# Chapter 2
# Cloning and Expression of β-Glucosidase from Cassava in *Pichia pastoris* GS115

**Dongheng Guo, Hongming Tian, Yanshan Xu and Suiping Zheng**

**Abstract** β-Glucosidases have been widely applied in the synthesis of alkyl polyglucosides. Studies have shown that cassava β-glucosidase has great synthesis ability. This study has realized the secretion of cassava β-glucosidase gene (*mebgl*) in *Pichia pastoris* GS115. The recombinant yeast expression vector pPICZαA-MEBGL has been successfully constructed. The constructed plasmid was linearized and integrated into *P. pastoris* GS115 strain by electroporation. Positive clones were selected on YPDZ plates and then cultured in shake flask. The supernatant was collected and used for alkyl polyglucoside synthesis. The synthesis reaction was conducted under conditions: 40 °C, pH 5.0, 10 % water content, while the conditions have not been optimized. The results of these studies have indicated that 5′AOX promoter was the best one for expression; in the shake flask fermentation, the strain had the maximum hydrolysis activity of 60 U/L, while the optimal temperature was 35 °C, the optimal pH was 6.0, *p*NP-Glc was used as substrate; the molecular mass of the recombinant monomer protein was estimated to be 70 kDa; the recombinant protein showed high ability to transfer glucose from *p*NP-Glc to n-hexyl alcohol with high yields of 60 %, and the yields would substantially increase after optimized. This recombinant cassava β-glucosidase is expected to promote the industrialization process of alkyl polyglucoside enzymatic synthesis.

**Keywords** Cassava β-glucosidase · *Pichia pastoris* GS115 · Secretory expression · Enzymology properties

## 2.1 Introduction

β-Glucosidases (E.C. 3.2.1.21) catalyze the hydrolysis of β-D-glucosidic linkages between β-D-glucose and aglycone or sugar. β-Glucosidases exhibit similar specificity for β-glucoside substrates, have a similar molecular weight (about 55–65 kDa)

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and pH optima (between pH 5 and 6) [1], and are evolutionarily related. However, they may have different substrate specificities, suggesting their diverse biological functions. According to the classification of glycosyl hydrolases based on amino acid sequence similarities [2], β-glucosidases belong to GH1, GH3, GH5, GH9, GH30, GH116 families. A large proportion of β-glucosidases belong to glycoside hydrolase families 1 and 3 (GH1 and GH3), and hydrolyze their substrates via double-displacement mechanism [3]. The crystal structures of β-glucosidases belonging to GH1 family have been reported. But to GH3 family, only four crystal structures have been reported. β-Glucosidases that belong to GH1 and GH3 family can catalyze reverse hydrolysis and transglycosylation reactions, leading to synthesis of oligosaccharides and alkyl polyglucosides.

Cassava β-glucosidase belongs to GH1 family, it can transfer a nonreducing glucosyl group from a glycoside or carbohydrate to water (hydrolysis) or another alcohol (transglycosylation) via retaining mechanism. Cassava β-glucosidase possess $(\beta/\alpha)_8$ barrel structure. It has two carboxylic acid residues on β-strands 4 and 7, which act as the general acid–base catalyst and the nucleophile, respectively [4]. Site-directed mutagenesis is carried out to study the function of active-site amino acid residues based on a homology model, while the MODELLER program is used [5]. The enzyme activity is destroyed when Glu-413 is changed to Gly, which is consistent with that it being the catalytic nucleophile. The Gln-339/Glu mutation also destroys activity, confirming a function in positioning the catalytic diad. The Phe-269 contributes to the cyanogenic specificity of the cassava β-glucosidase. The recombinant cassava β-glucosidase is successfully expressed in *Saccharomyces cerevisiae*. Its Michaelis constants for the natural substrate linamarin ($K_m$ = 1.06 mM) and the synthetic *p*-nitrophenyl β-D-glucopyranoside (*p*NP-Glc; $K_m$ = 0.36 mM) are very similar to the plant enzyme. Linamarase gene is intracellular expressed in *Pichia pastoris* GS115 [6]. SDS/PAGE analysis shows that the molecular weight of recombinant protein is around 71 kDa. The optimal temperature of recombinant protein is about 37 °C, and the optimal pH is about 5. The $K_m$ is 1.70 mmol/L, and $V_{max}$ is 8.36 μmol/(min mg), while *p*NP-Glc is used as the substrate.

The cassava β-glucosidase has very low hydrolysis activity, but shows high synthesis ability [7]. The alkyl polyglucosidase synthesis ability of β-glucosidases from cassava, Thai rosewood, and almond has been compared. Cassava β-glucosidase shows greater ability to transfer glucose from *p*NP-Glc to secondary alcohol acceptors than other β-glucosidases, and it is the only one to synthesize tertiary alkyl β-glucosides with high yields. However, it could not catalyze the synthesis of alkyl polyglucosides through reverse hydrolysis reaction. Cassava β-glucosidase requires active glycosyl donors and could not use mono- or disaccharides as sugar donors in alkyl polyglucoside synthesis.

