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Characterization and Optimization of Amylase Production in WangLB, a High Amylase-Producing Strain of *Bacillus*

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Abstract The costs of amylase represent ca. 24 % of the expenditures in the starch industry and an increase in amylase production and/or activity will greatly cut down on production costs. In the present study, we obtained a high amylase-producing strain of bacteria, WangLB, and identified it as a member of the *Bacillus* genus based on 16S rDNA analysis. The fermentation conditions for amylase production in the strain were optimized, and the maximum amylase activity we obtained was 26,670 ± 1390 U/mL, under the optimized conditions of 48-h incubation in liquid starch medium, 35 °C, pH 10, 1 % *v*/*v* inoculum concentration, 20 g/L starch concentration, and 0.1 % *w*/*v* peptone. The influences of 16 small organic inducers on amylase production were tested, and the results showed that 20 mmol/L alanine greatly enhanced amylase production to 290 % of the baseline level. We also conducted an amylase enzymology analysis. The molecular weight of the amylase was 55 kD, determined by SDS-PAGE. The optimum temperature and pH for the amylase were 55 °C and pH 9, respectively. The enzyme also showed high activity over a wide range of temperatures (50–85 °C) and pH values (3–10), and the activity of the amylase was Ca^{2+} independent. The kinetic parameters K_m and V_{max} were 0.37 ± 0.02 mg/mL and 233 U/mg, respectively. Finally, the amylase was applied to the hydrolysis of five different brands of starch. It was found that the hydrolyzability of the substrate by amylase increased along with starch solubility.

Keywords Bacillus sp · Amylase · Fermentation parameters · Enzymology

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Introduction

α-Amylase (1,4-α-D-glucan glucanohydrolase [E.C. 3.2.1.1]) is an endo-acting enzyme that randomly hydrolyzes α-(1 → 4) glycosidic linkages in starch to produce a mixture of oligosaccharides [1, 2]. Currently, α-amylase is widely applied in industries including the food, textile, paper, detergent, brewing, and medical industries, and it comprises 25–30 % of the world's enzyme consumption [3, 4]. Although α-amylase can be obtained from a variety of natural resources, such as animals, plants, and microorganisms, the bacterial and fungal amylases dominate commercial production because of their easy manipulation, cost-effective and efficient production, and thermostability [5, 6]. Among them, the *Bacillus* species, such as *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. methylotrophicus*, are the most frequently used bacterial workhorses for amylase production. The estimated value of the world amylase market was approximately US$ 2.7 billion in 2013 and was estimated to increase by 4 % annually through 2012 [3].

Recently, amylases with desirable features, including improved activity, substrate specificity, thermal stability, a wide pH profile, and proper resistance against denaturing agents and heavy metals, have drawn considerable attention from the industry. Consequently, there has been ongoing interest in the isolation and optimization of new amylase-producing *Bacillus* strains suitable to different industrial applications [4]. For example, the mesophilic *B. licheniformis* NCTB 6346 produces an extremely thermostable α-amylase that retains more than 98 % of its original activity after incubation at 85 °C for 60 min [7]. The amylase secreted by *B. licheniformis* SKB4 displays the highest activity at 90 °C [8]. The amylase produced by *Bacillus* sp. KR-8104 has an optimum pH of 3.38–4 [9]. These strains are suitable for the starch industry, because their high thermostability and high optimum temperature characteristics reduce cooling costs and decrease viscosity, allowing accelerated mixing and pumping, as well as a reduced risk of microbial contamination. Moreover, their stability at low pH values matches the starch slurry pH used in starch hydrolysis, of approximately 4.5 [10]. The amylase produced by *B. methylotrophicus* P11-2 [11] and *B. amyloliquefaciens* TSWK1-1 [12] show Ca^{2+} independence, which precludes the formation of calcium oxalate, a substance blocking process pipes and heat exchangers, unlike with Ca^{2+}-dependent amylases [13–17]. In addition, α-amylases with optimum pH values higher than 8 are well suited for applications in detergent production, such as for dishwasher and laundry detergent formulations [18, 19].

Despite these advances, there is still a great deal of room for improvement. For example, amylases with high optimum temperatures usually require high temperatures for strain growth and enzyme production, which involves special process designs and extra energy costs [13, 20]. Therefore, there has been high demand for the screening of new mesophilic strains that produce amylase with high thermostability and at high optimum temperatures. At the same time, the cost of amylase usually represents 24 % of the total process cost [21], leading to growing interest in the reduction of amylase costs by elevating enzyme production yield and/or activity.

In the present study, we report a high amylase-producing strain of *Bacillus*, WangLB, which achieved a maximum amylase production of 26,670 ± 1390 U/mL after 48 h of incubation. This activity of 26,670 ± 1390 U/mL represents an 8- to 7000-fold increase over production rates reported in the literature, including those of *B. subtilis* WBS (6.24 U/mL) [14], *Bacillus* sp. KR-8104 (3.824 U/mL) [9], *B. licheniformis* and *B. licheniformis* AI20 (100 and 384 U/mL, respectively) [1, 17], and *Bacillus cereus* BRSC-S-A26MB (3197.15 U/mL) [22]. The optimum temperature for the amylase production in WangLB was 35 °C, and the amylase showed higher than 90 % activity throughout the 55–85 °C range. Moreover, the amylase is

Ca^{2+} independent and showed high pH stability over a broad range of pH values from 3–10 (>85 %). These advantages indicate that *Bacillus* sp. strain WangLB has great potential for industrial applications.

Materials and Methods

Growth Media

Luria-Bertani (LB) media (1 L) was composed of 10 g peptone, 5.0 g yeast extract, and 5.0 g NaCl, to which deionized water was added for a final volume of 1 L. Liquid starch medium (1 L) was composed of 20 g starch, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$·7H2O, 0.5 g NaCl, and 0.01 g $FeSO_4·7H_2O$, to which deionized water was added for a final volume of 1 L.

