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A novel organic solvent-stable alkaline protease from organic solvent–tolerant *Bacillus licheniformis* YP1A

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ABSTRACT

An organic solvent-stable alkaline protease producing bacterium was isolated from the crude oil contaminant soil and identified as *Bacillus licheniformis*. The enzyme retained more than 95% of its initial activity after pre-incubation at 40 °C for 1 h in the presence of 50% (v/v) organic solvents such as DMSO, DMF, and cyclohexane. The protease was active in a broad range of pH from 8.0 to 12.0 with the optimum pH 9.5. The optimum temperature for this protease activity was 60 °C, and the enzyme remained active after incubation at 50–60 °C for 1 h. This organic solvent-stable protease could be used as a biocatalyst for organic solvent-based enzymatic synthesis.

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1. Introduction

Proteases are among the most important hydrolytic enzymes and have numerous applications in industrial sectors like the detergent industry, food industry, and leather industry [1]. Under normal aqueous conditions, proteases catalyze the hydrolysis of peptide bonds, but the reaction is reversed (i.e., peptide bonds are synthesized) in water-restricted media. The advantages associated with the application of enzymes to the synthesis of peptides include the enantio selectivity, dispensation with side-chain protection, and the use of wild, nonhazardous reaction conditions [2]. The major limitation for using proteases in synthetic chemistry is the significantly reduced activity of these enzymes under anhydrous conditions. Several methods have been developed to enhance the stability of enzymes in the presence of organic solvents (reviewed by Khmelnitsky et al. [3]). If proteases were naturally stable in the presence of organic solvents, they would be very useful for synthetic reactions.

Solvents are usually highly toxic to microorganisms, but organic solvent–tolerant microbes have also been reported in recent years [4,5]. It was hypothesized that extracellular enzymes secreted by organic solvent–tolerant microorganisms were stable in the presence of organic solvents. On the basis of this hypothesis, organic solvent–tolerant microorganisms were isolated and screened for production of solvent-stable extracellular enzymes such as lipases and proteases [6,7,8,9]. Most of the reported organic solvent-stable protease producers were *Pseudomonas* strains [9,10,11,12]. The reported solvent-stable protease activity declined significantly at pH values above 10.0.

In this paper, we reported a novel solvent-stable alkaline protease produced by solvent–tolerant *Bacillus licheniformis* YP1A. This extracellular protease YP1A can tolerate extra-alkaline conditions (pH 12.0). The isolation, identification, and characterization of this bacterium and the organic solvent stability of the protease were studied.

2. Experimental

2.1. Materials

Trypsin (EC 3.4.21.4) and casein acid hydrolysate were purchased from Sigma (St. Louis, MO, USA). Media components (peptone, yeast extract, starch, degossypolled cottonseed meal) were purchased from ShengXing Biotechnology Corporation (Nanjing, China). All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Isolation of organic solvent–tolerant bacteria strain and protease producers

Soil samples were collected from the areas contaminated by crude oil and chemicals. A small amount of soil was suspended in sterilized distilled water and 100 μl of the resulting suspension was added to a modified LB liquid medium, which contained (g/l): tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0; $MgSO_4 \cdot 7H_2O$, 0.5. Cyclohexane and toluene were added to the medium at concentrations of 30% and 10% (v/v) separately, and the cultivation vessel was plugged with a chloroprene–rubber stopper to prevent evaporation

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of the organic solvent. Cultivations were conducted in 250 ml Erlenmeyer flasks containing 30 ml medium at 30 °C. Incubation was carried out with agitation at 200 rpm for 2 days. Cultures were then acclimated by repeated transfers using the same medium. Samples of the repeated batch cultures were diluted and spread on LB medium, and colonies grown on the plates were further purified by repeated streaking.

Protease producers among the purified colonies were detected by plating on alkaline casein agar containing (g/l): peptone, 5.0; beef extract, 5.0; NaCl, 5.0; casein, 10.0; and bacteriological agar, 12.0. The pH of the medium was adjusted to 9.5 with 1 mol/l NaOH. Microbes showing clear zones were isolated as protease producers; the strain showing the maximum zone diameter was selected as a potent producer of the proteolytic enzyme and maintained on LB agar slants at 4 °C for further study. The strain was designated as YP1A. The identification of strain YP1A was based on BIOLOG Microbial Identification System (BIOLOG USA) and alignment of the sequence of 16S-rDNA (EF105377, GenBank) using the BLAST algorithm on the NCBI BLAST server.

