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Isolation and identification of an alkaliphilic *Bacillus flexus* XJU-3 and analysis of its alkaline amylase

Jian Zhao, Xiaojun Lan, Jun Su, Lei Sun, Erkin Rahman*

(*College of Life Science and Technology*, *Xinjiang University*, *Urumqi* 830046, *China*)

Abstract: [objective] To isolate and identify a new *Bacillus* strain capable of growing under highly alkaline conditions as alkaline amylase producers and to characterize its enzymatic properties. **[Methods]** The isolates were sampled from alkaline sewage in Shihezi city, Xinjiang and screened by plating them on the amylase agar medium depending on the halo zone diameter. The alkaline amylase producer with best enzymatic activity was designated as XJU-3. XJU-3 was identified by its physiological and biochemical characteristics, 16S rDNA sequence homology, and the content of its major cellular fatty acids. **[Results]** XJU-3 was a Gram-positive, spore-forming, aerobic, motile rod alkaliphilic bacterium. It can grow at a broad range of pH (4.0–12.5) in Luria broth medium and its optimum growth was at pH 10 and 37°C. Its NaCl tolerance was up to 15%. Its major cellular fatty acids were anteiso-C15:0 and iso-C15: 0. Comparative 16S rRNA gene sequence analyses showed that XJU-3 was most closely related to *Bacillus flexus*, with 99% similarity. The genomic DNA (G+C) content of our isolate was 39.13 mol %. XJU-3 produced extracellular alkaline amylase, and its maximal enzyme activity was observed at 40℃ and pH 10.0. More than 70% of the enzymatic activity was remained at pH 13.0. The enzyme activity was strongly enhanced with the presence of Co^{2+} and Mg^{2+}. **[Conclusion]** The strain XJU-3 was confirmed as *B. flexus*. Owing to its excellent pH tolerance, the kinds of major cellular fatty acids, and several phenotypic characteristics that were different from the description of the reference strain, the strain was further classified as a new variant of the species *B. flexus*. The enzymatic properties of XJU-3 alkaline amylase indicated its potential in industrial applications.

Keywords: *Bacillus flexus*; alkaline amylase; (G+C) content; 16S rDNA; Phylogenetic analysis

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1 INTRODUCTION

Microorganisms that prefer unusual extreme environments to normal conditions play an important role as investigative tools for studying the relationships and interactions between environmental factors and microbial life[1]. Organisms which display pH maxima for growth in the alkaline pH region, usually between 9 and 10 are properly defined as alkalophiles. Alkaliphilic *Bacillus* strains grow well in extremes of alkalinity higher than pH9[2]. The alkaliphilic *Bacillus* is a potentially rich source of useful enzymes[3]. Alkaliphilic microorganisms produce extracellular enzymes with potential applications in processes that demand extreme conditions. Alkaliphilic *Bacillus* species have attracted much interest due to their ability to produce extracellular enzymes that are active and stable at high pH, including alkaline amylase, protease and carboxymethylcellulase[4]. The unusual properties of these enzymes offer a potential opportunity for their utilization in processes demanding such extreme conditions[5]. An application of great impact has been the inclusion of enzymes in laundry and dishwashing detergents, leather tanning, paper pulp bleaching, production of cyclodextrins and wastes from food processing industries. Among the isolated enzymes, highly

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* Corresponding author. Tel: +86-991-8583450; E-mail: erkin1106@163.com

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alkaline amylases were most studied because of their industrial importance[6].

Amylases (E.C.3.2.1.1) are enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units. Amylases constitute a class of industrial enzymes, which alone form approximately 25% of the enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals [7,8]. Because of the industrial importance of amylases, there is ongoing interest in the isolation of new bacterial strains producing amylases suitable for new industrial applications, such as alkaline amylase for the detergent industry[9]. Amylases with pH values higher than 8.0 have potential applications for starch saccharification in starch and textile industries and also are an ingredient in detergents for automatic dishwashers and laundries[10]. Thus, the potential of alkaline amylases for industrial applications have stimulated the search for microbial strains expressing activities with desired properties[5].