16S rDNA Identification

Bacteria were grown in LB medium for 24 h at 37 °C, and genomic DNA was then isolated using the Geneaid DNA Extraction Kit purchased from FroggaBio (Toronto, Canada), following the manufacturer's protocol. The 16S rDNA was amplified using HAD-1 (5′-GACTCCTACGGGAGGCAGCAGT-3′) and E1115R (5′-AGGGTTGCGCTCGTTGCGGG-3′) primers (Eurofins Scientific, Toronto, Canada). The PCR reaction mixture contained 25 μL PCR Master Mix (2×) (FroggaBio), 2 μL HAD-1 primer, 2 μL E1115R primer, 4 μL genomic DNA template, and 17 μL sterilized water. The PCR program used was as follows: primary denaturation for 5 min at 94 °C, followed by 35 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing for 30 s at 55 °C, and extension at 72 °C for 1 min, and then concluding with a final extension step at 72 °C for 10 min. The PCR product was visualized on a 1 % agarose gel to confirm size, quantity, and purity. The PCR product was further purified by a PCR purification kit (FroggaBio) and sequenced by The Centre for Applied Genomics (TCAG, Toronto, Canada).

The bacterium was identified by using the 16S rDNA sequence as input for the Basic Local Alignment Search Tool (BLAST) program of the NCBI database (http://blast.ncbi.nlm.nih.gov/) to obtain the possible genus. The 16S rDNA data were then submitted to the NCBI GenBank database with accession no. KU310971. The phylogenetic tree of the strain was constructed using the MEGA 5.1 program.

Optimization of Fermentation Parameters for Amylase Production

The effects of incubation time, temperature, pH, starch concentration, inoculum amount, nitrogen sources, and small organic molecular inducers on amylase production were investigated by testing the amylase activity of the fermentation supernatant under various experimental conditions. In all the tested conditions, the strains were inoculated in 50-mL liquid starch medium and cultured in 250-mL shaking flasks with a rotating speed of 200 rpm. Amylase activity was assayed by measuring the release of reducing sugar using the 3,5-dinitrosalicylic acid (DNS) method [23]. Briefly, the crude enzyme solution was diluted 100 times, and then 10 μL of diluent was mixed with 0.1 mL of 1 % (*w*/*v*) starch and bathed at 40 °C for 20 min, followed by the addition of 0.3 mL DNS reagent. The reaction mixture was heated in a boiling water bath for 5 min. After cooling, 0.3 mL of the solution was injected into

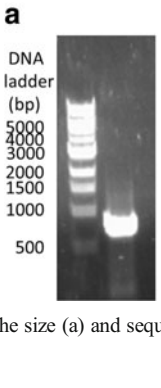
a 96-well ELISA plate to determine the absorbance at 520 nm. One unit of "amylase activity" was defined as the amount of the enzyme in 1 mL fermentation broth that hydrolyzed 1 μmol reducing sugar (in this case maltose) per minute under standard assay conditions.

The strain was first cultured in LB media at 37 °C for 24 h, and then inoculated in the liquid starch medium for different purposes. The basic fermentation conditions were 1 % (*v*/*v*) inoculation concentration, 30 °C, pH 7, 200 rpm, and 20 g/L starch, unless otherwise stated. The incubation time was first optimized by testing the amylase activity at 0, 24, 48, 72, and 96 h. In addition, the protein content in the supernatant was measured via the Bradford method [24] at the same incubation times. The amylase activity and protein content both peaked at 48 h (Fig. 1a), and the optimal incubation time of 48 h was therefore used in the subsequent tests.

The influence of temperature on amylase production was investigated by culturing the strain at 25, 30, 35, 37, and 40 °C. The effect of pH was investigated by culturing the strain at different pH values in the 3–11 range. The pH of the medium was adjusted through addition of HCl and NaOH. The impact of four nitrogen sources, yeast extract, peptone, urea, and ammonium nitrate, on the strain was determined by adding 0.2 % *w*/*v* of each nitrogen source to the liquid starch medium as the sole nitrogen source. The effect of the peptone concentration on amylase production was also tested at different concentrations in the range 0.1–0.5 % *w*/*v*. To determine the effect of the starch concentration, the strain was incubated in the liquid starch medium with starch concentrations of 10, 20, 30, 40, and 50 g/L. To evaluate the influence of the inoculum concentration, the strain was inoculated at 0.1, 0.3, 0.5, 0.7, 1.0, and 2.0 % *v*/*v* concentrations. The impact of 16 small molecular inducers (arabitol, sorbitol, galactose, xylose, mannose, glucose, mannitol, xylitol, adonitol, cellobiose, alanine, asparagine, galactose, sucrose, cysteine, and lactose) was determined by individually adding the inducers to the liquid starch medium at a concentration of 5 mmol/L. The effects of asparagine and alanine concentrations were further tested within the concentration range of 5–25 mmol/L.

Enzymology Characterization of Amylase

Separation and purification of amylase was performed by first culturing the amylase-producing strain in the LB medium at 37 °C for 24 h, and then using it to inoculate the liquid starch media, which was then cultured under the optimized conditions (35 °C, pH 10, 0.1 % *w*/*v*, peptone, 20 g/L starch, 1 % *v*/*v* inoculum concentration) for 48 h. The fermentation broth was centrifuged at 12,000×*g* and 4 °C for 10 min. Next, the supernatant was filtered with a polyethersulfone membrane (0.22 μm; Sterlitech Corporation, Kent, WA, USA), followed by dialyzation with a dialysis tube (molecular weight cutoff, 1 kDa) at 4 °C for 24 h. The



b

1 gaaggttttc ggatcgtaaa gctctgttgt tagggaagaa caagtgccgt tcaaataggg
61 crgcaccttg acggtaccta accagaaagc cacggctaac tacgtgccag cagccgcggt
121 aatacgtagg tggcaagcgt tgtccggaat tattgggcgt aaagggctcg caggcggttt
181 cttaagtctg atgtgaaagc ccccggctca accggggagg gtcattggaa actggggaac
241 ttgagtgcag aagaggagag tggaattcca cgtgtagcgg tgaaatgcgt agagatgtgg
301 aggaacacca gtggcgaagg cgactctctg gtctgtaact gacgctgagg agcgaaagcg
361 tggggagcga acaggattag ataccctggt agtccacgcc gtaaacgatg agtgctaagt
421 gttagggggt ttccgcccct tagtgctgca gctaacgcat taagcactcc gcctggggag
481 tacggtcgca agactgaaac tcaaaggaat tgacgggggc ccgcacaagc ggtggagcat
541 gtggtttaat tcgaagcaac gcgaagaacc ttaccaggtc ttgacatcct ctgacaatcc
601 tagagatagg acgtcccctt cgggggcaga gtgacaggtg gtgcatggtt gtcgtcagct
661 cgtgt

