g/L) and yeast extract (10 g/L) as carbon and nitrogen sources, respectively. The initial pH was 7. The experiments were performed in triplicate and the data presented in the figure are the average of three parallel experiments. Error bars are shown for standard deviation.

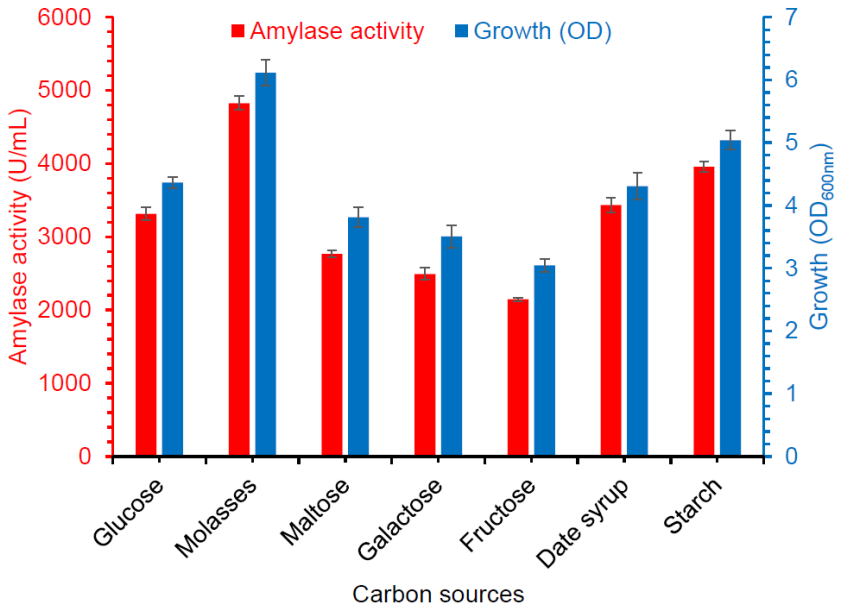

Figure 3. The effect of the carbon source (20 g/L initial concentration) on α-amylase production and *Bacillus* sp. BCC 021-50 growth at 37 °C, initial pH of 7.0, for 60 h. The experiments were performed in triplicate and the data presented in the figure are the average of three parallel experiments. Error bars are shown for standard deviation.

Agro-industrial residues, including wheat straw, rice straw, corn stover, sugarcane bagasse, molasses, and date syrup, could be used as alternate energy sources instead of pure sugars to produce commercially-important products to solve the disposal problem of residues and reduce the cost of the fermentation medium. The carbon source is one of the expensive materials used in the fermentation

medium and the purpose of our study was to reduce the fermentation cost by replacing pure sugars with inexpensive agro-wastes (molasses and date syrup). In this study, we evaluated molasses and date syrup as cost-effective energy sources. Abdullah et al. [28] evaluated various agro-industrial residues as carbon sources (coconut oil cake, rice bran, vegetable waste, banana peel, and wheat bran) for amylase production and the highest amylase titer was obtained when wheat bran was supplemented in the fermentation medium.

Several organic and inorganic compounds, for instance, ammonium nitrate, ammonium chloride, ammonium sulfate, urea, yeast extract, casein, tryptone, meat extract, and peptone, were tested as nitrogen sources and the results are shown in Figure 4. The maximum α-amylase titer was noted in mineral medium containing 10 g/L of peptone as the sole nitrogen source. Based on the results obtained, it could be hypothesized that bacterial cells secrete the highest amylase concentration when grown on organic nitrogen sources as compared to inorganic nitrogen sources. Our results are in line with the results presented by Dar et al. [29] for amylase production from *Penicillium chrysogenum* using peptone as an organic nitrogen source. Bozic et al. [22] obtained the highest amylase concentration from *Bacillus licheniformis* when tryptone was used as the sole nitrogen source. Ashwini et al. [30] reported amylase production from *Bacillus* sp. when starch and yeast extract were used as carbon and nitrogen sources, respectively. Different approaches, as reported [19], have obtained the highest amylase titers when ammonium sulfate was supplemented in the fermentation medium as inorganic nitrogen source. Generally, carbon and nitrogen intracellular concentrations differ from strain to strain due to the preference of the metabolic cycle in each strain.