The strain XJU-3 was originally isolated from alkaline sewage in the city of Shihezi, Xinjiang Uygur Autonomic Region of China. It showed very high alkali-tolerance and was identified as a new member of *Bacillus flexus*. This article deals with the isolation and characterization of XJU-3 and some properties of its extracellular alkaline amylase. The above features could favorably overcome the current industrial drawbacks of commercially exploited saccharifying enzymes.

2 MATERIALS AND METHODS

2.1 Isolation and screening of the amylase-degrading bacteria

Fourteen *Bacillus* spp. capable of growing highly alkaline conditions were isolated from alkaline sewage (pH11.0) in Shihezi city, Xinjiang. Screening for starch hydrolysis activity among the isolated colonies was performed by plating them on the amylase agar medium containing (W/V%): soluble starch 1.0, peptone 1.0, yeast extract 0.5, K_2HPO_4 0.1, $MgSO_4{\cdot}7H_2O$ 0.02, NaCl 1.0, agar 1.8, pH 10.0 and incubating for 24 h at 37°C. Staining of the plates with iodine reagent was carried out to reveal any starch hydrolysis halos[6]. The diameter of the halo zone versus the diameter of the colony was used as a semiquantitative method for the selection of the starch hydrolyzing strains. Depending on the zone diameter and clearance isolate XJU-3 was selected as a good alkaline amylase producer.

2.2 Physiological and morphological characteristics of the strain

Cell morphology was studied using light microscopy and transmission electron microscopy (TEM). Phenotype tests were performed according to [11] and the methods described in the Genus *Bacillus*[12].

2.3 Amplification of 16SrRNA gene and sequence analysis

Genomic DNA was extracted from the isolate and purified according to Saito and Miura (1963)[13]. Universal 16S rDNA PCR forward primer 8-27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-CTACGGCTACCTTGTTACGA-3′) were used to amplify 16S rDNA genes[14]. The sequences were edited with MEGA (a freeware program available in the webpage http: //www.megasoftware.net/ MEGA3.1)[15]. 16S rRNA gene sequence data of the isolate have deposited at GenBank database with accession number EF157300. A phylogenetic tree was drawn from neighbor-joining analysis (Kimura 2- parameter model) of the 16s RNA sequences, to depict the relationship between strain XJU-3 and closely related taxa.

2.4 Genomic (G+C) content

The (G+C) content of the DNA was determined by high performance liquid chromatography of the derived deoxyribonucleosides as described by Tamaoka and Komagata[16].

2.5 Analysis of cellular fatty acids

The cellular fatty acids of the isolated XJU-3 were analyzed in accordance with the procedures described in the User's Manual of the Sherlock-MIDI Automated Microbial Identification System (Sherlock Microbial Identification System, version 4.0, MIDI Inc., Newark, DE, USA)[17].

2.6 Characteristics of amylase

2.6.1 Enzyme production medium: The medium for enzyme production comprised (W/V%): starch 2.0, peptone 1.0, yeast extract 1.0, K_2HPO_4 0.1, and $MgSO_4{\cdot}7H_2O$ 0.02. The medium (50 mL) was inoculated with 0.5 mL of the inoculum with an optical density of 0.6 at 600 nm and incubated at 37°C. Samples were harvested till 72 h by centrifugation at 6000×*g* for 10 min and the cell free supernatant was assayed for amylase activity as described below.

2.6.2 Enzyme assay: The amylase activity was estimated on the basis of the reduction in blue color intensity resulting from enzymatic hydrolysis of starch[18,19] .The reaction mixture containing 1% starch (0.1 mL), 0.3 mL of 0.05 mol/L glycine NaOH buffer (pH 10.0) and 0.1 mL of culture filtrate was incubated at 37°C for 15 min in a water bath. The reaction was terminated by adding 1 mL chilled 1 mol/L HCl. To this mixture, 2 mL of diluted iodine was added and the volume was made to 10 mL with double distilled water. A substrate control-lacking enzyme was also kept with each set of reaction. The amylase activity was estimated after appropriate dilution and absorbance was read at 620 nm against a substrate blank in a SpectumLab53 spectrophotometer. One dextrinizing unit of amylase activity is defined as the amount of enzyme that results in 10%

decline in the optical density of the starch iodine complex at 620 nm when compared to substrate blank.

