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Proceeding Paper

Study of the Amylolytic Activity of Thermophilic Bacteria Isolated from an Algerian Hot Spring (Azzaba, Skikda) †

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Abstract: Thermostable amylases are among the most widely used and desirable enzymes in the food industry. Indeed, they guarantee faster reactions at high temperatures, enhanced substrate solubility and reduced microbial contamination and cooling costs. The objective of this work is to study the amylase activity of three strains of aerobic thermophilic bacteria isolated from the hot spring of Hammam Salhine, located in the wilaya of Skikda, Algeria. The three extracellular amylase-producing strains were subjected to the quantification of amylase activity. They presented medium to high activity, with significantly the best production for the AS1 strain with an activity of 10.62 ± 1.289 U ($p > 0.05$). Monitoring the kinetics of AS1 amylase activity reveals that the maximum enzymatic activity was reached after 52 h with a value of 53.665 ± 2.534 U. The maximum growth was reached after 54 h of fermentation at an OD of 0.865 ± 0.081 at 600 nm. The study of the effect of the variation in physicochemical parameters on the activity of AS1 amylase extract shows that the enzymatic activity was maximal at a temperature of 100 °C, a pH of 8.0 and in the absence of NaCl. The amylase extract of this strain showed significant thermostability at 100 °C.

Keywords: bacteria; thermophiles; amylase activity; enzyme assay; thermostability

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1. Introduction

Thermostable amylases are among the most widely used and desirable enzymes in the food industry, thanks to their unique reaction properties at elevated temperatures and their stability under a wide range of industrial bioprocess [1,2]. Indeed, they guarantee faster reactions at high temperatures, enhanced substrate solubility and reduced microbial contamination and production costs [1]. Thermophilic microorganisms inhabiting natural hot environments are an important source for the isolation of these innovative biocatalysts [3].

The objective of this work is to study the amylase activity of three strains of aerobic thermophilic bacteria coded AS1, AS3 and AS7, previously isolated from the hot spring of Hammam Salhine, located in the wilaya of Skikda, Algeria [4].

2. Materials and Methods

Three strains of spore-forming thermophilic bacteria coded AS1, AS2 and AS3 were generously provided by the METEX team at the BIOQUAL laboratory (INATAA, Constantine1). They were previously isolated from the hot spring of Hammam Salhine, located in

the wilaya of Skikda, Algeria, and phenotypically characterized [4]. After strain revival, the verification of the purity of their cultures and their production of extracellular amylases [5] and the optimal temperature, pH and NaCl concentration values for the growth of the strains were determined on a liquid medium [6]. The quantification of amylase production was carried out following the method in [7]. After, the monitoring of the kinetics of amylase activity of the best-producing strain AS1 and the study of the effect of the variation in physico-chemical parameters on the activity of its amylase extract were undertaken following the method in [8]. Data were analysed statistically using GraphPad Prism software version 9.5 (GraphPad Software, San Diego, CA, USA). A one-way variance (one-way ANOVA) was performed to compare the means. In the cases of a significant difference ($p < 0.05$), Tukey's test was performed to separate the means. Values were reported as mean ± standard deviation.

3. Results

3.1. Strain Revival and Screening of Extracellular Amylase Production

The AS1, AS3 and AS7 strains were revitalized by subculturing after 24 h incubation at 60 °C on nutrient broth from cultures stored at −41 °C or on nutrient agar from plates stored at 4 °C. After that, their purity was repeatedly verified with microscopic observation (Figure 1). The three bacterial strains presented a homogeneous appearance with Gram-positive rod-shaped cells grouped in chains (AS1) or isolated (AS3 and AS7). The strains were further confirmed as extracellular amylase producers. In fact, the amylolytic activity was manifested by the appearance of clear zones around producing colonies cultivated on 1% (*w*/*v*) starch agar and incubated at 60 °C for 72 h.