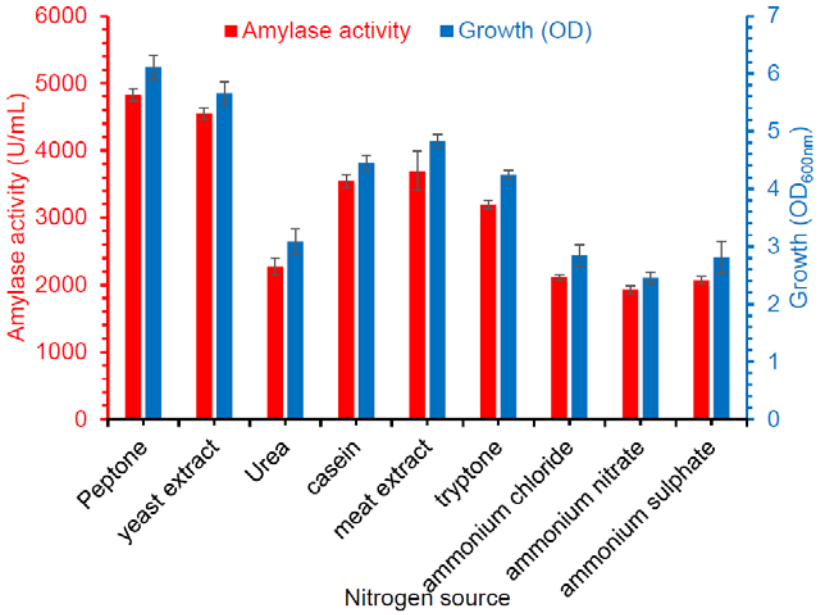

Figure 4. The effect of the nitrogen source (10 g/L initial concentration) on the biosynthesis of amylase from thermophilic marine *Bacillus* sp. BCC 021-50. The experiment was performed in a molasses (20 g/L) mineral medium, and the culture was incubated at 50 °C for 36 h. The experiments were performed in triplicate and the data presented in the figure are the average of three parallel experiments. Error bars are shown for standard deviation.

Figure 5 demonstrates the influence of initial pH (5.0 to 11.0) on amylase synthesis with molasses and peptone as the sole carbon and nitrogen source in mineral medium, incubated at 50 °C for 36 h. Amylase concentration significantly changed with the initial pH values, and the maximum yield was found at pH 8.0 and activity decreased with increasing pH values. Amylase production from *Bacillus cereus* under solid-state fermentation was reported in alkaline pH [31]. In contrast to our results, Dar et al. [29] obtained maximum amylase concentration in acidic pH (6.00) from

Penicillium chrysogenum. Thermo-alkaliphilic microbial strains are considered as industrially-important strains due to their commercial value, especially alkaline pH-stable amylase, which could be used in detergent formulations.

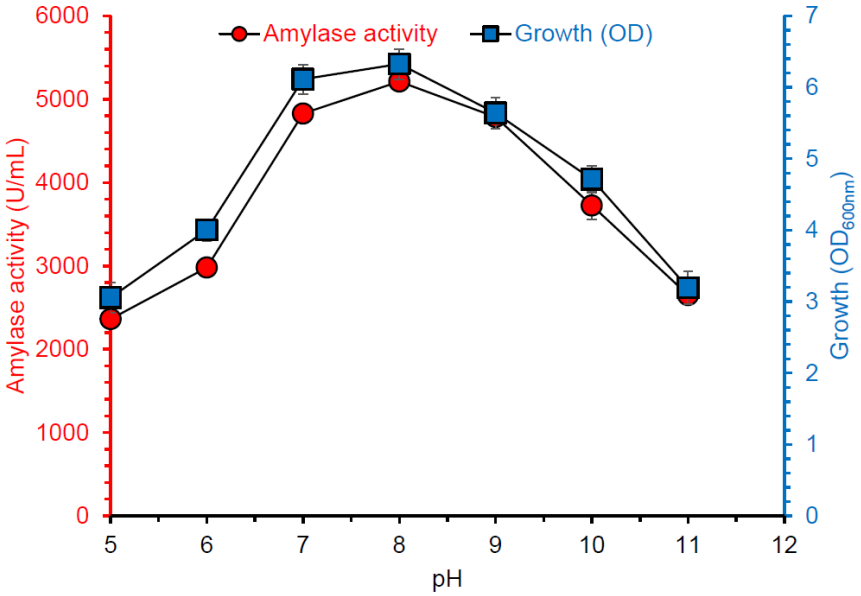

Figure 5. The effect of the initial pH on amylase production and microbial growth (*Bacillus* sp. BCC 021-50) at 50 °C for 36 h in a mineral medium containing molasses (20 g/L) and peptone (10 g/L) as carbon and nitrogen sources, respectively. The experiments were performed in triplicate and the data presented in the figure are the average of three parallel experiments. Error bars are shown for standard deviation.