All the experiments were carried out in triplicates and results presented are the mean of three values. The standard deviation was within 5%.

2.6.3 Partial purification: The cell free supernatant fluid was precipitated using ammonium sulphate to 85% saturation. The precipitate was dissolved in 0.2 M glycine NaOH buffer (pH 10.0) and dialysed overnight against the same buffer[19]. The dialysate was used for all enzyme characterization studies.

2.6.4 Effect of pH on enzyme activity and stability: The pH optimum of the enzyme was determined by varying the pH of the assay reaction mixture using the following buffers (0.2 mol/L): Na_2HPO_4/citrate phosphate buffer (pH 3.0–8.0), glycine/NaOH buffer (pH 9.0–10.0), Na_2HPO_4/NaOH buffer (pH 11.0) and KCl/NaOH buffer (pH12.0–13.0). To determine the stability of amylase, the enzyme was pre-incubated in different buffers (pH 7.0–13.0) for 30 and 60 min. The residual enzyme activity was determined as described earlier.

2.6.5 Effect of temperature on enzyme activity and stability: The temperature optimum of the enzyme was evaluated by measuring the amylase activity at different temperatures (40°C –90°C) in 0.2 mol/L glycine/NaOH buffer (pH 10.0). The effect of temperature on amylase stability was determined by measuring the residual activity after 20, 40 and 60 min of preincubation in 0.2 mol/L glycine/NaOH buffer (pH10.0), at temperatures ranging from 40°C to 70°C.

2.6.6 Effect of metal ions on enzyme activity: For determining the effect of metal ions on amylase activity, enzyme assay was performed after pre-incubation, at 40°C (optimum) for 60 min, of the enzyme with various metal ions each at a concentration of 50 mmol/L. The enzyme assay was carried out in the presence of $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, $FeSO_4$, $CoCl_2$, $MnSO_4{\cdot}4H_2O$, $ZnSO_4{\cdot}7H_2O$, $CuSO_4$ and EDTA.

3 RESULTS

3.1 Morphological, physiological and biochemical properties

From a sample of alkaline sewage (pH11.0) collected from Shihezi city, Xinjiang, we have screened a novel alkaline amylase-degrading microorganism. Depending on the zone diameter and clearance, the isolate XJU-3 was selected as a good alkaline amylase producer. The isolate has deposited in the China Center for Type Culture Collection (CCTCC AB 207167).

The isolate was able to grow and produce extracellular amylase when it was cultured in amylase induction medium. It was a Gram-positive, aerobic, motile, spore-forming, alkaliphilic bacterium, and its cells was rod-shaped [(0.9–1.4) μm×(2.0–4.3)μm] with non-fla gella under transmission micrograph (Fig. 1). It grew at a wide range of pH values (4.0–12.5) in LB medium, with the optimum being at pH 10. Growth occurred at 10°C–60°C, with the optimum growth temperature being at 37°C. Its NaCl tolerance was high up to 15 %. It showed obviously catalase-positive and oxidase-negative reactions but did not reduce nitrate to nitrite. Typical phenotypic and chemotaxonomic properties of are summarized in Table 1. The differences were ob-

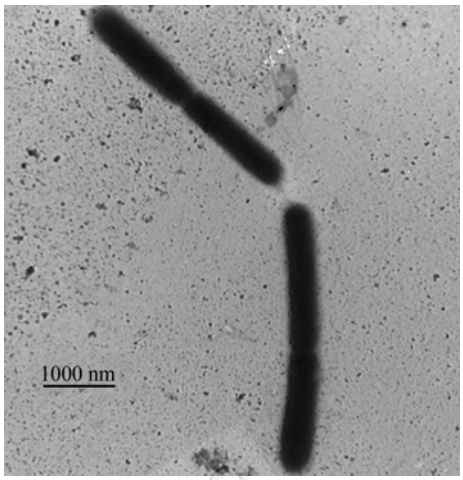

Fig. 1 Transmission micrograph of *Bacillus flexus* XJU-3.

