A new Bacillus strain degrading starch, named Bacillus sp. UEBS-S, was isolated from a southern Tunisian area. Amylase production using solid-state fermentation on millet, an inexpensive and available agro-resource, was investigated. Response surface methodology was applied to establish the relationship between enzyme production and four variables: inoculum size, moisture-to-millet ratio, temperature, and fermentation duration. The maximum enzyme activity recovered was 680 U/g of dry substrate when using $1.38 \times 10^9$ CFU/g as inoculation level, 5.6:1 (ml/g) as moisture ratio (86%), for 4 days of cultivation at 37°C, which was in perfect agreement with the predicted model value. Amylase was purified by Q-Sepharose anion-exchange and Sephacryl S-200 gel filtration chromatography with a 14-fold increase in specific activity. Its molecular mass was estimated at 130 kDa. The enzyme showed maximal activity at pH 5 and 70°C, and efficiently hydrolyzed starch to yield glucose and maltose as end products. The enzyme proved its efficiency for digesting raw cereal below gelatinization temperature and, hence, its potentiality to be used in industrial processes.

Key words: Bacillus sp., amylase, solid-state fermentation, millet, characterization

α-Amylases (E.C. 3.2.1.1) hydrolyze α-1-4-glycosidic bonds in starch, glycogen, and other related carbohydrates starch to yield diverse products including dextrins and progressively smaller polymers of glucosyl units. Being one of the major industrial enzymes with approximately 25% of the enzyme market, amylases have found numerous applications in the food, chemical, textile, laundry, baking, and energy sectors [21, 32].

Amylases are commonly produced under submerged conditions, but solid-state fermentation (SSF) is often employed to produce larger amounts [37]. Many agro-industrial residues such as wheat bran, coconut oil cake, rice, sweet potato, or sweet sorghum have been used as supporting insoluble materials for bacterial or fungal SSF [28, 38, 43]. For optimal usage, these matrices should provide all the needs for growth without supply of additional nutrients [7, 37].

Among agricultural crops, pearl millet is an attractive matrix for SSF. This insoluble starchy substrate, usually used in animal feed, has recently been shown to efficiently promote growth of several types of organisms in semi-SSF systems, in which additional nutrient sources were supplied for growth. For instance, Haq et al. [23] reported that addition of pearl millet to a rich medium containing other starch resources at 1% (w/v) allowed doubling of the amylase production level by Bacillus licheniformis. However, raw pearl millet has never been tested as a unique source of nutrients for the production of starch-degrading enzymes from bacterial or fungal origin. Owing to the expanding development of the starch industry, there is still a demand for enzymes produced at a low cost with improved properties such as high efficiency, Ca$^{2+}$ independency, stability, and raw starch digestibility [25]. In recent years, worldwide interest has been focused on the raw-starch-digesting amylases, which would be of value to simplify the starch process conversion, and to reduce the resulting production cost [20]. It was reported that fungi such as Aspergillus sp. [37] and Bacillus sp. were good producers of raw-starch-digesting amylases [36, 40].
In this investigation, a statistical approach based on Dohlert design was used to optimize raw-cereal-digesting alpha-amylase production under SSF conditions. Millet, a local and economical starchy product, was used as the crude substrate. The enzyme was also purified and characterized.

**Material and Methods**

**Characterization of Millet**

The millet seeds were harvested, washed, dried (at 50°C for 48 h), and crushed. Crushed millet having a particle size between 500 and 1,000 μm was used as solid substrate for amylase production. It was stored at 4°C for subsequent analyses. The total nitrogen and lipid, moisture, and ash contents of crushed millet were analyzed [2]. The total protein content was estimated by multiplying the total nitrogen content by a factor of 5.7. The starch content was determined using the enzymatic colorimetric method [12].

