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Purification and characterization of a novel α-amylase from a newly isolated 
Bacillus methylotrophicus strain P11-2

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Abstract

An aerobic bacterial strain P11-2 with high amylolytic activity was isolated from soil sample collected from wheat field of Jiyuan, China. The strain was identified as *Bacillus methylophilicus* by morphological and physiological characteristics as well as by analysis of the gene encoding the 16S rRNA. The α-amylase was purified to homogeneity by a combination of 80% (NH$_4$)$_2$SO$_4$ precipitation, DEAE FF anion exchange, and superdex 75 10/300 GL gel filtration chromatography. The purified α-amylase exhibited specific activity of 330.7 units/mg protein that corresponds to 13.1 fold purification. The relative molecular mass of the α-amylase was 44.0 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The optimal pH and temperature for enzyme activity were 7.0 and 70 °C, respectively. The α-amylase activity was stimulated by Mg$^{2+}$, Ba$^{2+}$, Al$^{3+}$ and DL-dithiothreitol (DTT), however, Ca$^{2+}$ almost had no activation or inhibition on the α-amylase. After 4 hours of reaction toward soluble starch, the end products were glucose, maltose and maltotriose. The 10 residues of the N-terminal sequence of the purified α-amylase was SVKNGQILHA, which showed no homology to other reported α-amylases from *Bacillus* strain.

Key Words

α-Amylase; *Bacillus methylophilicus* strain P11-2; Purification; Characterization
1. Introduction

α-Amylase (EC 3.2.1.1, α-1,4-glucan-4-glucanohydrolase) catalyzes the hydrolysis of α-1,4-glycosidic bonds of starch, glycogen and various related polysaccharides in a random manner and releases different sizes of oligosaccharides in an α-anomeric configuration [1]. α-Amylases represent one of the most important industrial enzymes, they have a wide variety of applications in different industrial fields, such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries. These enzymes account for about 30% of the world’s enzyme production [2].

α-Amylases occur in animals, plants, and microorganisms, but only microbial α-amylases are used in industrial applications [3]. They have been reported from a wide variety of microorganisms, such as from several species of Aspergillus [4, 5], from several species of genus Bacillus [2, 6, 7] and Streptomyces [8, 9]. Up to date, microbial amylases have completely replaced chemical hydrolysis in the starch processing industry [10].

For commercial purposes, α-amylases are mainly derived from the genus Bacillus [3, 11]. Different species of the genus Bacillus produce α-amylases with many different properties. Some Bacillus stains produce thermostable α-amylases, others produce α-amylases with acid-resistant property. B. subtilis [12], B. stearothermophilus [13], B. licheniformis [14] and B. amyloliquefaciens [15] are known to be good producers of α-amylase with different properties, and have been widely used for commercial production of α-amylase.

Both genetic manipulation and media optimization have been used to improve
α-amylase properties that is suitable for specific industrial application or get a high yield of enzymes in culture. However, the discovery of new bacterial strains that produce α-amylases with special properties remains a very important way of advancing the field [1].

In this article, we report the discovery of a Ca\(^{2+}\)-independent α-amylase from the newly isolated \textit{B. methylotrophicus} strain P11-2. We also report purification and characterization of the α-amylase produced by \textit{B. methylotrophicus} strain P11-2.

2. Materials and Methods

2.1. Microorganism

The strain P11-2 used in this study was isolated by screening a soil sample collected from wheat field in Jiyuan, Henan Province, China. The soil sample (1 g) was suspended in 9 ml of sterile distilled water and serial dilutions were conducted. Aliquots (0.1 ml) of appropriate dilutions were surface plated on LB agar plate containing soluble starch (1\% w/v). Plates were then incubated at 30 °C for 4–5 days. Colonies exhibiting halo of starch hydrolyzing activity were picked. The strain P11-2 that produced relatively high level of amylase was used for further study.

The 16S rDNA of strain P11-2 was amplified by PCR. Two universal 16S rDNA primers were used for the 30-cycle amplification PCR: 27F (5'-AGAGTTTGATCCTGGCTCA-3'), and 1492R (5'–GGTACCTTGTTACGACTT-3') [16]. The 16S rDNA products were cloned into pGEM-T vectors (Promega, Madison, WI, USA) and sequenced on an Applied...
Biosystems (Foster, Calif., USA) Automatic Sequencer. The 16S rDNA sequences were aligned and compared with sequences deposited in the GenBank database using the BLAST program [17].