Fig. 1 The size (a) and sequence of the 16S rDNA of strain WangLB

solution was then transferred to another dialysis tube with a molecular weight cutoff of 50 kDa and concentrated by 10 % (*m*/*v*) polyethylene glycol at 4 °C for 24 h. The enzyme was then purified by the trichloroacetic acid (TCA)/acetone protein precipitation method [24]. Solutions of TCA/acetone (*v*/*v*: 1:4) were added to the concentrated enzyme solution with a volume ratio of 4:1 to precipitate the enzyme at −20 °C for 12 h. After precipitation, the enzyme solution was centrifuged at 12,000×*g* for 10 min. The protein pellet was washed by 100 % acetone three times, and then dried to produce the crude enzyme for the following experiments. The purified amylase was measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine molecular weight and activity.

The SDS-PAGE analysis was conducted following the method described by Laemmli [25], using a Bio-Rad electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). A protein marker and the purified amylase were run simultaneously in 10 % SDS-PAGE to determine the molecular weight of the amylase. The gel was then stained in Coomassie Brilliant Blue R-250 (panel a) to determine the molecular weight of the enzyme. The gel containing 0.25 % starch was utilized to detect the amylase activity (panel b). The gel was washed by 2 % Triton X-100 and rinsed with distilled water three times. The gel was then transferred to 100 mM PBS buffer (pH 7) and incubated at 55 °C for 20 min. The resulting gel was stained with 0.1 % Congo red solution and then destained with 1 M NaCl to visualize the clear bands of amylase activity.

The enzymology characteristics of the purified amylase were investigated by various means, using amylase activity as the response variable, which was assayed by the DNS method described above. The effect of temperature was measured in a range from 25 to 90 °C, at intervals of 5 °C. The impact of pH was measured in a pH range of 3–12. The buffer solutions used were as follows: pH 3–5 in 100 mmol/L citrate buffer, pH 6–8 in 100 mmol/L PBS buffer, pH 9–11 in 100 mmol/L glycine + NaOH buffer, and pH 12 in 100 mmol/L KCl + NaOH buffer. To evaluate the effect of metal ions, amylase activity was measured in the presence of 2 and 5 mmol/L Cu^{2+}, Ca^{2+}, Co^{2+}, K^{+}, Na^{+}, Mn^{2+}, Mg^{2+}, and Zn^{2+}, respectively. The influence of detergents (SDS, Triton X-100, and Tween 20) and organic solvents (methanol and ethanol) were tested by individually adding these chemicals to the reaction mixture with final concentrations of 2 and 5 %, respectively.

The kinetic parameters of the amylase were determined with the Michaelis–Menten equation, as described in our previous work [26]. The starch concentration varied from 0.2–3 mg/mL. The reciprocal of the reaction velocity ($1/V$) was plotted as a function of the reciprocal of the starch concentration ($1/[S]$) to make a Lineweaver–Burk plot, which enabled us to calculate the Michaelis constant (K_m) and maximum velocity (V_{max}) from the slope and intercept of the plot.

Application of Amylase for Hydrolysis of Different Types of Starch

The purified amylase was further tested by applying it to the hydrolysis of five different brands of starch using the DNS method described above. The five brands of starch substrate were S1 from Real Canadian Superstore with item no. of 64548 (Thunder Bay, ON, Canada), S2 from Wal-Mart (Great Value, Thunder Bay, ON, Canada), S3 from Real Canadian Superstore with item no. of 64549, S4 from BDH Chemicals (Toronto, ON, Canada), and S5 from Acros Organics (Toronto, ON, Canada). The relative solubilities of each of the five brands of starch were determined by individually measuring the OD600 values of 1 % *w*/*v* starch solution prepared in water. Thus, the higher the OD600 value, the lower the solubility of the starch. In addition, the reducing sugar contents of the five brands of starch were determined by the DNS method described above, except that the amylase was not added.

Results and Discussion

Previously, we screened a high amylase-producing bacterium, WangLB, from the forest soil in Thunder Bay, ON, Canada. In this work, we first identified the possible genus of the strain through 16S rDNA sequencing, and then optimized the fermentation parameters for amylase production. Next, the enzymology characteristics of the amylase were evaluated and comprehensively compared with values in the literature. Finally, the amylase was applied for the hydrolysis of the five brands of starch from different commercial sources.

16S rDNA Identification and Phylogenetic Analysis of the Strain

The 16S rDNA of bacteria, coding for 16S rRNA, has been widely utilized in the identification of bacterial genera and the construction of phylogenetic trees because of its slow evolutionary rate [27, 28]. In this study, the 16S rDNA of the WangLB strain was amplified through PCR and the desired DNA fragment of ca. 700 bp was detected by DNA electrophoresis (seen in Fig. 1a). The 16S rDNA was sequenced by TCAG, and the result is shown in Fig. 1b. The 665-bp segment of 16S rDNA was then analyzed by the nucleotide BLAST feature of the NCBI database to obtain possible identities based on homology. From the BLAST results, WangLB was identified as a strain of *Bacillus* with 99 % similarity. The sequence of the 16S rDNA was successfully uploaded to the NCBI GenBank database with accession no. KU310971.

The sequence of the strain was also compared with previously published 16S rDNA sequences from the *Bacillus* genus in the GenBank database to construct a phylogenetic tree by applying the neighbor-joining method. As shown in Fig. 2, the *Bacillus* sp. strain WangLB was 98 % similar to *B. amyloliquefaciens*, *B. methylotrophicus*, *B. vallismortis*, *B. subtilis*, and *B. tequilensis.* Based on the above results, the strain in this study was identified as *Bacillus* sp. strain WangLB.