2.2.2. Effect of organic solvents on the growth of bacteria

The strain YP1A was cultured in the modified LB liquid medium supplemented with different concentrations of organic solvent, and all cultivation flasks were plugged with chloroprene rubber stoppers. During the cultivation, 1.0 ml cultures were diluted and the optical density of the culture was determined using spectrophotometer at 660 nm wave length. One absorbance unit at 660 nm corresponded to 0.39 mg dried cell mass per milliliter. The absorbance of cell density less than 0.1 were considered as no cell growth. Values were the average of three independent measurements.

2.2.3. Effect of initial medium pH on the growth of bacteria

The strain YP1A was cultured in the modified LB liquid medium with the initial pH ranging from 7.0 to 10.5, adjusted with NaOH. After 24 h of cultivation, the growth of YP1A was determined as described above.

2.2.4. Culture conditions for protease production

Stock culture of YP1A was inoculated into the modified LB medium and then incubated at 30 °C and 180 rpm overnight. Portion of the culture (0.3 ml) was used to inoculate 30 ml of protease production medium in a 250 ml Erlenmeyer flask. The protease production media consisted of (g/l): starch, 5.0; yeast extract, 1.0; NaCl, 5.0; degossypolled cottonseed meal, 10.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5. The pH was adjusted to 7.0 with 1 mol/l NaOH. The incubation was done at 30 °C in an orbital shaker at 180 rpm. After 48 h of growth, the cells were harvested by centrifugation at 10,000 rpm and 4 °C for 10 min, and the supernatant was used as crude protease.

2.2.5. Assay of extracellular protease activity

Caseinolytic activity was measured by a slight modification of the Keay and Wildi method [13]. 0.5 ml of diluted crude enzyme was pre-incubated at 40 °C for 5 min. The reaction was started by addition of 1.0 ml casein 1.0% (w/v), pH 9.0. The reaction mixture was then incubated at 40 °C for 10 min and terminated by the addition of 1.5 ml 0.4 mol/l trichloroacetic acid (TCA). A vortex mixer was used to ensure complete mixing at various stages of these assay procedures. This mixture was further incubated at 40 °C for 20 min, followed by centrifugation at 10,000 rpm for 10 min. The supernatant was harvested, and 5.0 ml of 0.55 mol/l Na_2CO_3 and 1.0 ml folin ciocalteau reagent: water (1:3 v/v) were added to 1.0 ml of the supernatant to yield a blue color. The colored mixture was incubated in an incubator at 40 °C for 20 min before the absorbance was read at 660 nm. A "blank" was prepared by adding TCA at zero time. One unit (U) of protease activity was equivalent to 1 μg of tyrosine liberated per minute under the conditions described above. The amount of tyrosine was calculated from the tyrosine standard curve. Protein concentration of the supernatant was determined by Bradford method.

2.2.6. Organic solvent stability of protease

Two milliliters of diluted crude protease and trypsin (Sigma Chemical Co.) were incubated in the absence or presence of 2.0 ml of organic solvent at 40 °C with constant shaking at 180 rpm for 1 h. The residual activities were determined by the procedure described above.

2.2.7. Determination of optimum temperature and thermal stability of protease

The protease activity was measured at various temperatures with 40 °C as a control, casein used as the substrate. The thermal stability was studied by incubating the enzyme at 50, 60, and 70 °C. Appropriate aliquots were withdrawn at different time intervals and the residual activities were determined at 40 °C.