3.3. Characterization of Crude Amylase of Bacillus sp. *BCC 021-50*

The characterization of enzymes is significantly important for industrial applications and varies due to the specific enzyme requirement for each application. Crude amylase stability in terms of pH, temperature, surfactants, and oxidizing and bleaching agents is beneficial for various applications. The effect of pH on crude enzyme activity was monitored at various pH ranges (6.0–11.0). Soluble starch was used as a substrate, the reaction was incubated at 35 °C, and the pH activity profile of the crude amylase is shown in Figure 6. Amylase activity increased until pH 7.50 and gradually decreased with increasing pH. Results are in accordance with Goyal et al. [32] with maximum amylase activity at pH 7.0 from *Bacillus* sp. I-3. Previous studies [33] have characterized amylase from thermophilic *Bacillus* KSM-K38 in the pH range of 8.0–9.5. Our crude amylase was stable in a wide pH range of 7.0–10.0. Similar results are reported by Hagihara et al. [33] for amylase stability in the pH range of 6.0–11.0.

Maximum amylase activity was monitored at 70 °C as shown in Figure 7. The relative activities at 30 and 85 °C were about 70.32% and 73.48%, respectively. Similar results are reported by Goyal et al. [32], where maximum amylase activity was noted at 70 °C. The enzyme was thermo-stable up to 75 °C and retained 89.84%, 76.92%, and 59.89% initial activity after 1 h incubation at 65, 70, and 75 °C, respectively, as shown in Figure 7. Yang et al. [34] purified and characterized amylase in terms of thermostability and observed maximum stability from 50 to 60 °C, whereas Vieille et al. [35] investigated amylase from *Pyrococcus furiosus*, which was stable at higher temperatures 80–100 °C.

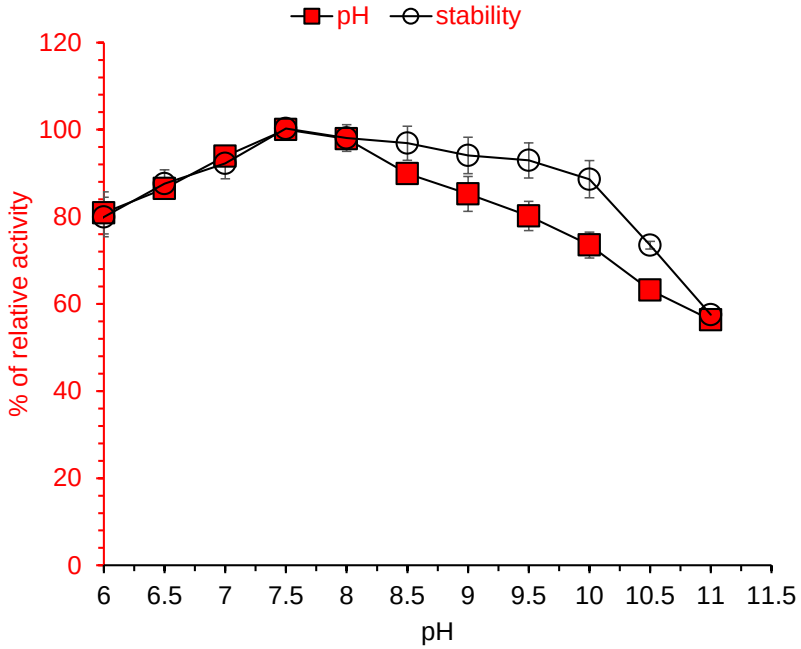

Figure 6. The effect of pH on amylase activity and stability produced by *Bacillus* sp. BCC 021-50. The pH of the enzyme reaction was adjusted in the range of 6–11 with the addition of different buffers. Results are the average of triplicate experiment.

