Production Purification and Characterization of Alkaline β-Glucosidase Isolated from Agrobacterium sp. PJD – 1 – 1

YAO Jian1 Chen Qing-long1 Zhong Guo-xiang1 Gui Lun1 Bi Wen-hui2


Abstract In order to screen novel β-glucosidase producing strains from environment one targeted novel strain PJD–1–1 producing β-glucosidase were isolated from putrefied sugarcane leaves with screening and spreading plate. 16S rDNA analysis revealed it was a novel Agrobacterium sp. When the strain was incubated at initial pH 7.0 20 °C with lactose as carbon and NaNO3 as nitrogen sources the maximum enzyme activity was 3.92 U/mg. β-glucosidase from this strain was purified using (NH4)2 SO4 precipitation followed by dextran gel-filtration chromatography and ion-exchange chromatography. A purifying fold of 4.85 with gaining rate of 8.0% was obtained. SDA-PAGE analysis of the purified enzyme showed that it was a clear and pure band with molecular mass of ca. 40 kDa. The most optimum activity of the enzyme was at 50 °C and pH at 8.0. The enzyme could maintain stability under the conditions below 50 °C. Hg2+ and Ag+ heavily inhibited the enzyme activity suggesting that the active catalytic sites of the enzymes might possess thiol radical. Ba2+, Ca2+, Pb2+, Co2+, Zn2+, Mn2+, Na+, K+, EDTA and urea had no obvious effects on the enzyme activity. It is concluded that the novel strain Agrobacterium sp. PJD-1-1 producing β-glucosidase was successfully screened from putrefied sugar cane leaves. The produced enzyme had thermal stability, alkaline feature and metal ions tolerance made it useful in the food and broad potential applications in other fields.

Keywords alkalinity; β-glucosidase; ion-exchange chromatography; metal ions; Agrobacterium sp. PJD-1-1
Cellulose, a major component of plant cell walls, is the most abundant polysaccharide in nature and a virtually inexhaustible source of renewable bioenergy. The hydrolysis of cellulose depends on at least three enzymes including endoglucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). The endoglucanase randomly attack cellulose in amorphous zones and release oligomers while the cellobiohydrolase liberate cellobiose from reducing and non-reducing ends, and the β-glucosidase hydrolyzes the dcellobiose and cellobiooligosaccharides to glucose, which is essential for the final step of cellulose saccharification because it reduces the inhibition of endoglucanase and cellobiohydrolase and release oligomers while the cellobiohydrolase liberates cellobiose from reducing and non-reducing ends.

Furthermore, β-glucosidase plays important roles in many biological processes such as biogenesis of various functional molecules from glycoside precursors and cyanide-based biological defense mechanisms in plants. In addition, β-glucosidase has potential applications in the pharmaceutical cosmetic and detergent industries. To date, many β-glucosidases from different microorganisms have been isolated and investigated for their physicochemical properties such as β-glucosidase from Penicillium italicum, Aspergillus fumigatus, and Saccharomyces cerevisiae, Chaetomella raphigera, Myceliophthora thermophila, Colletotrichum graminicola, Fusarium proliferatum, Thermoanaerobacterium thermosaccharolyticum, Oenococcus oeni, ATCC BAA-4163, and Bifidobacterium breve UCC2003. However, the properties of β-glucosidases from these microorganisms did not always meet the requirements for a given application. In this study, an Agrobacterium sp. strain that produces β-glucosidase with novel properties was isolated from putrefied sugarcane leaves and its production of β-glucosidase was optimized. The enzyme was purified and its biochemical properties including molecular mass, optimum pH and temperature, and metal ions were investigated.

1 Materials and methods

1.1 Materials

1.1.1 Sample sources Furthered sugarcane leaves were used for isolating β-glucosidase producing strains.

1.1.2 Medium (g/L) ① Screening medium: lactose 5.0, NaNO₃ 2.0, K₂PO₄ 1.0; KCl 0.5, MgSO₄ 0.5, FeSO₄ 0.01; esculin hydrate 1.0; ammonium iron (III) citrate 2.5; NaNO₃ 0.5, NaNO₂ 2.0, K₂PO₄ 1.0; KCl 0.5, MgSO₄ 0.5; FeSO₄ 0.01; 15°C sterilization for 20 min; ② Fermentation medium: lactose 5.0, NaNO₂ 2.0, K₂PO₄ 1.0, KCl 0.5, MgSO₄ 0.5; FeSO₄ 0.01; 15°C sterilization for 20 min.