Optimization of Fermentation Parameters for Amylase Production

Incubation time is a crucial factor for the production of amylase. The amylase activity and total protein content in the supernatant of *Bacillus sp.* strain WangLB were measured and are presented in Fig. 3a. The amylase activity reached a maximum of 26,670 ± 1390 U/mL after 48-h incubation, at which time the total protein content in the supernatant also peaked with a value of 28.0 ± 1.7 μg/mL. The activity was much higher (ca. 8–7000-fold) than other amylase-producing *Bacillus* sp. strains reported in the literature, as listed in Table 1. For instance, the highest activity of amylase produced by *B. subtilis* WBS was 6.24 U/mL (ca. 1/4274 of that of WangLB) [14]; the amylase produced by *Bacillus* sp. KR-8104 had a maximum activity of 3.824 U/mL (ca. 1/6974 of that of WangLB) [9]; the maximum activities of amylase produced by *B. licheniformis* and *B. licheniformis* AI20 were 100 and 384 U/mL, respectively (ca. 1/26 and 1/69 of that of WangLB) [1, 17]; the amylase produced by *B. cereus* BRSC-S-A26MB showed the maximum activity of 3197.15 U/mL (ca. 1/8 of that of WangLB) [22].

The effect of temperature on the production of amylase by strain WangLB was studied at different temperatures ranging from 25 to 40 °C, as shown in Fig. 3b. The strain showed stable production of amylase within 30–37 °C, with a maximum at 35 °C. At temperatures higher than 37 °C, the amylase production dramatically declined. In the literature, the optimum temperature for amylase production was in the range of 37 − 50 °C as listed in Table 1. The

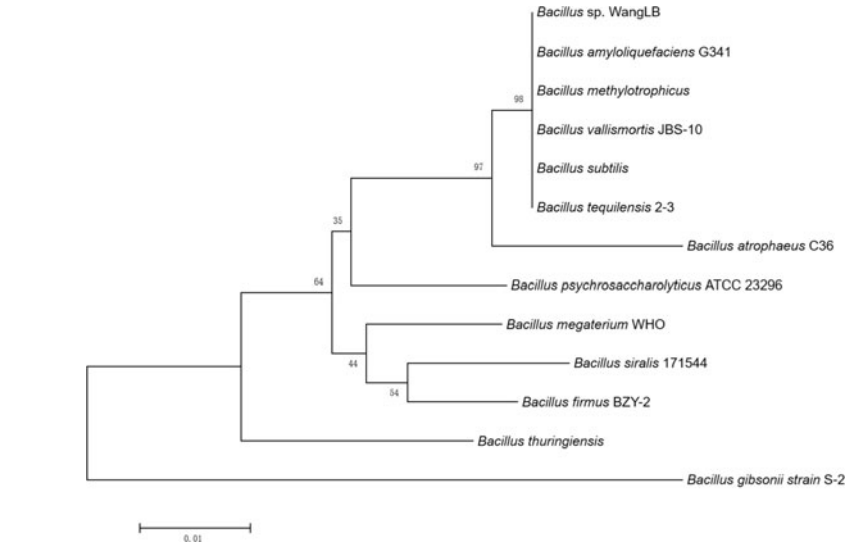


Fig. 2 Phylogenetic tree of the *Bacillus* sp. WangLB

relatively low optimal temperature of strain WangLB could enable producers to save on the costs of energy consumption and avoid the use of special equipment that tolerates high production temperatures.

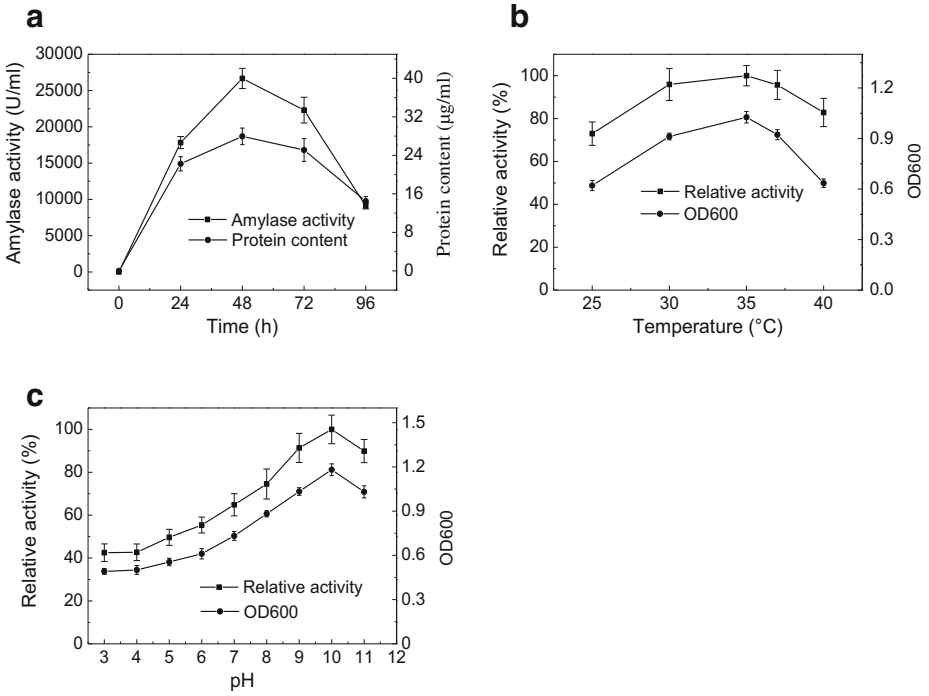


Fig. 3 Effects of incubation time (**a**), temperature (**b**), and pH (**c**) on the amylase production and strain growth of *Bacillus* sp. WangLB

Table 1 Comparison of various biochemical properties of α-amylase from *Bacillus* sp. WangLB with other *Bacillus* species