2.2. Culture conditions

A single colony from nutrient agar plate was transferred into 5.0 ml liquid nutrient medium and incubated at 37 °C overnight. Then, a 1.0 ml of the seed culture was inoculated to 100 ml with sterile nutrient medium [in g/L: soluble starch 15, peptone 10, yeast extract 5, NaCl 1, K₂HPO₄ 1, MgSO₄·7H₂O 0.5. pH7.2.] in 500 ml Erlenmeyer flasks and incubated at 37 °C with shaking (200 rpm). Growth was monitored by measuring absorbance at 600 nm. Amylase production was observed by measuring amylase activity in the culture supernatant.

2.3. Enzyme assay

A 200 μl reaction mixture containing 1% (w/v) soluble starch and 20 μl of the enzyme sample in 20 mM Tris-HCl buffer (pH 7.5) was incubated at 50 °C for 10 min and the amount of reducing sugars released was measured using 2,5-dinitrosalicylic acid (Analytical grade) reagent. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar as glucose equivalents in 1 min under the assay conditions. Protein was quantified by Bradford’s method using bovine serum albumin as the standard protein [18].

2.4. Purification procedure

Culture broth was centrifuged (10000 rpm, 4 °C, 10 min) to remove cells and debris and the supernatant was collected. Solid ammonium sulfate was added to the
supernatant with a saturation of 80% under gentle stirring, and then kept at 4 °C overnight. The suspension was centrifuged at 10000 rpm for 30 min at 4 °C. The precipitate was then dissolved in 20 mM Tris-HCl buffer (pH 7.5), and centrifuged (10000 rpm, 4 °C, 10 min) again. The supernatant was dialyzed against 20 mM Tris-HCl buffer (pH 7.5) to remove the salts. The solution was applied to a Hiprep DEAE FF column (1 ml) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The unbound proteins from the column were removed by washing with the same buffer at a flow rate of 1 ml/min. Proteins bound to the column were eluted with 20 ml 20 mM Tris-HCl buffer (pH 7.5) containing 0 to 1 M NaCl at the same flow rate, and fractions of 2.0 ml were collected with online monitoring of protein elution at 280 nm. Each fraction was assayed for α-amylase activity and fractions with enzyme activity were pooled. The pooled solution was condensed by freeze-drying, then dissolved in 20 mM Tris-HCl buffer (pH 7.5). A 0.5 ml aliquot of the solution was loaded onto a superdex 75 10/300 GL (GE Healthcare) gel filtration column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 1.0 ml in each tube were collected and assayed for α-amylase activity. Fractions with α-amylase activity showing single protein band on denaturing polyacrylamide gel electrophoresis were pooled and used as the purified enzyme.

2.5. Electrophoretic analysis

Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [19]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 dissolved in a water/methanol/acetic
acid (50:40:10) solvent for 1 h, then destained in the same solvent. The zymogram was carried out according to Mitsunaga et al. [20]. After electrophoresis, gel was incubated in a 1% soluble starch solution in 20 mM Tris-HCl buffer (pH 7.5) for 30 min at 37 °C, then discarded the substrate solution and the gel was stained with a solution of 1.3% I$_2$ and 3% KI for detection of activity.

2.6. Enzymatic reaction product analysis

A 5.0 U of the purified amylase was incubated with 1% (w/v) soluble starch in 20 mM Tris-HCl buffer (pH 7.5) at 40 °C and at different time intervals. Hydrolysis products were subjected to analyzed by High-performance liquid chromatography (HPLC) with a Shodex SH1011 column (8×300 mm; Waters, USA) at 50 °C using a 0.01 N of sulfuric acid as mobile phase at a flow rate of 1.0 ml/min and the products were detected using refractive index detector (Waters 2414) maintained at 50 °C. Authentic chromatographic grade glucose and maltose were used as standards for identification of the hydrolysis products in the reaction mixture.