Table 1 Characteristics of *Bacillus flexus* XJU-3 and *B.flexus* DSM 1320^T

Characteristic	*B.flexus* XJU-3	*B.flexus* DSM 1320^T *
Colony color	Cream	Cream
Gram reaction	+	+
Spore position	C	ND
Tolerance of pH4-12.5	+	ND
Growth in NaCl at:		
5% (w/v)	+	+
8% (w/v)	+	+
15% (w/v)	+	ND
Growth at:		
40°C	+	+
50°C	+	–
60°C	+	–
Catalase	+	ND
Oxidase	–	ND
Nitrate reduction	–	–
Hydrolysis of:		
Casein	+	+
Starch	+	+
Urea	+	-
Tween20,40,60,80	+	ND
Acid production from:		
D-Fructose	+	+
D-Glucose	+	+
Glycerol	+	+
D-Lactose	–	+
Maltose	+	+
D-Mannitol	+	+
D-Raffinose	–	+
Sucrose	+	+
D-Xylose	+	–
D-Trehalose	+	+

C, central; +, positive; –, negative; ND, no data. * data for reference strain B. flexus DSM 1320^T from Lim J-M *et al.*(2006)[20]

the isolate and reference strain *B. flexus* DSM 1320^{T}[20] served that our isolate could grow at 50°C–60°C and assimilate urea. Both strains showed nearly identical results on the carbohydrate utilization tests. And they also could use glycerol, D-Glucose, Maltose, D-Fructose, D-Mannito, starch, D-Trehalose and sucrose. However, D-Xylose could be used by strain XJU-3 but can not be used by *B. flexus* DSM 1320^{T}. At the same time, D-Lactose and D-Raffinose could be used by *B. flexus* DSM 1320^{T} but can not be used by strain XJU-3.

3.2 16S rDNA sequences analysis and (G+C) content

For further analysis, we amplified complete 16S rDNA sequence (1451 nucleotides) of the strain XJU-3 with GenBank accession number was EF157300. In order to understand the phylogenetic position of our isolate, we constructed a phylogenetic tree based on comparison of 16S rDNA sequences of our isolate and correlative taxa (Fig. 2). XJU-3 has 99% sequence similarity with all known strains of *B. flexus* and formed a tight cluster with them. The (G+C) content of the genomic DNA of strain XJU-3 (39.13 mol %) was almost identical to that of *B. flexus* (37–39 mol %). All obtained data of 16S rDNA sequences similarity, phylogenetic tree and (G+C) content of the genomic DNA demonstrated that the isolate XJU-3 is a new strain of *B. flexus*.

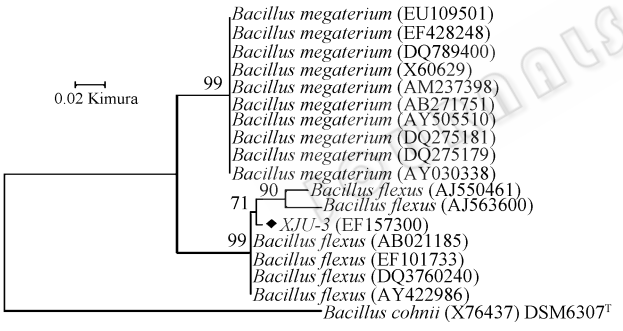

Fig. 2 Phylogenetic tree drawn from neighbor-joining analysis (Kimura 2-parameter model) of the 16s rDNA sequences, depicting the relationship between strain XJU-3 and closely related bacillus. With 1000 bootstrap replicates are shown. Numbers in parentheses represent the sequences accession number in GenBank. The number at each branch point is the bootstrap support value (%).

3.3 Cellular fatty acids analysis

The cellular fatty acid profile of strain XJU-3 was characterized by saturated branched fatty acids such as anteiso-C15:0 (20.98%), iso-C15:0 (44.83%) as the major fatty acids. The fatty acid profile of strain XJU-3 was similar to that of its close relative, *B. flexus* DSM 1320^{T}, but the fatty acid compositions are somewhat different (Table 2), the primary component of cellular fatty acid in XJU-3 is an iso-C15:0, and the secondary component is an anteiso-C15:0, Whereas the primary component in *B. flexus* DSM 1320^{T} is an anteiso-C15:0, and the secondary component is an iso-C15:0.