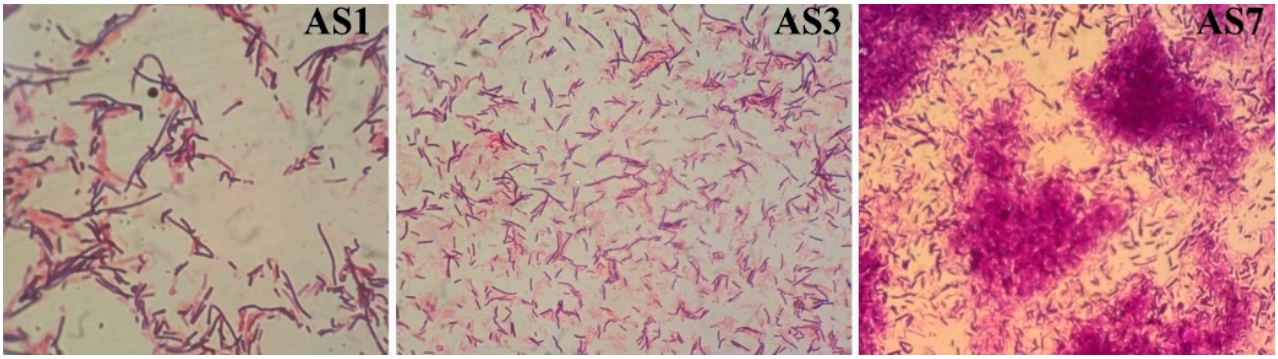

Figure 1. Observation of strains under an immersion photonic microscope (×1000) after Gram staining.

3.2. Determination of Physiological Growth Optima

Analysing the results in Figure 2 discloses that the three strains are moderately thermophilic (optimum temperature of 55 °C) and neutrophilic (optimum pH of 6.0 or 8.0). Strains AS1 and AS7 are non-tolerant to salinity (optimum at 0% *w*/*v* NaCl), while AS3 is halotolerant (optimum at 2%).

3.3. Amylolytic Activity Assay

According to the results obtained (Figure 3), the three strains present with medium to high activity, with significantly the best production for AS1, with an activity of 10.62 ± 1.289 U ($p < 0.05$). It was, therefore, selected for further work.

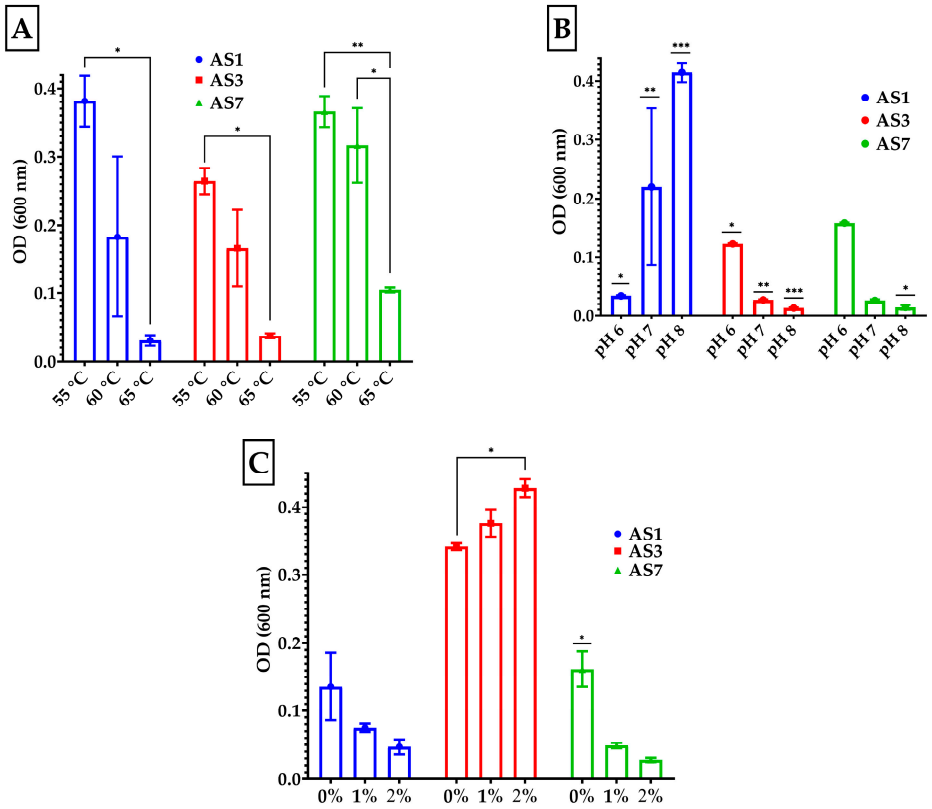

Figure 2. Morphological and growth optima. (**A**) Temperature, (**B**) pH, (**C**) NaCl. The starred bars indicate significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