2.7. Effect of pH and temperature on enzyme activity and stability

The optimal pH for enzyme activities was determined at 50 °C at various pH levels (pH 3.0–12.0). Citric acid phosphate buffer was used for pH 3.0–5.0, sodium phosphate was used for pH 6.0–7.0, Tris–HCl was used for pH 7.5–9.0, and sodium carbonate–bicarbonate was used for pH 10.0–12.0. For pH stability, residual activity was measured at pH 7.5 after 1 h of incubation at 40 °C in the pH buffers being tested. Enzyme activities were tested at temperatures ranging from 30 °C to 80 °C in 20 mM Tris–HCl buffer (pH 7.5). The purified enzyme was incubated for 1 h at the tested
temperature, and residual activity was measured at 50 °C in 20 mM Tris–HCl buffer (pH 7.5).

2.8. Effect of different compounds on α-amylase activity

To investigate the effects of different metal ions and compounds on α-amylase activity, the purified α-amylase was preincubated for 1 h at 37 °C with 5 mM of various metal ions, 10 mM ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), Tween 80, SDS and DL-dithiothreitol (DTT), individually. Residual activity was determined under optimal enzyme assay conditions. Activity assayed in the absence of any additives was taken as 100%.

2.9. Determination of kinetic parameters

The hydrolysis of soluble starch was performed in 20 mM Tris–HCl buffer pH 7.0 at 70 °C (optimal enzyme assay conditions). Initial soluble substrate concentrations ranged from 0.5 to 4 mg/ml. The generated reducing sugars were determined at regular time intervals and expressed in equivalent micromoles of glucose by 1 ml of purified enzyme. The kinetic parameters Km and Vmax were estimated from Lineweaver-Burk plot.

2.10. N-terminal amino acid sequencing of the purified α-amylase

After SDS–PAGE, the α-amylase band was transferred onto a polyvinylidene fluoride (PVDF) membrane (Pall Corporation, USA). The α-amylase band on the PVDF membrane was cut out and dissolved in 20% (v/v) acetonitrile/water containing 0.001% trifluoroacetic acid (TFA). The N-terminal sequences of the purified α-amylase were determined by automated Edman degradation using a pulsed-liquid
sequence analyzer (ABI PROCISE™ 494 GC320078-1 protein sequencing system, Applied Biosystems, USA).

3. Results

3.1. Identification of the isolate P11-2

The soil isolated strain P11-2 is aerobic, motile, endospore-forming, Gram positive, and rod-shape. Colonies are creamy white, convex, wrinkly, non-transparent with irregular edges. Growth occurs at 20–43 °C (optimal temperature 37 °C), pH 2.0–11.0 (optimal pH 7.0) and 0–12% NaCl. It is positive for oxidase, catalase, nitrate reduction, Voges–Proksaer test, starch hydrolysis, and gelatin hydrolysis. However, H₂S formation, indole production, citrate utilization, lipid hydrolysis, urea hydrolysis are all negative. It utilizes various carbohydrates as carbon sources, such as glucose, sucrose, maltose, lactose, fructose, cellobiose, dextrin, etc.

Due to the morphological and physiological characteristics of strain P11-2, we supposed that it belonged to the genus *Bacillus*. To ensure the genetic background of strain P11-2, the gene encoding the 16S rRNA was partially amplified by PCR and its nucleotide sequence was determined. The 16S rRNA gene sequence determined for strain P11-2 was 1431 nucleotides long, and was deposited in GenBank under the accession number JQ246449.

Homology searches revealed that strain P11-2 was related to members of genus *Bacillus*, and showed highest sequence similarity (100%) to *B. methylotrophicus* CBMB205ᵀ. The 16S rRNA gene sequence of strain P11-2 was aligned automatically
using the CLUSTAL_W program to reference sequences of the genus *Bacillus*

obtained from the GenBank [21], and a phylogenetic tree was constructed based on the neighbor-joining method using the software MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 [22]. Phylogenetic tree (Fig. 1) indicated that strain P11-2 together with *B. methylotrophicus* CBMB205T formed a distinct linkage in the tree with 80% bootstrap support.

Compared strain P11-2 to the type strain *B. methylotrophicus* CBMB205T [23], there are some differences in physiological and biochemical characteristics, for example, strain P11-2 can grow in presence of NaCl up to 12.0%, whereas the type strain cannot grow in the presence of >4.0% (w/v) NaCl. However, most of the characters of strain P11-2 show good agreement with the type strain, like colony morphology, temperature and pH scope for growth, oxidase, catalase, nitrate reduction, gelatin hydrolysis, etc. Therefore, strain P11-2 was identified as *B. methylotrophicus* strain P11-2.