1.1.3 Reagent 4-Nitrophenyl-β-D-Galactopyranoside (pNPG) was purchased from Sigma-Aldrich.

1.2 Methods

1.2.1 Isolation of β-glucosidase producing Agrobacterium sp. PJD-4 strain Bacteria strains from putrefied sugarcane leaves were plated on screening medium plates and incubated at 30°C for 4 days. Isolates were maintained purely at 30°C after 4 times of subsequent transfers on the same plates. For the β-glucosidase production isolate the isolate was cultivated in the fermentation medium. Then the culture was incubated at 20°C (pH 7.0) for 5 days and the culture supernatant was used for enzyme activity.

1.2.2 DNA extraction and 16S rDNA sequence analysis Genomic DNA extraction was carried out according to a method described previously. Amplification of the 16S rDNA was performed by using 27f (5'-AGAGTTTGATCCTGCTAG-3') and 1492r (5'-TACCTGTGACCTTGT-3').
ucts were ligated to pGEM-T easy vector and transformed into E. coli DH5α. The insert DNA sequencing was performed using a BigDye sequencing kit and ABI 377 DNA sequencer (Applied Biosystems Inc.). The sequence was analyzed using the gapped BLAST (http://www.ncbi.nlm.nih.gov) search algorithms and aligned to their nearest neighbors. Phylogenetic tree was constructed by neighbor-joining method using Molecular Evolutionary Genetics Analysis 5.0 software (MAGE ver. 5.0).

1.2.3 Enzyme assay β-Glucosidase activity was determined according to the method described previously with minor modification. The reaction mixture containing 100 μL of purified β-glucosidase and 100 μL of 10 mmol/L pNPG in 50 mmol/L phosphate buffer (pH 8.0) was incubated at 50 °C for 15 min. Then the reaction was terminated by adding 200 μL of 200 mmol/L Na₂CO₃. The absorbance of p-nitrophenol released was measured at 410 nm using a spectrophotometer. One unit of β-glucosidase activity was defined as the amount of enzyme required to release 1 μmol/L of p-nitrophenol in 1 min under specific conditions.

1.2.4 Optimization of β-glucosidase production

β-Glucosidase production by Agrobacterium sp. PJD-1-4 under different incubation time (0-8 days) in shake (120 r/min) culture conditions was investigated. Various carbon sources (0.5% w/w) such as pectin, lactose, cellulose, avicel, xylose, carboxymethylated cellulose (CMC) as well as various nitrogen sources (0.2% w/w) such as NH₄Cl, urea, NaNO₃, beef extract, yeast extract, peptone (NH₄)_2SO₄ and different concentrations of lactose ranging from 0.25% to 2.5% (w/w) were tested for β-glucosidase production. Based on the conditions described above, a set of experiments was conducted at different temperatures (15-35 °C) and pHs (pH 5.0-9.0) to obtain maximum β-glucosidase production.

1.2.5 Purification of β-glucosidase

1. Ammonium sulfate precipitation and ultrafiltration: Culture filtrate containing β-glucosidase was saturated with gradient ammonium sulfate at 4 °C for 12 h. The precipitate was collected by centrifugation at 8,000 r/min for 20 min and suspended in 50 mmol/L phosphate buffer (pH 8.0).

2. Gel-filtration chromatography: Five milliliter of the concentrated sample was loaded on to Sephadex G-100 column with a bed size of 1.5 cm × 60 cm pre-equilibrated with 50 mmol/L phosphate buffer (pH 8.0). The column was eluted with the same buffer at a flow rate of 1 mL/min. Fractions of one milliliter size were collected and estimated at 280 nm for protein content and at 410 nm for β-glucosidase activity. The fractions containing β-glucosidase activity were pooled.

3. Ion exchange chromatography: Further purification was carried out on DEAE Sepharose Fast Flow column (GE Healthcare) pre-equilibrated with 10 mmol/L phosphate buffer (pH 8.0). The column was eluted using step gradients of 0.2, 0.4, 0.6, 0.8, 1.0 mol/L NaCl in 10 mmol/L phosphate buffer (pH 8.0) at a flow rate of 0.75 mL/min. All fractions were analyzed for β-glucosidase activity and fractions corresponding to the activity peaks were pooled and concentrated.