3. Results and discussion

3.1. Isolation of organic solvent–tolerant microorganisms

Solvent–tolerant bacteria were screened from soil samples collected from the crude-oil-contaminated area. Cyclohexane and toluene were added to the medium at the beginning in order to enrich solvent–tolerant microbes. As a result, 47 organic solvent–tolerant strains were isolated. Eight protease producers were selected from these strains on the basis of the size of the clear zone formed on casein agar plates. Strain YP1A, which showed the maximum zones diameter, was selected for further study. Table 1 shows the morphological and biochemical characteristics of the strain YP1A. It is strictly aerobic, motile, gram positive, spore-forming, and rod-shaped. Strain YP1A was identified as *Bacillus licheniformis* combining the analysis of BIOLOG Microbial Identification System (SIM=0.62, 16–24 h) with the alignment of its 16S-rDNA sequence by BLAST algorithm (homology 99%). Most of the reported organic solvent-tolerant strains, which can produce solvent-stable protease, were from *Pseudomonas* genus [9,10,11,12] and only one was *Bacillus cereus* [14]. *Bacillus licheniformis* YP1A could be considered as a novel

Table 1
Partial morphological and biochemical characteristics of strain YP1A

Characteristic	Results
Shape	Rods
Gram stain	Positive
Cell dimensions (μm)	0.06–0.08 by 1.5–3.7
Spore	+
Motility	+
Aerobiosis	+
Catalase activity	+
Hydrolysis of gelatin	+
Hydrolysis of starch	+
Sucrose	+
Maltose	+
D-Sorbitol	+
α-D-Lactose	–
D-Galactose	–
D-Xylose	+
D-Arabinose	–
Mannitol	+
Propionate	+
Voges–Proskauer test	+

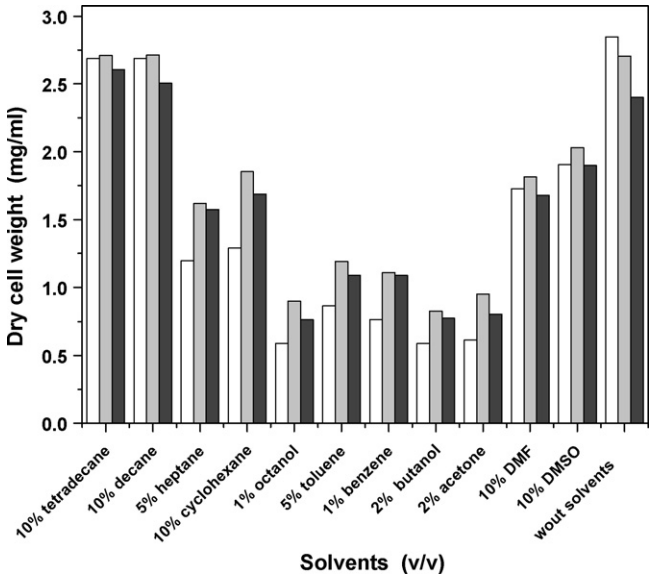

Fig. 1. Effect of organic solvent on the growth of strain YP1A. Strain YP1A was cultivated in a 250-ml Erlenmeyer flask containing 30 ml of the modified LB medium in the presence or absence of organic solvent at 30 °C. The initial concentration of solvents was 10% (v/v). The concentrations of solvents less than 10% were the maximum concentration under that strain YP1A could grow. The biomass of strain YP1A were detected at 12 h (□), 24 h (▨) and 36 h (■) separately.

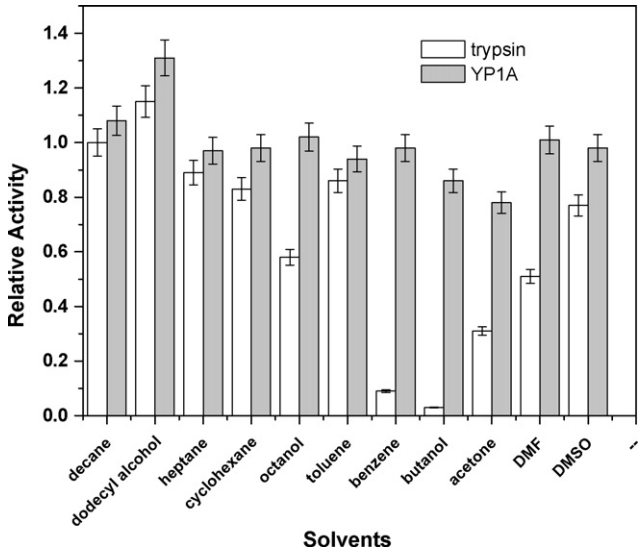

Fig. 2. Effect of organic solvents on the protease stability. An aliquot of 2.0 ml enzyme solution (cell-free supernatant or trypsin solution) was incubated in 2.0 ml organic solvent at 40 °C for 1 h with shaking and the remaining activity was measured. Trypsin was dissolved in 0.05 mol/l Tris–HCl buffer (pH8.0) at the concentration of 0.01 mg/ml with activity of 25,400 U/mg. The specific activity of cell-free supernatant (0.077 mg/ml) was 3166 U/mg. The protease activity of the non-solvent containing control was taken as 1.0.

strain producing solvent-stable protease, which may increase our knowledge on the biodiversity of solvent-stable protease producers.