### 3.2. Production of α-amylase

The courses of the growth and α-amylase production by *B. methylotrophicus* strain P11-2 are shown in Fig. 2. α-Amylase production was initiated during the exponential growth phase of the strain and began to increase rapidly at the end of exponential growth phase. α-Amylase production reached its maximum (144 U/ml) at the third day of cultivation, which correspond to the end of stationary growth phase.

### 3.3. Purification of α-amylase

The separation of the α-amylase produced by *B. methylotrophicus* strain P11-2 was
performed using ion exchange and gel filtration chromatography on an ÄKTA prime plus system as described in “Materials and methods”. The results of the purification procedure are summarized in Table 1. The α-amylase was purified 13.1-fold with recovery of 7.0% and specific activity of 330.7 units/mg protein. The purified enzyme was analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 3, the enzyme appeared as a single band on SDS-PAGE with an estimated relative molecular mass of 44 kDa. Also, zymographic activity staining revealed the activity band for the amylase at the corresponding position on SDS-PAGE.

3.4. Effects of temperature and pH on the α-amylase activity

The optimal reaction temperature for the α-amylase was about 70 °C (Fig. 5a), the α-amylase activity decreased sharply at temperatures above 70 °C. The enzyme activity was stable when the temperature was below 50 °C. However, the enzyme was almost inactivated at temperatures over 70 °C with 1 h incubation. The α-amylase had high activities between pH 6.0 and 8.0, with maximal activity at pH 7.0 (Fig. 5b). The α-amylase increased from pH 3.0 to 6.0, and decreased from pH 8.0 to 12.0. The enzyme showed good pH stability in the pH range 6.0–9.0, retaining over 80% of its original activity in this pH range at 40 °C for 1 h. However, the α-amylase activity decreased sharply at pHs below 4.0 and above 10.0, only about 20% activity was retained at pH 3.0 to 4.0 and pH 10.0 to 12.0. It retained about 60% of its maximal activity at pH 5.0.

3.5. HPLC analysis of hydrolysis products of α-amylase

The end products of starch hydrolysis by the α-amylase of strain P11-2 were analyzed
by HPLC. As shown in Fig. 4, at an early stage (1 h), the hydrolysis products were maltose, maltotriose and maltotetraose, and with a trace amount of glucose. As the incubation time prolonged, the amount of glucose, maltose and maltotriose increased, but the amount of maltotetraose decreased. After 3 hours of incubation, the amount of maltotetraose was hard to be detected, which was hydrolyzed to smaller one by the α-amylase of strain P11-2. The main products were glucose, maltose and maltotriose, the contents of the three were about 15.29%, 59.44% and 25.27%, respectively, at the time of 16 hours of incubation. However, during the whole process of cultivation, the soluble starch could not be completely hydrolyzed to the three main products by the α-amylase of strain P11-2, at the time of 16 hours of incubation, the degree of hydrolysis was about 84.6%.

### 3.6. Effects of metal ions and chemicals on the α-amylase activities

The effect of various metal ions and chemicals on the amylolytic activity of the purified enzyme was studied. The compounds tested had different effects on the enzyme activity. As shown in Table 2, the purified α-amylase was activated by Mg$^{2+}$ (182%), Ba$^{2+}$ (135%) and Al$^{3+}$ (111%). Ca$^{2+}$ and Fe$^{2+}$ almost had no activation or inhibition on the α-amylase with a relative activity of 96% and 93%, respectively. The following metal ions had slight inhibitions on enzyme activity: Zn$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, Co$^{2+}$ and Fe$^{3+}$. On the other hand, Hg$^{2+}$ completely inhibited the α-amylase activity, the chelating agent EDTA inhibited the enzyme with 78% activity lost. PMSF and Tween 80 had a slightly inhibitory effects on the α-amylase activity. Interestingly, significant increase (162.1%) in α-amylase activity was observed by adding reducing agents
3.7. Kinetic constants

The kinetics of the purified α-amylase was analyzed using soluble starch as substrate. In the optimal enzyme react conditions, the purified α-amylase showed a typical Michaelis-Menten reaction rate. The $K_m$ and $V_{max}$ Kinetic values calculated from the Lineweaver-Burk plot were 1.2 mg/ml and 204 U/mg protein, respectively.