Bacillus species	Amylase production			Amylase enzymology						References
	Activity (U/ml)	Optimum temperature (°C)	Optimum pH	MW (kD)	Optimum temperature (°C)	Optimum pH	Ca^{2+} dependence	K_m (mg/mL)	V_{max} (U/mg)	
Bacillus sp. WangLB	26670	35	10	55	55-80	9–10	−	0.37	233	This work
Bacillus sp. ANT-6				94.5	80	10.5	+			[13]
Bacillus sp. KR-8104	3.824	37			70	4				[9]
Bacillus sp. KR-8104	3.824	37			60	3.38				[9]
B. subtilis WBS	6.24				40	7	+			[14]
B. subtilis DR8806				76	70	5	+			[15]
B. subtilis AS-S01a				21	55	9		1.9	198.21	[18]
B. licheniformis	100	40	7				+			[16]
B. licheniformis AI20	384			55	60-80	6–7.5	+	0.709	0.454	[17]
B. licheniformis AS08E				55	80	10				[1]
B. licheniformis SKB4		42	6.5		90	6.5		6.2	1.04	[8]
B. megaterium VUMB109				150						[31]
B. methylotrophicus P11-2				44	70	6–9	−	1.2	204	[11]
B. amyloliquefaciens P-001	55	42	9		60	6.5	+			[3]
B. amyloliquifaciens TSWK1-1		50	7	43	70	7	−			[12]
B. cereus BRSC-S-A26MB	3197									[22]

pH also plays a significant role in the production of amylase by strain WangLB. As shown in Fig. 3c, the optimum pH for amylase production was pH 10. Within a pH range of 9–11, strain WangLB could still produce amylase at about 90 % of its maximum rate (Fig. 3c). The optimum pH for amylase production ranged from 6.5 to 9 for different *Bacillus* species, as listed in Table 1.

The impacts of four nitrogen sources, yeast extract, peptone, urea, and ammonium nitrate, were investigated at a concentration of 0.2 % *w/v*. As shown in Fig. 4a, the addition of ammonium nitrate, yeast extract, and peptone to the liquid medium promoted the growth of *Bacillus* sp. WangLB, while the addition of urea slightly inhibited the strain's growth. However, only the addition of peptone remarkably elevated the amylase production to 138 ± 10 %, while the other three nitrogen sources, i.e., ammonium nitrate, urea, and yeast extract, reduced the amylase production to 74, 74, and 59 %, respectively. Given that peptone could elevate amylase production, the effect of peptone concentration on enzyme production was further investigated. It was found that the maximum enzyme production of 149 % was reached when 0.1 % *w/v* peptone was used in the liquid medium (Fig. 4b). Similarly, the amylase production by *B. licheniformis* was found to be elevated by ca. 1-fold after the addition of 0.5 % *w/v* peptone [16]. However, the amylase production by *B. amyloliquefaciens* P-001 was increased by 0.2 % *w/v* urea and inhibited by 0.2 % *w/v* peptone [3].

Because starch is the sole carbon source in the liquid medium available for strain growth, the amount of starch plays a crucial role in the production of amylase. Therefore, the influence

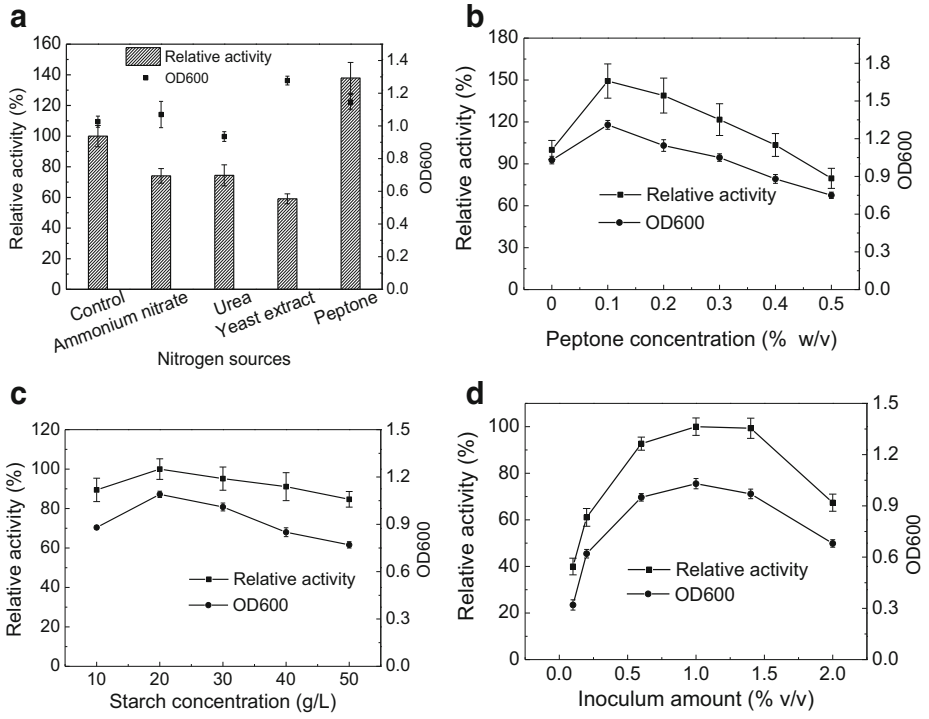


Fig. 4 Effects of four nitrogen sources (**a**), peptone concentration (**b**), starch concentration (**c**), and inoculum amount (**d**) on the amylase production and strain growth of *Bacillus* sp. WangLB

of starch amount on amylase production was evaluated by changing the concentration of starch from 10 to 50 g/L. As shown in Fig. 4c, the relative activity of amylase achieved the maximum value at 20 g/L starch concentration.

The WangLB strain was grown in different inoculum concentrations in the range of 0.1–2 % *v*/*v*. As shown in Fig. 4d, the amylase activity peaked at 1 % *v*/*v* inoculum concentration, and within the 0.6–1.4 % *v*/*v* inoculum concentration range, the amylase activity was higher than 92 %. However, amylase production greatly decreased when the *v*/*v* inoculum concentration was higher than 1.4 % *v*/*v*, which may have resulted from the fact that the overgrowth of the bacteria produced anaerobic conditions during the fermentation process, thereby consuming the majority of substrate available for growth and metabolic processes [29, 30].

Sixteen small organic inducers, arabitol, sorbitol, galactose, xylose, mannose, glucose, mannitol, xylitol, adonitol, cellobiose, alanine, asparagine, galactose, sucrose, cysteine, and lactose, were added to the liquid starch medium at a final concentration of 5 mmol/L to test whether they can stimulate the production of amylase. As can be seen in Fig. 5a, the addition of asparagine, alanine, and lactose dramatically raised amylase production to 124, 122, and 120 %, respectively. Mannose, sucrose, glucose, sorbitol, mannitol, galactose, and cellobiose moderately increased the amylase production from 103–116 %, while galactose, cysteine, adonitol, xylose, arabitol, and xylitol reduced the amylase activity to 66–98 %. The corresponding protein contents were also tested, and the data are also shown in Fig. 5a. The addition of cellobiose and alanine increased the protein contents from 28 to 31.1 and 33.3 μg/mL, respectively, while cysteine reduced the protein content to ca. 15 μg/mL. Other inducers showed a slight influence on the protein content. In particular, alanine increased both the amylase activity and protein content.