3.2. Effect of organic solvents and pH on the growth of bacteria

Fig. 1 showed the comparison between the growth of the strain YP1A in the liquid medium containing various organic solvents and in medium without organic solvent. The parameter log *P* is the logarithm of the partition coefficient, *P*, of the solvent between octanol and water. This parameter is used as a quantitative measure of the solvent polarity. The observation showed that organic solvents with log *P* above 5.5, such as tetradecane (log *P* = 7.6) and decane (log *P* = 5.6) had little effect on the growth of strain YP1A. Strong inhibition on growth was observed in those solvents whose log *P* values were ranging from 0 to 3.0, such as octanol (log *P* = 2.92), toluene (log *P* = 2.5), benzene (log *P* = 2.0) and butanol (log *P* = 0.8), at a concentration between 1% and 5% [v/v]. Physiological investigation of microbes has revealed a correlation between solvent toxicity and its log *P* value. The greater the polarity of a solvent, the lower its log *P* value and the greater its toxicity. Strain YP1A grew well in the presence of the water-miscible solvents dimethylformamide (DMF, log *P* = −1.0) and dimethylsulphoxide (DMSO, log *P* = −1.35) at a concentration of 10% [v/v]. Several studies have been conducted on the solvent tolerance of bacteria [5]. However, the tolerance of bacteria to hydrophilic solvents, such as DMF or DMSO, has been rarely reported. Since the water-miscible solvents DMF and DMSO are usually used as media in organic reactions, strain YP1A might have potential applications in biotransformation reactions.

The growth of strain YP1A in liquid medium at different pH (pH 7.0–10.5) was studied. The optimal pH range for YP1A growth was 7.5–9.5. Strain YP1A could tolerate high alkaline conditions, and more than 80% of the biomass remained when pH was ranging from 10.0 to 10.5, which suggested strain YP1A is highly tolerant to both organic solvents and alkaline conditions.

3.3. Effect of organic solvents on protease stability

The effects of various organic solvents on the stability of the crude protease were tested. Fig. 2 showed the remaining activity of the protease YP1A and trypsin in the presence of organic solvent. Trypsin was greatly inactivated in the presence of butanol (log *P* = 0.8), acetone (log *P* = −0.23), and benzene (log *P* = 2.0), while protease secreted by strain YP1A retained more than 80% of its initial activity in these organic solvents. The protease retained more than 95% of its initial activity even after treated with hydrophilic solvents DMSO and DMF. This data suggested that protease YP1A can tolerate a variety of organic solvents. Moreover, when solvents such as decane and dodecyl alcohol with log *P* values above 4.0 were added to the crude protease, the enzyme activity levels were 1.1 and 1.2 times the control, respectively.

The way that solvents affect enzymatic activity is to interact directly with the essential water surrounding the enzyme molecule. Highly polar solvents are capable of thirstily absorbing the essential water from the enzyme, entailing the loss of catalytic properties [15]. Generally, the enzymatic activity is low in polar solvents with log *P* < 2, is moderate in solvents with log *P* = 2–4, and is high in polar solvents with log *P* > 4 [16]. Proteases from *Bacillus* spp., like subtilisin, are not naturally tolerant to hydrophilic solvents. Thus, genetic modification to enhance the resistance of subtilisin E to polar organic media has been reported [17]. When protease is used in kinetic- and equilibrium-controlled synthesis, the use of homogeneous aqueous-organic mixtures with highly solvating organic media such as DMF, DMSO, acetonitrile, or MeOH is a preferred approach [18]. The solvent-stable protease produced by *Pseudomonas aeruginosa* PST-01 has been reported to catalyze peptide synthesis in monophonic aqueous-organic solvent systems; the equilibrium yield of Cbz–Arg–Leu–NH_2 increased with an increase in the concentration of DMF or DMSO [19]. The activity of protease YP1A remained high after it was treated with various 50% (v/v) solvents; especially its high tolerance for the water-miscible solvents DMF and DMSO. This characteristic makes it an ideal catalyst for kinetic- and equilibrium-controlled synthesis.