3.8. N-terminal sequence

N-terminal amino acid sequencing of the purified α-amylase resulted in unequivocal determination of the first 10 amino acid residues, which is SVKNGQILHA. When compared to the National Center for Biotechnology Information (NCBI) protein database and SWISS-PROT by BLAST search, the purified α-amylase sequences did not show significant homology to known microbial protein. The purified α-amylase reported here from *B. methylotrophicus* strain P11-2 represented therefore a novel α-amylase.

4. Discussion

Species from the genus *Bacillus* produced a large variety of exoenzymes, such as α-amylase [24], cellulose [25], xylanase [26], protease [27, 28], lipase [29, 30], and a lot of them have been commercialized. The strain P11-2 is a promising α-amylase producing *Bacillus* strain, it was identified by morphological and physiological characteristics as well as by analysis of the gene encoding the 16S rRNA. The results showed that it belonged to *Bacillus methylotrophicus* [23]. To our knowledge, this
work is the first to report on the enzyme production by *B. methylotrophicus*.

The molecular weight of α-amylases produced by species of genus *Bacillus* usually range from 22.5 kDa to 68 kDa [10, 31], the relative molecular weight of *B. methylotrophicus* strain P11-2 is 44 kDa from the SDS-PAGE, which fell into this scope. The closest α-amylase is the α-amylase produced by *B. amyloliquifaciens* TSWK1-1 with molecular weight of 43 kDa [32] and the α-amylase produced by *B. subtilis* BF7658 with molecular weight of 48 kDa [33].

The Km value of α-amylase from *B. methylotrophicus* strain P11-2 towards soluble starch is lower than that of *B. circulans* GRS 313 [34], *B. subtilis* KCC103 [35], *Bacillus* sp. DR90 [36], *B. subtilis* KIBGE HAS [12] and *B. subtilis* strain AS-S01a [37], this means that the α-amylase from *B. methylotrophicus* strain P11-2 possesses considerably more starch-hydrolysing activity compared with the activity of α-amylases from many other *Bacillus* strains, furthermore, the fermentation time for the reach of maximum α-amylase activity is short (3 days). These features make *B. methylotrophicus* strain P11-2 an attractive α-amylase producer for potential applications in industry.

Most of α-amylases are metalloenzymes, which require calcium ions (Ca$^{2+}$) for their activity, structural integrity and stability [10]. This is due to the formation of a calcium-sodium-calcium metal triad in the main Ca$^{2+}$ binding site, bridging domains A and B of the enzyme [38]. But in the case of α-amylase produced by *B. methylotrophicus* strain P11-2, calcium ions had no activation or inhibition on the α-amylase activity. Therefore, the α-amylase of strain P11-2 seemed to act in a
Ca\textsuperscript{2+}-independent manner. This is in line with some other \(\alpha\)-amylases produced by Bacillus spp. [39, 40]. However, the chelating agent EDTA inactivated the enzyme, suggesting that the \(\alpha\)-amylase of strain P11-2 is a metalloenzyme, Mg\textsuperscript{2+} or Ba\textsuperscript{2+} instead of Ca\textsuperscript{2+} could be essential for the activity of this metalloenzyme. There was a significant stimulation of activity with DTT, indicating that one or more thiol groups are important for the activity.

The main end products of soluble starch hydrolysis by the \(\alpha\)-amylase of strain P11-2 were glucose, maltose and maltotriose. Therefore, the \(\alpha\)-amylase of strain P11-2 is classified as a saccharifying-type \(\alpha\)-amylase, which typically produces glucose, maltose and maltotriose from soluble starch [41]. The end products of soluble starch hydrolysis by the \(\alpha\)-amylase of strain P11-2 resemble the products by the \(\alpha\)-amylase of B. licheniformis NCIB 6346, which produced glucose, maltose, maltotriose and maltopentaose from starch, however, the former produced maltose as the major product, for the latter, it was maltopentaose [42]. For \(\alpha\)-amylases from Bacillus spp., there are few reports on the end products of soluble starch hydrolysis containing glucose, except that the end products of soluble starch hydrolysis from \(\alpha\)-amylase of Bacillus subtilis AX20 contained 70-75% glucose [43]. For the \(\alpha\)-amylase of strain P11-2, after 4 h of incubation, the composition of glucose in the end products raised to 15.29\%, that is a rare phenomenon for \(\alpha\)-amylase.