The effects of alanine and asparagine concentrations on amylase production were also evaluated (Fig. 5b, c). At 5 mmol/L asparagine, amylase production showed a maximum of 121 %, and at 20 mmol/L alanine, amylase activity reached a maximum of 290 %, indicating that alanine can greatly increase amylase production.

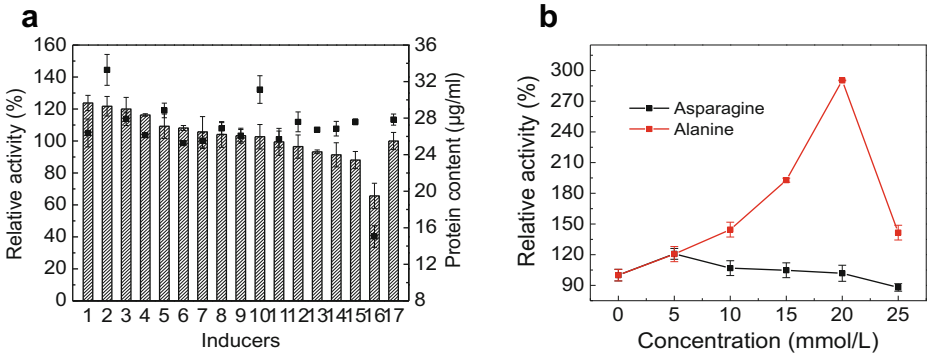


Fig. 5 Effects of 16 small molecular inducers on the amylase production by *Bacillus* sp. WangLB. **a** Relative activity of amylase in the presence and absence of 16 inducers. (1) asparagine, (2) alanine, (3) lactose, (4) mannose, (5) sucrose, (6) glucose, (7) sorbitol, (8) mannitol, (9) galactose, (10) cellobiose, (11) control, (12) galactose, (13) adonitol, (14) xylose, (15) arabitol, (16) xylitol, (17) cysteine. **b** Influences of asparagine and alanine concentrations on the amylase production

Enzymology Characterization of Amylase

The amylase secreted by *Bacillus* sp. strain WangLB was subjected to the TCA/acetone protein precipitation method for separation and purification. The purified amylase was tested by SDS-PAGE to determine its molecular weight. As shown in Fig. 6, after purification, only one band was observed, with a molecular weight of 55 kD, which is shown in panel a. This enzyme also showed a white band in the corresponding place of panel b, indicating that the enzyme had the activity of starch hydrolysis. These results collaboratively demonstrated that the purified enzyme was amylase, with a molecular weight of 55 kD. In the literature, the molecular weight of amylase produced by different *Bacillus* sp. varied from 21 to 150 kD as listed in Table 1, and the molecular weight of the amylase produced by *Bacillus* sp. strain WangLB was the same as those of *B. licheniformis* AI20 and *B. licheniformis* SKB4 [1, 17].

Experiments were conducted to investigate the effect of temperature on amylase activity, and the results are shown in Fig. 7a. It can be seen that the optimum temperature for amylase activity was 55 °C. This enzyme also showed good activity within the temperature range of 50–85 °C, with relative activity higher than 90 %. Even at 90 °C, this amylase showed 80 % activity after 20-min incubation. In the literature, the optimum temperature for amylase activity from various *Bacillus* sp. ranged from 40–90 °C, as listed in Table 1. For instance, the amylase from *B. subtilis* DR8806 showed an optimum temperature of 70 °C, and it retained ca. 90 % activity within the 40–70 °C range [4]; the amylase produced by *B. licheniformis* AI20 achieved higher than 80 % activity within the 50–90 °C range, with the optimum temperature at 70 °C [17]; and the amylase from *B. methylotrophicus* P11-2 showed maximum activity at 70 °C. However, when the temperature increased to 80 °C, the activity dramatically decreased to ca. 35 % [11].

The effect of pH on amylase activity was investigated, and the results are depicted in Fig. 7b. It was observed that the amylase showed higher than 85 % relative activity in the pH range 3–10, with the optimum pH at 9. In the literature, the amylase from *B. subtilis* DR8806 showed an

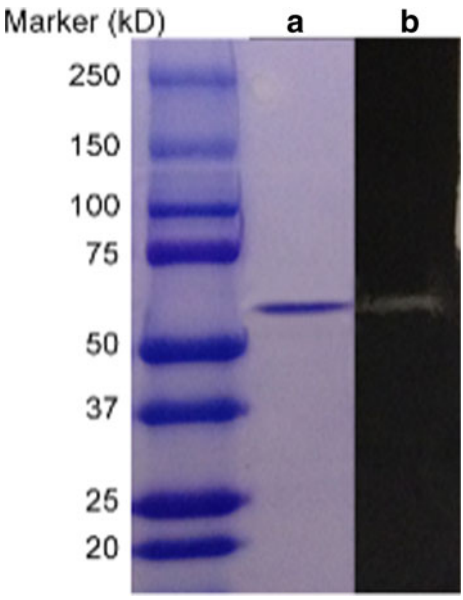


Fig. 6 SDS-PAGE of the supernatant produced by *Bacillus* sp. WangLB. **a** Regular SDS-PAGE; **b** determination of amylase activity. Therefore, the molecular weight of amylase could be read from the image