1.2.6 Enzyme characterization

The molecular mass and effect of temperature, pH and additives on β-glucosidase activity were studied and the activity was assayed as described above unless otherwise stated. All the experiments were done in triplicate for each tube at each sample condition. 1. Protein determination and SDS-PAGE analysis: Protein concentrations were determined according to Bradford’s method with bovine serum albumin (BSA) used as standard. The molecular mass of purified β-glucosidase was analyzed by SDS-PAGE according to the method of Laemmli with a 12% separating gel and 5% concentrating gel used in this study. After electrophoresis, the SDS-PAGE gel was stained with Coomassie brilliant blue G-250 for molecular mass analysis. The molecular weights of the protein were determined by the standard protein mixture of 205, 97, 66, 45 and 25 kDa. 2. Optimum temperature and pH for the enzyme activity: the optimum temperature of β-glucosidase was determined at a temperature...
The optimum pH was determined by measured β-glucosidase activity in a pH values ranging from 5.5 to 9.5 at 50℃. The following buffers were used: 50 mmol/L sodium acetate buffer (pH 4.0-6.0); 50 mmol/L phosphate buffer (pH 5.5-8.0); 50 mmol/L Tris-HCl buffer (pH 7.5-9.0); 50 mmol/L glycine-NaOH buffer (pH 8.5-10.0). Then the residual activity was measured as described above.

Effect of temperature on the stability of β-glucosidase:
The thermostability of β-glucosidase was determined by incubating the purified enzyme in phosphate buffer (50 mmol/L, pH 8.0) at different temperatures (30, 35, 40, 45, 50, 55, 60, 65, and 70℃) for 1 h. Then the residual activity was measured as described above.

Effects of various chemical additives on β-glucosidase activity:
The effects of various metal ions (Na⁺, K⁺, Mg²⁺, Ca²⁺, Mn²⁺, Ba²⁺, Fe²⁺, Co²⁺, Ni²⁺, Pb²⁺, Cu²⁺, and Fe³⁺) and ethylene diamine tetraacetic acid (EDTA), urea, and sodium dodecyl sulfate (SDS) on β-glucosidase activity were investigated. The enzyme was incubated with 1 mmol/L of the respective chemicals at room temperature for 0.5 h. Then the residual activity was assayed as described above.

## Results

### 2.1 Isolation and identification of β-glucosidase producing bacteria

A β-glucosidase producing bacteria strain named PJD-I-4 was isolated from putrefied sugarcane leaves. Comparison of almost complete 16S rRNA sequence of PJD-I-4 against the GenBank database indicates that PJD-I-4 was included in the genus *Agrobacterium*. Strain PJD-I-4 shares highest percentage nucleotide sequence similarities with *Agrobacterium* sp. 2382 (identity of 100%).

A phylogenetic tree illustrating the relationship of strain PJD-I-4 to related bacterial species was shown in Fig. 1 and the selected strain was identified as *Agrobacterium* sp. PJD-I-4 on the basis of 16S rRNA sequence.

![Fig. 1 Phylogenetic tree derived from the analysis of 16S rDNA sequence using neighbor-joining method showing the relationship among Agrobacterium sp. PJD-I-4 and representatives of some related taxa](http://www.cnki.net)
2.2 Optimization of parameters for the enzyme production

Various carbon sources such as pectin, lactose, cellulose, avicel, xylose, CMC and nitrogen sources such as NH₄Cl, urea, NaNO₃, beef extract, yeast extract, peptone (NH₄)₂SO₄ were tested for β-glucosidase production by Agrobacterium sp. PJD-1-1. All substances induced β-glucosidase production at different levels. β-Glucosidase activity reached the highest level with lactose as carbon source followed by avicel, cellulose, pectin, CMC and xylose (Fig. 2A). Lactose at the concentration of 0.5% showed the maximum enzyme production and further increase caused a decrease in enzyme production (Fig. 2B). With lactose as carbon source, NaNO₃ as nitrogen source showed maximum enzyme production, while other inorganic nitrogen sources result in least production of the enzyme. Among the organic nitrogen sources, peptone was found to be the best for enzyme production while urea resulted in least enzyme production (Fig. 2C). Besides carbon and nitrogen sources, initial pH, incubation temperature and incubation time also influenced enzyme production. Initial pH of 7.0 was found to be the optimum pH for enzyme production an increase and decrease in pH would cause the decrease in the enzyme production (Fig. 2D). Temperature of 20 ℃ was found to be the optimum temperature for enzyme production and higher temperature was found to reduce the enzyme production (Fig. 2E). The Agrobacterium sp. PJD-1-1 was found to have maximum enzyme production when it was incubated at pH 7.0 and 20 ℃ for 5 days longer incubation time would decrease enzyme activity (Fig. 2F).