5. Conclusions

In conclusion, the present study described the purification and characterization of
α-amylase from a newly isolated *B. methyloptrophicus* strain P11-2. Some characters of the α-amylase from *B. methyloptrophicus* strain P11-2 are similar to α-amylases from other strains of *Bacillus* spp., but most characters are different, such as molecular weight, the end products of starch hydrolysis, the effects of metal ions on the enzyme, and the first 10 amino acid residues of the enzyme. Therefore, the α-amylase produced by *B. methyloptrophicus* strain P11-2 should be a novel α-amylase. It could be attractive for practical applications in biotechnological processes.

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Figure Legends:

Figure 1 Phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic position of strain P11-2 to some other related members of the genus Bacillus. Reference sequences were retrieved from GenBank under the accession numbers indicated in parentheses after the strain designation. Numbers at nodes are percentage bootstrap values based on 1,000 replications; only values greater than 50% are shown. Bar, 0.005 substitutions per nucleotide position.

Figure 2 Time course of the growth of B. methylotrophicus strain P11-2 (filled squares) and the production of α-amylase (filled circles) during cultivation.

Figure 3 SDS-PAGE and zymogram analysis of the purified α-amylase from B. methylotrophicus strain P11-2. Lane M, molecular weight markers; lane 1, purified α-amylase eluting from superdex 75 10/300 GL column; lane 2, activity staining of the purified enzyme.

Figure 4 HPLC product analysis of soluble starch by the purified α-amylase from B. methylotrophicus strain P11-2 after incubation for 1 hour (A), 2 hours (B), 3 hours (C) and 16 hours (D). Peak 1 to Peak 5 refers to glucose, maltose, maltotriose, maltotetraose and the remaining unhydrolyzed substrate, respectively.

Figure 5 Effects of temperature (A) and pH (B) on activity (solid lines) and stability (dot lines) of the purified α-amylase. Relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. For determining the stability, the activity of the enzyme without any treatment was taken as 100%.
Table 1: Purification steps of $\alpha$-amylase from *B. methylotrophicus* strain P11-2

<table>
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<th>Purification steps</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
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<th>Yield (%)</th>
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<td>(NH$_4$)$_2$SO$_4$ precipitation</td>
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Table 2 Effect of various metal ions and chemical reagents on α-amylase activity of strain P11-2

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</table>
Figure 1

Bacillus amyloliquefaciens subsp. amyloliquefaciens DSM 7<sup>T</sup> (FN597644)

Bacillus siamensis PD-A10<sup>T</sup> (GQ281299)

Bacillus methylotrophicus CBMB205<sup>T</sup> (EU194897)

Bacillus methylotrophicus strain P11-2 (JQ246449)

Bacillus vallismortis DSM 11031<sup>T</sup> (AB021198)

Bacillus subtilis subsp. inaquosorum BGSC 3A28<sup>T</sup> (EU138467)

Bacillus tequilensis 10b<sup>T</sup> (HQ223107)

Bacillus atrophaeus JCM 9070<sup>T</sup> (AB021181)

Bacillus licheniformis ATCC 14580<sup>T</sup> (AE017333)

Bacillus aerius 24K<sup>T</sup> (AJ831843)

Bacillus vietnamensis 15-1<sup>T</sup> (AB099708)
Figure 2

[Graph showing α-amylase activity and OD600 over cultivation time (h).]
Figure 3

![Image of a gel electrophoresis with bands at kDa markers and lanes labeled M, 1, 2.]
Figure 4
Figure 5

A

Relative activity vs. Temperature (°C)

B

Relative ε vs. pH
Highlights

• This is the first to report on the enzyme production by *Bacillus methylo trophicus*.

• The α-amylase produced by strain P11-2 was purified to homogeneity.

• The end products of enzyme reaction toward soluble starch are different from others.

• The N-terminal sequence of the α-amylase showed no homology to other α-amylases.

• Based on its characters, α-amylase of strain P11-2 should be a novel α-amylase.