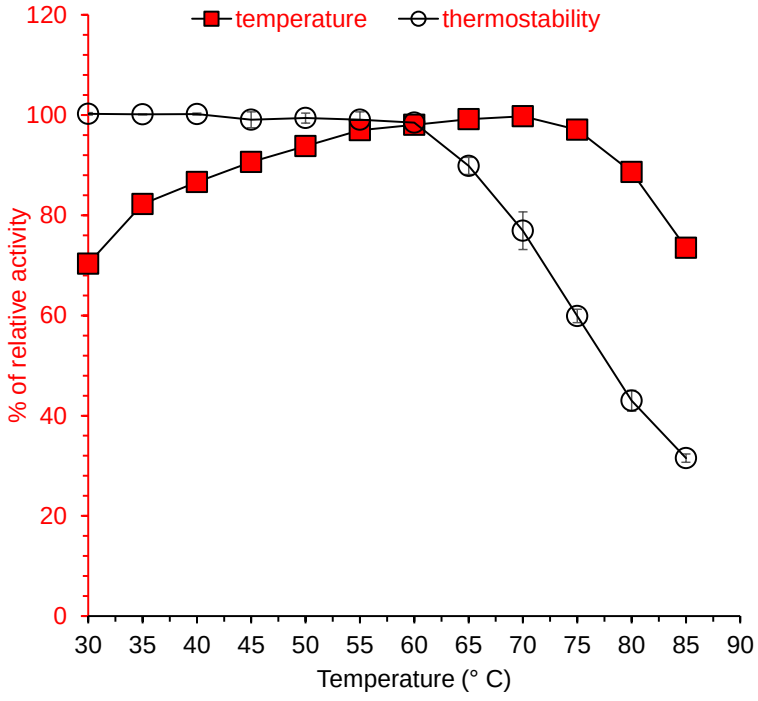

Figure 7. The effect of temperature and thermostability on crude amylase produced by *Bacillus* sp. BCC 021-50. The enzyme was mixed with substrate and incubated at different temperatures for 30 min. Percentage of relative activity was measured under assay conditions. Thermostability of amylase was determined by incubating the enzyme at different temperatures without the addition of substrate for 1 h. Percentage of relative activity was checked under assay conditions. The results are the average of triplicate experiments and the data presented in the figure are the average of three parallel experiments. Error bars are shown for standard deviation.

For the sake of detergent application, we need to check the stability of amylase in the presence of detergents. Generally, washing is carried out in the range of 30–40 °C so, we used a temperature of 40 °C in this experiment. The amylase was stable against all surfactants, especially 0.1% SDS (*v*/*v*), which increased amylase activity up to 141.2 % when incubated at 40 °C for 1 h. However, with 0.2% SDS (*v*/*v*) activity reduced to 122.7%, and the results are depicted in Figure 8. Overall, amylase relative activity was enhanced by addition of all tested surfactant, oxidizing, and bleaching agents, and these results indicate the suitability of amylase for biotechnological applications.

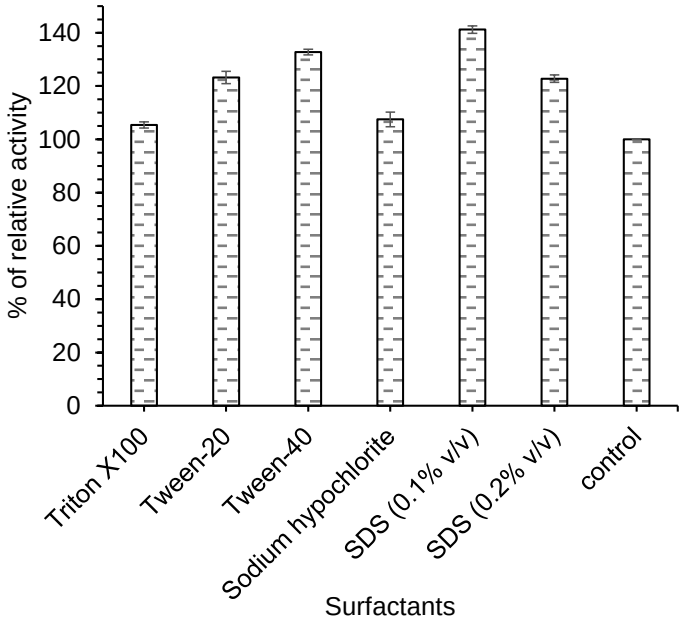

Figure 8. The effect of detergents, surfactant, and oxidizing agents on the crude amylase produced by *Bacillus* sp. BCC 021-50 after 1 h at 40 °C, pH 7.5. The results are shown as the percentage of relative activity compared to that of control (no additive). The experiments were performed in triplicate and the data presented in the figure are the average of three parallel experiments. Error bars are shown for standard deviation.

4. Conclusions

High fermentation medium cost is one of the financial barriers in amylase production from microbial sources. Therefore, in this study, agro-industrial residues were used and marine microorganisms were isolated and screened for amylase production. The highest amylase concentration of 5211 U/mL was obtained after 36 h incubation at 50 °C, pH 8.0, with 20 g/L molasses as the substrate and 10 g/L peptone as a nitrogen source. Crude amylase was characterized to optimize enzyme substrate reaction conditions and maximum amylase activity was noted at 70 °C and pH 7.50. However, our results show clear advantages of enzyme stability in alkaline pH, high temperature, and stability in the presence of surfactant, oxidizing, and bleaching agents. These results are significant and contribute towards the development of an economical amylase production process using agro-industrial residues.

Supplementary Materials: The following are available online at www.mdpi.com/2311-5637/3/2/25/s1.

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Author Contributions: Imrana Khushk, Altaf Ahmed Simair, and Abdul Sattar Qureshi performed the experiments; Abdul Sattar Qureshi, Altaf Ahmed Simair, and Changrui Lu designed the experiments for this study; Abdul Sattar Qureshi, Altaf Ahmed Simair, Khalil Ahmed Ansari, and Haider Ali Chaudhry compiled data and prepared the graphs and tables; and Altaf Ahmed Simair and Abdul Sattar Qureshi wrote the manuscript, and all authors have revised manuscript and agreed for submission.

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

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