Fig. 2 Effect of carbon sources (0.5%) (A), lactose concentration (B), nitrogen sources (C), pH (D), temperature (E) and incubation time (F) on enzyme production

2.3 Partial purification of β-glucosidase

The strain Agrobacterium sp. PJD-1-1 secreted β-glucosidase into the culture filtrate when grown in liquid medium and the extracellular extract was harvested after 5 days incubation. The partial purification process was summarized in Table 1. In first step of the purification with ammonium sulfate fractionation about 75.5% of total β-glucosidase activities were recovered in the fraction of 20%-60% ammonium sulfate. This step removed the greater part of the contaminants and decreased total protein amount from 12.09 mg to 5.072 mg. The precipi-
tate with ϒ-glucosidase was dissolved in 10 mmol/L phosphate buffer (pH 8.0) and further purified with gel-filtration chromatography and ion exchange chromatography, retaining 10.6% of the activity from the previous step. SDS-PAGE analysis of the purified enzyme showed the presence of a single band with an apparent molecular mass of ca. 40 kDa when stained with Coomassie brilliant blue (Fig. 3), so further purification steps were not required.

### Table 1  Purification of ϒ-glucosidase from Agrobacterium sp. PJD-1-1

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein /mg</th>
<th>Total activity /U</th>
<th>Specific activity /([U mg(^{-1})])</th>
<th>Yield /%</th>
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<tr>
<td>Cell extract</td>
<td>12.090</td>
<td>47.40</td>
<td>3.92</td>
<td>100.0</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4)</td>
<td>5.072</td>
<td>35.80</td>
<td>7.06</td>
<td>75.5</td>
</tr>
<tr>
<td>Sephadex G100</td>
<td>0.489</td>
<td>5.64</td>
<td>11.53</td>
<td>11.9</td>
</tr>
<tr>
<td>DEAE Sepharose</td>
<td>0.210</td>
<td>3.80</td>
<td>19.00</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Fig. 3  SDS-PAGE analysis of the purified enzyme

2.4  Effect of pH and temperature on ϒ-glucosidase activity

The effect of pH and temperature on ϒ-glucosidase activity was measured at a pH range of 5.5 to 9.5 and a temperature range of 25 to 60 °C. The enzyme showed its highest activity at pH of 8.0 (Fig. 4A) and temperature of 50 °C (Fig. 4B). Thermal stability analysis revealed that the enzyme was stable below 50 °C, no obvious loss of activity was found when incubated below 50 °C, and it kept more than 70% of its activity after incubation at 60 °C for 30 min (Fig. 4C).

Fig. 4  Effect of pH (A), temperature (B) on enzyme activity and temperature on the stability (C) of enzyme

2.5  Effects of different chemicals on ϒ-glucosidase activity

Effects of different chemicals on activity of ϒ-glucosidase was tested by pre-incubating the enzyme with 1 mmol/L of each chemical respectively at room temperature for 30 min. Ba\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), and urea only slightly affected the enzyme activity compared with that in the absence of any added chemicals (Table 2). The relative activity of the enzyme was decreased to 64.1% and 21.2% by the presence of Ni\(^{2+}\), Cu\(^{2+}\) respectively. Hg\(^{2+}\) and Ag\(^{+}\) strongly affected the enzyme activity to 11.2%
and 9.4% respectively.

Table 2 Effect of chemicals on β-glucosidase activity from Agrobacterium sp. PJD-1-1

<table>
<thead>
<tr>
<th>Chemicals (1 mmol • L⁻¹)</th>
<th>Relative activity /%</th>
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<tr>
<td>Control</td>
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<tr>
<td>Ba²⁺</td>
<td>96.8</td>
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<tr>
<td>Ca²⁺</td>
<td>92.3</td>
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<tr>
<td>Mg²⁺</td>
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<tr>
<td>Ni²⁺</td>
<td>64.1</td>
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<tr>
<td>Co²⁺</td>
<td>85.2</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>83.1</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>21.2</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>11.2</td>
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<tr>
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<td>Urea</td>
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<tr>
<td>EDTA</td>
<td>89.9</td>
</tr>
<tr>
<td>SDS</td>
<td>84.1</td>
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3 Discussion