Table 2 Cellular fatty acid profiles of *B. flexus* xju-3 and *B.flexus*DSM 1320^{T}

Fatty acid	*B.flexus* xju-3	*B.flexus*DSM 1320^{T}
Straight-chain fatty acids		
C15:0	–	–
C16:0	6.39	1.2
Branched fatty acids		
iso-C14:0	6.97	1.6
iso-C15:0	44.83	32.2
anteiso-C15:0	20.98	52.4
iso-C16:0	2.92	8.6
iso-C17:0	2.98	2.1
anteiso-C17:0	2.54	3.4
Unsaturated fatty acids		
C16:1ω7c alcohol	0.72	ND

Values are percentages of total fatty acids. ND, Not detected. Fatty acids representing less than 0.5 % are not included.

3.4 Characteristics of amylase

3.4.1 pH optimum and stability: Optimum pH of XJU-3 amylase is shown in (Fig. 3-A). Determination of enzyme activity at 40°C at pH values ranging from 3.0 to 13.0 showed the amylase to be active in a wide range of pH values with maximum production being at pH 10.0 (14.63 U/mL). More than 70% of enzyme activity was found between pH 9.0 and 13.0. A significant decline of enzyme activity was observed in the acidic pH and only 50% residual activity was retained at pH7.0, whereas 90% retained at pH 11.0. The pH stability was determined at 40°C with different pH values (pH 7.0–13.0). Amylase was stable between pH 8 and 13, and more than 60% of the activity was retained after incubated for 1h (Fig. 3-B). It is clearly evident that amylase produced by XJU-3, which was designated as XJU-3 amylase, is very stable to extremely alkaline environment.

3.4.2 Temperature optimum and stability: Using starch as substrate, the optimum temperature of the amylase was 40°C (Fig.3-C). Under some constant temperature, we found that XJU-3 amylase was very stable at 50°C. Even more than 60% of amylase activity still observed when it was incubated at 60°C for 1 h (Fig.3-D).

3.4.3 Effect of different metal ions and chelating agents: The effects of various metal ions and chelating agents on enzyme activity were examined at pH 10 and 40°C (Table 3). Slight effects were observed in the presence of Zn^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+} and chelating agents of EDTA only inhibited activity of the enzyme to 52, 78, 39, 54 and 65%, respectively. In contrast, Mg^{2+}, Co^{2+} obviously increased the enzyme activity to 138 and 123%, respectively.