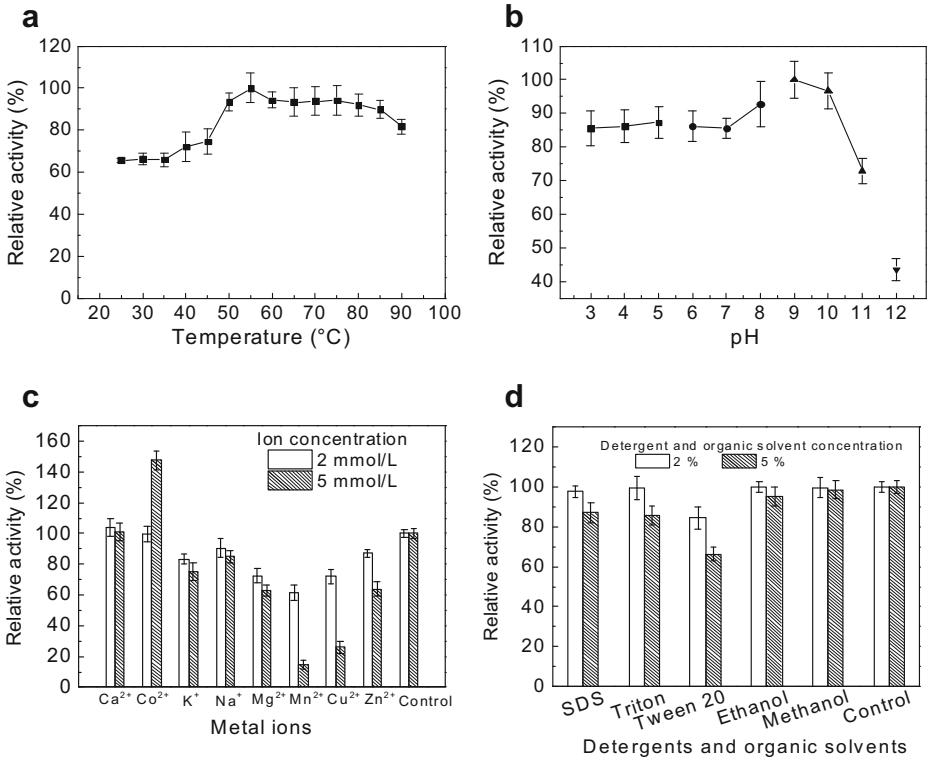


Fig. 7 Effects of temperature (**a**), pH (**b**), metal ions (**c**) and detergents and organic solvents (**d**) on the activity of amylase. For the effect of pH, the buffer solutions used were pH 3–5 in 100 mmol/L citrate buffer, pH 6–8 in 100 mmol/L PBS buffer, pH 9–11 in 100 mmol/L glycine + NaOH buffer, and pH 12 in 100 mmol/L KCl + NaOH buffer

optimum pH of 5, and it retained ca. 80 % activity within the pH range 4–9 [4]; the amylase produced by *B. licheniformis* AI20 maintained higher than 80 % activity within the pH range 5.4–9.6, with the optimum pH of 7.5 [17]; the amylase from *B. methylotrophicus* P11-2 had the highest activity at pH 8 and showed higher than 80 % activity over the pH range 5–9 [11].

These results indicate that the amylase from the WangLB strain has much broader temperature and pH stability than those reported in the literature, and therefore has potential applications in the related industries.

The influences of different metal ions (at concentrations of 2 and 5 mmol/L) on amylase activity were determined, and the results are presented in Fig. 8a. The addition of Ca^{2+} had almost no effect on amylase activity, indicating that the amylase produced by the WangLB strain is Ca^{2+} independent. In the literature, many of the *Bacillus* sp. amylases are reported to be Ca^{2+} dependent; the addition of Ca^{2+} has significant effects on the amylase activity [13–17]. However, one of the major problems of the starch industry related to the calcium requirements of amylase production is the formation of calcium oxalate, a substance that may block process pipes and heat exchangers. As a result, the demand for calcium-independent amylase has received increasing attention in recent years [11, 12], implying that the amylase from the WangLB strain has a great potential for use in the starch industry.

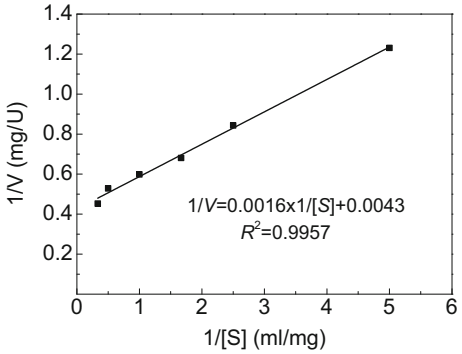


Fig. 8 Kinetic parameters K_m and V_{max} obtained from the Lineweaver–Burk plot

The addition of 2 mmol/L Co^{2+} did not have any influence on the activity, while 5 mmol/L Co^{2+} elevated the activity to 148 %. The other ions, K^+, Na^+, Mg^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}, suppressed the activity to different extents. These results were similar to those reported in the literature. For instance, the *B. amyloliquefaciens* TSWK1-1 amylase was inhibited by Mg^{2+}, Cu^{2+}, and Na^+ [12]; the *Bacillus* sp. ANT-6 amylase activity was suppressed by Zn^{2+} and Na^+ [13]; the *B. megaterium* VUMB109 amylase was inhibited by Mn^{2+} and Zn^{2+} [31]; and the *B. subtilis* DR8806 amylase was inhibited by Mg^{2+}, Mn^{2+}, and Zn^{2+} [15]. However, the amylase activity of *B. megaterium* VUMB109 was found to be elevated by Mg^{2+} and K^+ [31], and the amylase from *B. subtilis* DR8806 could be elevated by K^+ and Na^+, while suppressed by Co^{2+} [15].

Figure 7d shows the impacts of detergents and organic solvents on amylase activity. Triton X-100 and SDS at concentrations of 2 % did not affect the amylase activity, whereas when the concentrations were elevated to 5 %, the activity was reduced to ca. 86 %. Tween 20 at concentrations of 2 and 5 % greatly inhibited the activity, to 84 and 66 %, respectively. In the literature, *B. subtilis* strain AS-S01a amylase activity was found to be unaffected by 1 % SDS, Triton X-100, and Tween 20 [18], whereas 1 and 5 mmol/L SDS and Triton X-100 dramatically suppressed the *B. subtilis* DR8806 amylase activity to 85, 45, 70, and 12 %, respectively [15]. The organic solvents methanol and ethanol (at 2 and 5 %) had almost no suppressive effects on the activity.