**Microorganism Isolation**

Soil samples were collected from the Ben Gardene area, in Southern Tunisia. One gram of each sample was dispersed into 5 ml of sterile water. An M1 medium, containing (g/l) Na₂HPO₄, 4; KH₂PO₄, 3; yeast extract, 1; peptone 1; NaCl, 0.5; and NH₄Cl, 1, was adjusted to pH 9 and supplemented with 10 g/l of commercial starch (Sigma, Saint Louis, MO, USA). It was further inoculated with 1 ml of soil suspension, and incubated at 45°C with shaking (180 rpm). A total of 100 strains were isolated and screened using the iodine staining assay. The ratio of the clear zone diameter to that of the colony was then determined, and four isolates exhibiting the highest ratio were tested for amylase production in liquid culture. Among them, one strain called UEB-S produced the highest level of extracellular amylase activity (2 U/ml) after 24 h incubation in a non-optimized medium and was retained for all subsequent studies.

**Phenotypic and Genomic Characterization of Strain UEB-S**

The UEB-S strain biochemical profile and physiological characteristics were determined based on biochemical tests associated with the carbohydrate fermentation pattern, using API 50 CH strips (bioMerieux, Saint Louis, MO, USA). It was further inoculated with 1 ml of soil suspension, and incubated at 45°C with shaking (180 rpm). The resulting solid suspensions were completely solubilized by the enzyme. Enzyme activity was determined as previously described [37]. The reaction mixture consisted of 0.5 ml of 1% soluble starch (w/v), 0.4 ml of 0.1 M acetate buffer (pH, 5.0), and 0.1 ml of crude enzyme extract. After 5 min of incubation at 70°C, the released reducing sugars (glucose equivalents) were measured by the dinitrosalicylic acid (DNS) method [44]. One unit (U) of amylolytic activity is defined as the amount of enzyme releasing 1 μmol of glucose equivalent per minute. Amylase yield was expressed as the total units of crude enzyme produced per gram of solid substrate (U/g).

**Experimental Design and Statistical Analyses.**

A Dohlert uniform shell design [11, 19, 41] was set up to determine the best experimental conditions for optimal enzyme production. The influence of four independent parameters including the inoculum volume (Uᵢ), the ratio of moisturizing agent to solid substrate (Uᵢ₁), the incubation temperature (Uᵢ₂), and the fermentation duration (Uᵢ₃) was examined. The Dohlert matrix consists of N experiments with N = K² + K + 1, where K is the number of variables. Since K = 4, the matrix comprised 21 experiments, which were uniformly scattered in the space of the coded variables (Xᵢ). The levels for the four variables (Table 1) were determined from the results of preliminary experiments (data not shown).

The Dohlert design experimental conditions (Table 2) were deduced from the experimental matrix, by converting coded variables Xᵢ into the real variables (which were experimentally used) Uᵢ using the relationship

\[ Xᵢ = (Uᵢ - Uᵢ₀) / ΔUᵢ \]

where Uᵢ₀ is the level of Uᵢ at the center of the domain and ΔUᵢ is the increment of Uᵢ corresponding to a variation of Xᵢ equal to 1.

**Table 1. Experimental domain for the Dohlert design.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Factor</th>
<th>Unit</th>
<th>U₀</th>
<th>ΔUᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁</td>
<td>U₁ : Inoculum</td>
<td>10⁷ CFU/g</td>
<td>0.80</td>
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</tr>
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<td>X₂</td>
<td>U₂ : Moisture-to-solid ratio</td>
<td>ml/g</td>
<td>4.00</td>
<td>3.00</td>
</tr>
<tr>
<td>X₃</td>
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<td>X₄</td>
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</tbody>
</table>

**Amylase Production from Bacillus UEB-S by Solid-State Fermentation**

Crushed millet (1 g) was transferred into a 250-ml Erlenmeyer flask. Water was then added (taking into account the inoculum volume) to reach the desired moisture-to-millet ratio (liquid-to-solid ratio; L/S). The mixtures were vigorously mixed and autoclaved at 121°C for 20 min. Solid-state fermentation experiments were performed using the conditions stated in Table 2. After fermentation, 10 ml of distilled water was added to the flask, and the content was stirred at 180 rpm, 30°C, for 30 min. The resulting solid suspensions were centrifuged at 8,000 × g for 10 min at room temperature, and the harvested supernatant was used as a crude enzyme extract.

**Determination of Enzyme Activity**

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