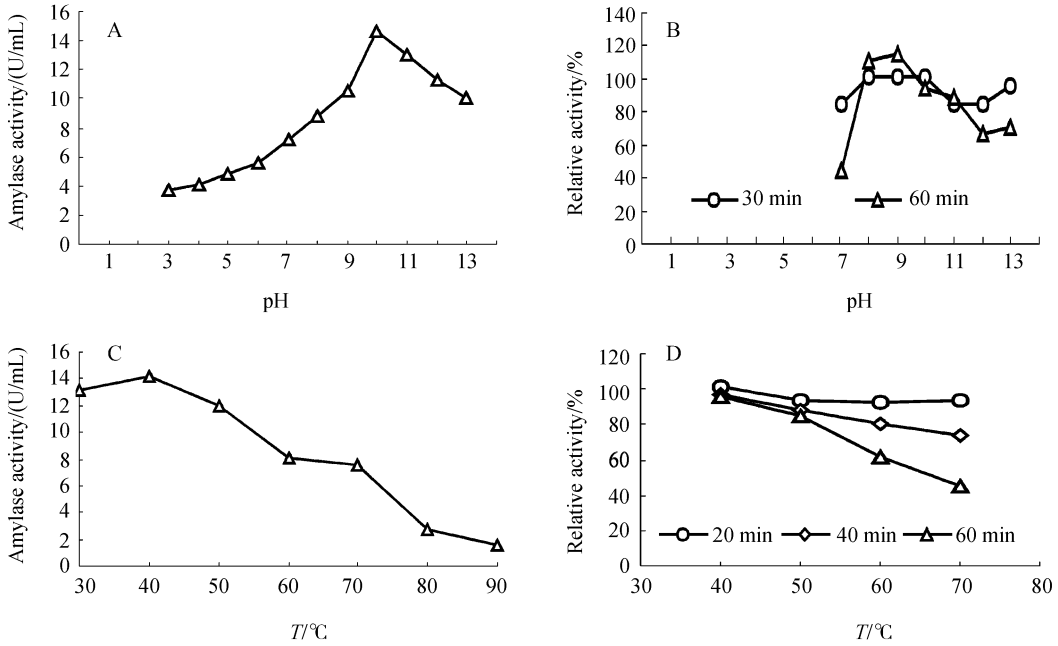

Fig. 3 The effects of temperature and pH on the amylase activity and stability. A: The effects of pH on the amylase activity, B: pH stability of the enzyme, C: The effects of temperature on the amylase activity, D: Thermal stability of the enzyme.

Table 3 Effects of metal ions

Cation/(50 mmol/L)	Relative activity /%
Control	100
Fe^{2+}	52
Co^{2+}	123
Cu^{2+}	78
Mn^{2+}	39
Zn^{2+}	91
Mg^{2+}	138
Ca^{2+}	54
EDTA	65

4 DISCUSSION

The isolate XJU-3 could grow and produce extracellular amylase when it was cultured in amylase agar medium. Combined with morphological, physiological and biochemical characteristics with 16S rDNA sequence analysis and the (G+C) content of the genomic DNA , the strain should be identified as *B. flexus*.

Owing to the primary component of cellular fatty acid, the 16S rDNA sequences, the hydrolysis of urea, the utilization of D-Xylose, D-Lactose and D-Raffinose, and the excellent pH tolerance, which were different from those of the reference strain *B. flexus* DSM1320^T and the description of the Manuals of Bergey[11], the strain was classified as a new member of *B. flexus*.

The alkaliphiles can grow at pH 9 and the optimum pH is 10.0–12.0, but they can grow weakly or not grow at pH6.5 approximately[21], while alkali-tolerant bacteria cannot grow at the pH over 10.5, but can grow at pH7.0–9.0[2]. According to the broad range of pH tolerance (from 4.0 to 12.5), we could not distinguish exactly that the strain was belonging to alkaliphiles or alkali-tolerant bacteria. Similar observations were also reported by Yixia *et al.* (2006) for *B. halodurans* XJU-1 (pH4.5–12.6) and *B. halodurans* XJU-80 (pH3.8–12.8)[22]. Furthermore, the isolate seemed to have some facultatively acid-tolerant characters, which also suggested that the isolate has adapted to various regulatory pathways for its survival under different pH environmental conditions during long stage of evolutionary process.

The optimal temperature and pH of amylase activity from XJU-3 was 40°C and pH 10.0, respectively. From the fact that its enzyme activity was sharply declined at the acidic pH, 50% residual activity was still retained at pH 7.0 and even 90% enzyme activity was observed at pH11.0, it seemed that the amylase was an alkaline enzyme. The optimum amylase activity was at pH10.0, and it showed better resistance to alkaline condition, which is comparable to that described for other *Bacillus*. Commercial saccharifying amylase from *B. licheniformis* NCIB 6346[23] showed maximum activity at pH7.0–9.0, and its activity was reduced markedly at lower or higher pH. *Micrococcus halobius OR-1* remained 60% of amylase activity at pH12.0[24]. However, amylase of XJU-3 was very stable at pH8.0–13.0, with 70% of its activity being remained after incubated for 1 h at pH13.0, and it was more stable under the alkaline condition, than that of *Bacillus sp.* L1711[5]. It is clear that the amylase of our isolate is more stable to extremely alkaline environment than all above mentioned amylase and this is a

desirable property for industrial starch liquefaction.