β-Glucosidase plays a major role in the conversion of cellululosic biomass to ethanol being the rate-limiting enzyme that determines the action of all cellulose components. A large number of β-glucosidases from microorganism have being produced purified and studied but most of these enzymes show maximal activity in the acidic pH range (pH 4.0–6.5) \[\text{17}\] . In this study a new strain Agrobacterium sp. PJD-1-1 producing β-glucosidase with its optimum activity at pH 8.0 was isolated. As carbon and nitrogen are energy sources which are essential for the growth of microorganisms. The effects of supplementation of different carbon and nitrogen sources viz. lactose, pectin, cellulose, avicel, xylose and CMC for carbon sources and NH₄Cl, urea, NaNO₃, beef extract, yeast extract, peptone (NH₄)₂SO₄ for nitrogen sources on production of β-glucosidase were evaluated. The resulted showed that lactose and NaNO₃ at the concentration of 0.5% and 0.2% separately obtained maximum enzyme production. Different to our results Saha et al. \[\text{18}\] reported that β-glucosidase from Candida peltata obtained its highest activity with xylose as carbon sources. Besides carbon and nitrogen sources initial pH incubation temperature and time were also tested for enzyme production. The Agrobacterium sp. PJD-1-1 in this study was found to have maximum enzyme production when it was incubated at pH 7.0 and 20 °C for 5 days. However the enzyme from Candida peltata increased up to 4 days when it was incubated at 28 °C after which it decreased gradually \[\text{18}\].

Upon fractionation of the β-glucosidase active fractions with ammonium sulfate 75.5% of total β-glucosidase activities were recovered in the fraction of 20%–60% ammonium sulfate. After gel filtration chromatography on Sephadex G-100 column the enzyme was found in fractions 20-60 tubes and pooled. Anion-exchange chromatography of the combined active fraction on a DEAE Sepharose column removed most of the contaminants and the effluent containing β-glucosidase activity was concentrated by ultrafiltration. The enzyme was purified 4.85-fold to homogeneity with overall enzyme yield of 8.0% and a specific activity of 19.0 U/mg proteins. SDS-PAGE analysis of the purified enzyme showed a single band with molecular mass of ca. 40 kDa which was lower than most of analogous β-glucosidases \[\text{19-24}\] . The optimum temperature of purified enzyme was 50 °C which was comparable to that of β-glucosidases from Sporobolus pararoseus \[55 °C \text{12,25} \] Rhynchosporus palmarum \[55 °C \text{16} \] Pichia etchellsii \[50 °C \text{22} \] Candida sake \[52 °C \text{22} \] and Aspergillus oryzae \[50 °C \text{22} \] higher than that of β-glucosidases from Wickerhamomyces anomalus \[35 °C \text{22} \] Pichia pastoris \[40 °C \text{26} \] and Galleria mellonella \[42 °C \text{22} \] but lower that of β-glucosidases from Thermoanaerobacterium thermosaccharolyticum \[70 °C \text{11} \] Penicillium italicum \[65 °C \text{5} \] Aspergillus unguis \[60 °C \text{11} \]. Most β-glucosidases characterized so far show maximal activity in the acidic pH range (pH 4.0–6.5) while the β-glucosidase...
screened in this study showed its highest activity at alkaline conditions with optimum pH of 8.0.

The effects of various mental ions and agents on the enzyme activity were tested. Ba\(^{2+}\), Ca\(^{2+}\), Pb\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Na\(^{+}\), K\(^+\), urea and EDTA were slightly affected the enzyme activity which was consistent with the properties of β-glucosidase from Aspergillus niger\(^{[20]}\). However, the activity of β-glucosidases from Penicillium funiculosum and Sporidiobolus pararoseus was increased by Mn\(^{2+}\), K\(^+\), and Na\(^{+}\)\(^{[20,24]}\). Zn\(^{2+}\) and SDS strongly inhibited the activity of β-glucosidase from Sporidiobolus pararoseus\(^{[24]}\), while K\(^+\), Ca\(^{2+}\), EDTA and Ba\(^{2+}\) enhanced the activity of β-glucosidase from a marine Streptomyces\(^{[24]}\). The activity of β-glucosidase in this study was heavily inhibited by Cu\(^{2+}\), Hg\(^{2+}\) and Ag\(^{+}\). Cu\(^{2+}\) and Hg\(^{2+}\) also inhibit HGT-BG\(^{[24]}\) and BglA\(^{[20]}\), suggesting that the active catalytic sites of these enzymes might possess thiolic groups that cause sensitivity to inhibition by Hg\(^{2+}\). However, the activity of β-glucosidase from Myceliophthora thermophila was activated by Cu\(^{2+}\)\(^{[24]}\).

In conclusion, a novel Agrobacterium sp. PJD-1-4 producing β-glucosidase was isolated. The high level of enzyme production on lactose and NaNO\(_3\) as carbon and nitrogen sources\(^{[4]}\) and it showed its optimum activity in the alkaline pH range and also showed considerable thermal stabilities\(^{[4]}\) which make it an interesting candidate for biological applications.

Reference


[17] Rajasree KP, Mathew GM, Pandey A et al. Highly glucose...