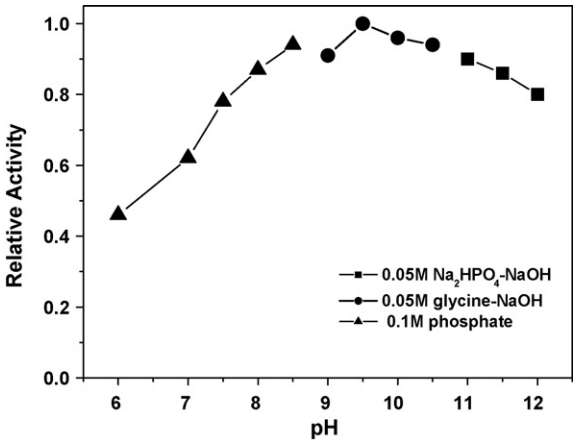

Fig. 3. Effect of pH on protease activity. The remained activity was measured by incubating the enzyme with the substrate at different pH values. The buffers used were 0.1 mol/l phosphate (▲) (pH 6.0–8.5), 0.05 mlo/l glycine-NaOH (●) (pH 9.0–10.5), and 0.05 mol/l Na_2HPO_4–NaOH (■) (pH 11.0–12.0). The activity at pH 9.5 (3554 U/mg) was taken as 1.0.

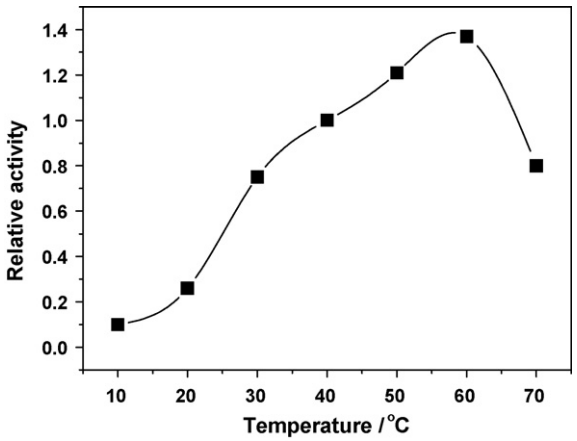

Fig. 4. Effect of temperature on protease activity. The diluted cell-free supernatant (0.045 mg/ml) was incubated with the substrate at different temperatures. The activity (3110 U/mg) at 40 °C was taken as 1.0.

3.4. The effect of pH on protease activity

The relative enzyme activities, measured at various pH levels, were shown in Fig. 3. The enzyme was active in a pH range of 7.5–12 and showed an optimum pH of 9.5 at 40 °C. Protease YP1A remained 80% of the maximal activity at pH 12.0. Organic solvent-stable proteases from *Bacillus cereus* BG1, *Pseudomonas aeruginosa* PseA, and *Pseudomonas aeruginosa* PST-01 were reported to have optimal pH levels between 8.0 and 9.0 and become completely inactive at pH values over 10.0 [10,11,20]. The tolerance of protease YP1A to both solvents and extra-alkaline conditions suggested that it was a novel protease.

3.5. Effects of temperature on enzyme activity and thermal stability of protease

The relative protease activities, measured at various temperatures, were shown in Fig. 4. The optimum temperature of protease YP1A was 60 °C. protease YP1A also showed considerable activity over the range of 40–60 °C. Protease YP1A retained 94% and 92% of its maximal activity after incubation at 50 and 60 °C for 60 min, respectively. However, 69% of activity was lost within 15 min of incubation at 70 °C.

4. Conclusion

This paper described the isolation of an organic solvent and alkaline-tolerant bacterium and the characterization of its secreted alkaline solvent-stable protease. The isolated protease producer strain YP1A was identified as *Bacillus licheniformis*. The protease was active in a broad range of pH 8.0–12.0. In addition, the protease YP1A retained more than 95% of its initial activity in the presence of 50% (v/v) organic solvents. These properties make this organic solvent-stable protease a promising biocatalyst for enzymatic synthesis in the presence of organic solvents.

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