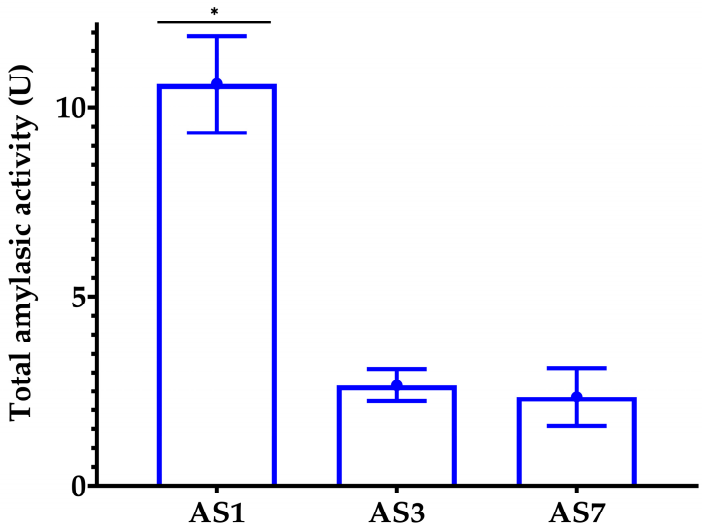

Figure 3. Results of total amylolytic activity assay for the three strains. One unit of amylase activity is defined as the amount of enzyme that releases 1 µmol of glucose-equivalent reducing sugar per minute under the conditions of the assay. The starred bar indicates a significant difference ($p < 0.05$).

3.4. Growth and Amylase Production Kinetics for the Best-Producing AS1 Strain

The study of the growth kinetics of strain AS1 and its total amylase activity production reveals that its maximum enzymatic activity was reached after 52 h with a value of 53.665 ± 2.534 U, and its maximum growth was reached after 54 h of fermentation at a DO of 0.865 ± 0.081 at 600 nm (Figure 4).

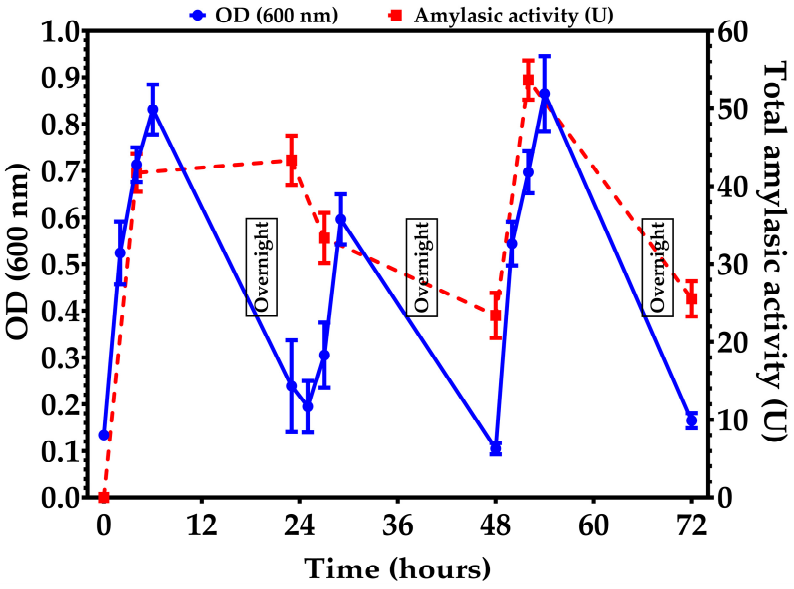

Figure 4. Growth and production kinetics of extracellular amylases in the AS1 strain.