E.C.M.J. Bernharsdotter reported that Co^{2+} was found to stimulate activity on amylases from *Bacillus sp.* L1711[5], and our data are in accordance with the results. Co^{2+} stimulates the enzymatic activity up to 123%. The effect of Mg^{2+} were irregularly varied, for instance, it had a potent inhibitory effect on the amylases of *B. subtilis* JS-2004[25] and *Bacillus* sp. L1711[5], whereas it could stimulate on the enzyme of XJU-3 to 138%. A stronger inhibitory effect was observed at the presence of Ca^{2+}, Cu^{2+}, and Mn^{2+}. Ca^{2+} plays an important role in the maintenance of enzymatic activity and structural integrity of amylases, including those of various alkaliphilic *Bacillus* species[18,26]. Amylases of *B. subtilis* JS-2004[25], *Bacillus sp*. TS-23[27] and ANT-6[28] were activated by low concentration of Ca^{2+}. Inhibition of amylase activity at high concentration of Ca^{2+} has been reported earlier also for *Bacillus sp.* L1711 amylase[5]. Similarly, in this study, amylase of XJU-3 was inhibited by 54% (50 mmol/L Ca^{2+}). Inhibition of catalytic activity of certain amylases at relatively high concentrations of Ca^{2+} ions has been ascribed to interference caused by the binding of the metal ion at the secondary binding site within the substrate binding cleft that involves the catalytic residues[29,30]. The inhibition of XJU-3 amylase by Cu^{2+} and Mn^{2+} could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased enzyme activity[25,31].

XJU-3 can grow at a broad range of pH values (4.0–12.5), and its alkalitolerance was obviously higher than previously reported *Bacillus sp*. To date, there wasn't any report about the properties of amylase produced by *B. flexus*, Moreover, XJU-3 amylase was most active at pH10.0 and showed better stability at alkaline condition (pH8.0–13.0). Thereby, our isolate may not only provide important clue for clarification of the mechanism of adaptation to extreme environment and stress response, but also is as significant as reference strain for the study of extreme organism and the industrial application of potential extremozymes. Along with these characteristics, XJU-3 amylase would certainly have a wider industrial application and have a prospect future. Moreover, we can screen amylase high-production strain through conventional mutative methods and gene engineering techniques to increase its output, and the correlative study is carrying out.

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一株碱性淀粉酶产生菌 *Bacillus flexus* XJU-3 的分离鉴定及酶学特性分析

赵建，兰小君，苏俊，孙磊，艾尔肯·热合曼*

(新疆大学生命科学与技术学院，乌鲁木齐 830046)

摘要: 【目的】从新疆石河子市一处碱性工业污水（pH11.0）分离、鉴定高产碱性淀粉酶菌株，并对其所产酶酶学特性进行研究。【方法】在碱性淀粉酶分离培养基上对所分离菌株进行筛选，分离到一株高产碱性淀粉酶菌株，并将其编号 XJU-3。应用生理生化试验，脂肪酸含量，16S rDNA 序列以及(G+C) mol%含量等方法对菌株进行鉴定，同时对 XJU-3 所产碱性淀粉酶的生物学特性进行研究。【结果】 XJU-3 可在 pH4.0~12.5 的 LB 培养基上生长,最适生长温度 37℃。16S rDNA 序列构建的系统进化树表明 XJU-3 与 *Bacillus flexus* 类聚在一起，且序列同源性为 99%。该菌产生的淀粉酶最适 pH10.0，最适温度 40℃，且在 pH9.0~13.0 内有较高活性和稳定性。Co^{2+}和 Mg^{2+}能明显提高酶的活性。【结论】XJU-3 被鉴定为 *Bacillus flexus*，由于 XJU-3 与 *B. flexus* DSM 1320^T 在尿素水解和优势脂肪酸含量上有差异，且具有宽范围 pH 耐受性，因此 XJU-3 被认为是 *B.flexus* 的一个新菌株。XJU-3 所产的碱性淀粉酶酶学特性良好，具有极大的工业应用潜力。

关键词: *Bacillus flexus*；（G+C）mol%含量；16S rDNA；系统进化树；碱性淀粉酶

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*通讯作者。Tel: +86-991-8583450; E-mail: erkin1106@163.com

作者简介：赵建(1974–), 女, 河南南阳人, 硕士研究生, 主要从事工业微生物的研究。E-mail：lmlm314@163.com

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