The enzyme kinetic parameters K_m and V_{max} in the Michaelis–Menten equation were obtained from the slope and intercept of the Lineweaver–Burk plot in Fig. 8. V_{max} represents the maximum reaction rate the system can reach at saturating substrate concentrations. K_m is the substrate concentration at which the reaction rate is half of V_{max}. Thus, K_m is negatively related to the affinity of the enzyme to the substrate. The K_m and V_{max} values of the amylase from the WangLB strain were calculated to be 0.37 ± 0.02 mg/mL and 233 U/mg, respectively. These two values were comprehensively compared with values in the literature. As listed in Table 1, K_m values in the literature ranged from 0.709 to 6.2 mg/mL, which are ca. 2–16-fold greater than the value found in the present study, indicating that of the amylase from the *Bacillus* sp. strain WangLB has much higher affinity for the starch substrate. The V_{max} values from the literature were between 0.454 and 204 U/mg (Table 1), suggesting that the amylase produced by *Bacillus* sp. strain WangLB shows relatively higher maximum velocity compared with the amylases from other *Bacillus* sp.

Application of Amylase for Hydrolysis of Different Types of Starch

In this section, the purified amylase from the *Bacillus* sp. strain WangLB was applied to the hydrolysis of five different types of starch. As shown in Fig. 9a, b, the amylase activity increased as the OD600 value of the 1 % *w*/*v* starch solution decreased. Because the OD600 value is negatively related to the solubility of the starch, it can be concluded that the hydrolyzability of the substrate by amylase is positively related to the starch solubility.

Previously, we screened the high amylase-producing strain *Aspergillus niger* WLB42, and the amylase of this fungus showed substantial product inhibition; when the reducing sugar content increased from 1.5 to 4.5 % *w*/*w*, the amylase activity was reduced to ca. 15 % of the original activity (the paper is under revision). In the present work, we also tested the product inhibition of the amylase from the WangLB strain. As shown in Fig. 9c, the reducing sugar content of S4 and S5 were 1.5 and 4.3 % *w*/*w*, respectively, while the amylase activities presented in Fig. 9a were almost the same. This indicates that that the amylase secreted by the *Bacillus* sp. strain WangLB has much less of a product inhibition effect. This property is also shown by the relatively low K_m value described above.

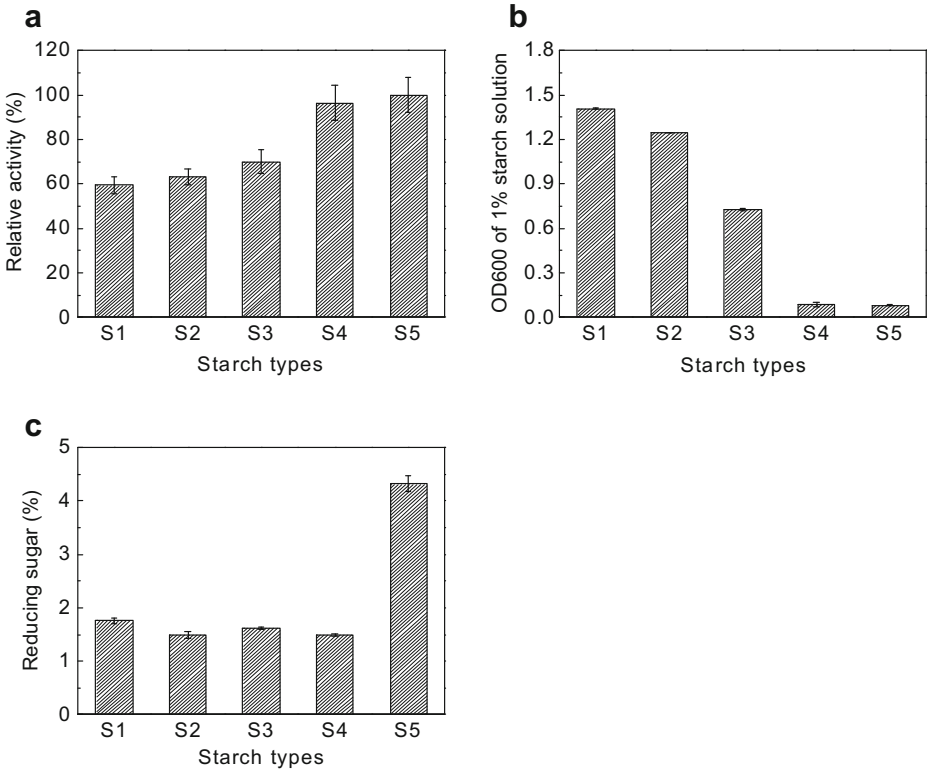


Fig. 9 Effects of five different brands of starch on amylase activity. **a** Relative amylase activity under five brands of starch substrates. **b** OD600 values of five 1 % (*w*/*v*) starch solutions. **c** Reducing sugar (maltose) content (% *w*/*w*) in five brands of starch. S1–S5 were purchased from Real Canadian Superstore with an item number of 64548, Great value from Wal-Mart, Real Canadian Superstore with an item number of 64549, BDH Chemicals, and Acros organics, respectively

Conclusions

In this study, a high amylase-producing bacterial strain, WangLB, was identified as a *Bacillus* sp. through the analysis of 16S rDNA. The optimal fermentation parameters for amylase production were found to be 48-h incubation in liquid starch medium at 35 °C, pH 10, 1 % *v*/*v* inoculum concentration, 20 g/L starch concentration, and 0.1 % *w*/*v* peptone. The maximum amylase activity obtained under the optimized conditions was 26,670 ± 1390 U/mL, about 8–7000-fold greater than the reported activities in literature. The effects of 16 small organic inducers on amylase production were tested and 20 mmol/L alanine was found to greatly enhance amylase production to 290 % of the control one. We also investigated the enzymology characteristics of the amylase. The molecular weight of the purified enzyme was 55 kD, as determined by SDS-PAGE. The optimum temperature and pH for the amylase activity were 55 °C and pH 9, respectively. The enzyme also showed good activity over a broad range of temperatures (50–85 °C) and pH values (3–10). The activity of the amylase was Ca^{2+} independent. The kinetic parameters K_m and V_{max} were determined to be 0.37 ± 0.02 mg/mL and 233 U/mg, respectively. Finally, the amylase was applied to the hydrolysis of five different brands of starch, and it was found that the more soluble the starch, the higher the hydrolyzability of the substrate by amylase. The low fermentation temperature, high amylase activity, and wide ranges of temperature and pH stability suggest that this enzyme has a great potential for industrial applications.

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