3.5. Study of the Effect of Variations in Physical–Environmental Factors on Amylase Activity

The study of the effect of the variation in physico-chemical parameters on the activity of the amylase extract of strain AS1 shows that the enzymatic activity was maximal at a temperature of 100 °C, a pH of 8.0 and in the absence of NaCl. The amylase extract of this strain showed significant thermostability at 100 °C (Figure 5).

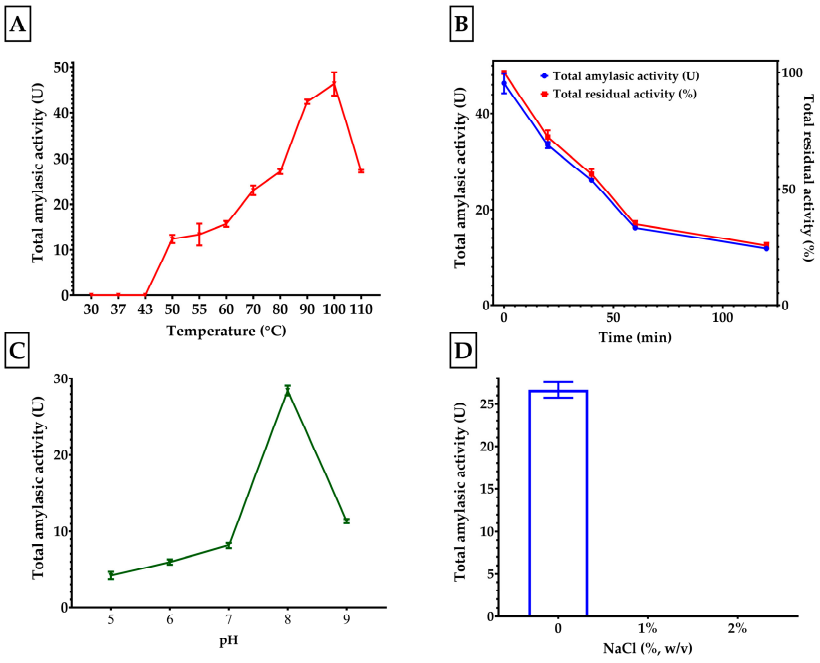

Figure 5. Study of the effect of the variation in physico-chemical parameters on the activity of the amylase extract of strain AS1. (**A**) Effect of T°; (**B**) thermostability; (**C**) pH variation; (**D**) NaCl concentration.

4. Discussion

Overall, all three bacteria showed amylolytic activity, with significantly the best production for the AS1 strain. Our results are promising compared to the findings of [8,9]. According to [10], the kinetics of amylase production are similar to classic microorganism growth kinetics. Classical bacterial growth and production kinetics with maximum

enzymatic activity were reached after 24 h [11,12]. However, the growth and amylase activity production kinetics of strain AS1 fluctuated, with the maximum enzyme activity (53.665 ± 2.534 U) after 52 h and maximum growth after 54 h of fermentation. This could be linked to several factors, like the composition of the medium, pH of the medium, incubation time, size of the inoculum and incubation temperature. The study of the effect of the variation in physico-chemical parameters on the activity of the amylase extract of strain AS1 shows that the enzymatic activity was maximal at a temperature of 100 °C, a pH of 8.0 and in the absence of NaCl. The amylase extract of this strain showed significant thermostability at 100 °C. The potential amylase activity of AS1, particularly at 100 °C, means that it can be used in a variety of industrial applications [13,14]. According to [15], an optimal pH of 8.0 indicates that enzymes can function under alkaline conditions, which represents a huge potential for the detergent industry [16]. The highest enzyme activity was recorded at 0% NaCl, while it was nil at 1 and 2% NaCl. The salt tolerance of amylases in this study indicates that the elevation of NaCl concentration strongly affects the enzymatic activity of the strain. This clearly indicates that the enzymes are non-tolerant to NaCl, which corresponds to the growth assay results of the AS1 amylase-producing strain [4].

5. Conclusions

In conclusion, our study confirms the production of extracellular amylases by three thermophilic bacterial strains isolated from the Hammam Salhine hot spring. Strain AS1 was found to be the highest amylase producer, with optimum activity at 100 °C and a pH of 8.0. This study opens the door for further research on the characterization of amylases and their producer bacteria, as well as their potential application in various industrial fields.

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