The *P. pastoris* expression system is widely used as eukaryotic expression system [8, 9]. The system has many advantages, such as relatively simple operation, fast growth, high expression, low miscellaneous proteins, postprocessing, protein

folding, and posttranslational modification. And the AOX1 promoter can ensure a high expression of exogenous genes with methanol as the sole carbon and energy source.

In this study, we constructed the recombinant expression vector pPICZαA-MEBGL. The constructed plasmid was integrated into *P. pastoris* GS115 strain. After screened by nutrient deficiencies plate and shake flask fermentation, we got the positive recombinant strains. And we carried out preliminary enzymatic properties study on the recombinant cassava β-glucosidase, such as hydrolysis and synthesis ability.

## 2.2 Materials and Methods

### *2.2.1 Materials, Strains, and Media*

*Escherichia coli* Top10F™ was used for the amplification of all plasmids. The *P. pastoris* GS115 strains were used as host strains for β-glucosidase expression. The cloning and expression vector pPICZαA was purchased from Invitrogen.

LB culture medium: 1 % tryprone, 0.5 % yeast extract, 1 % NaCl. Zeocin was added to the medium when the medium was used for screening. In the medium, the concentration of zeocin was 25 μg/mL. YPD, BMGY, BMMY reference Invitrogen company operating manuals. YPDZ medium, which the concentration of zeocin was 100 μg/mL, was used for screening.

### *2.2.2 PCR of Cassava β-Glucosidase Gene*

The Genebank ID of *mebgl* gene was gi:249261. The gene was synthesized after codon optimization. The oligonucleotide primers contain 5′ *Eco*RI and 3′ *Kpn*I restriction endonuclease sites. Upstream primer contains *Eco*RI restriction endonuclease site (outlined by underline), "His" label and protected bases: 5′CAGGAATTCCATCATCACCATCACCATACAGATGACGATGACGACAA 3′. Downstream primer contains *Kpn*I restriction endonuclease site and protected bases: 5′CGGGGTACCCTACATAACGTAAAACTTTC3′. The PCR amplification of *mebgl* gene was conducted under conditions: 2 μL 100 ng/μL template, 25 μL 2× KOD FX Buffer, 10 μL 2 mmol dNTPs, 2.5 μL 10 μmol/L each primer, 1 μL KOD FX DNA polymerase, 9.5 μL dd$H_2O$; initial denaturation at 94 °C for 5 min, 30 cycles (10 s at 94 °C, 30 s at 55 °C, and 96 s at 68 °C) were followed by a final incubation at 68 °C for 10 min.

### 2.2.3 Cloning and Construction of the Yeast Expression Vector

The PCR products were digested with *Eco*RI and *Kpn*I, gel purified, and subsequently inserted into the *Eco*RI–*Kpn*I sites in pPICZαA, giving pPICZαA-MEBGL. The recombinant plasmid was transformed into *E. coli* Top10F™ via $CaCl_2$ transformation method. Single colonies of *E. coli* Top10F™ that contained pPICZαA-MEBGL were grown in LB resistance screening medium, at 37 °C, 200 rpm for 14–16 h. The recombinant plasmid was extracted, confirmed by restriction digestion, and DNA sequencing.

### 2.2.4 Yeast Transformation and Screening

Electrotransformation was carried out with the Bio-Rad Gene Pulser system (Bio-Rad, Richmond, CA, U.S.A.) according to the manufacturer's instructions, 0.1 μg linearized plasmid was used. After that, the GS115 cells were coated in YPDZ plates. The plates were cultured at 30 °C for 3 days. Single colonies of *P. pastoris* GS115 were grown in YPDZ medium, and confirmed by colony PCR.

### 2.2.5 Expression of Cassava β-Glucosidase in Pichia pastoris GS115

The strains were cultured at BMGY, at 30 °C, 200 rpm for 20–24 h. Then the culture was transformed into BMMY which contained 2 % (v/v) methanol. The initial $OD_{600}$ was controlled at one. After that, the strains were cultured at BMMY for 144 h, at 30 °C, 200 rpm. 2 % (v/v) fermented liquid was extracted and 2 % (v/v) methanol was added every 24 h for the induction of the recombinant proteins.

### 2.2.6 Purification of Recombinant Cassava β-Glucosidase

The fermentation liquor was collected after the strains were cultured for 144 h. The supernatant was used for purification after filtered by 0.22 μm filter membrane. Then the supernatant was desalted through desalting crude (HiTrap™). After that, the supernatant was used for Ni-chelating affinity chromatography (HisTrap™FF). The recombinant protein was eluted by 10, 20, 50, 100, 200, 300 mM imidazole. Then the eluent was concentrated through 10 kDa filter membrane bag. The recombinant protein was used for further analysis.

### 2.2.7 SDS-PAGE Analysis

SDS-PAGE electrophoresis was performed on 6 % spacer polyacrylamide gel and 12 % (w/v) separation polyacrylamide gel. After electrophoresis, proteins were stained in 0.05 % (w/v) Coomassie Brilliant Blue R250 (Sigma-Aldrich) solution.

### 2.2.8 The Analysis of Hydrolysis and Transglycosylation Activities

The hydrolysis activity of cassava β-glucosidase was assayed with colorimetric method while *p*NP-Glc was used as substrate. The reaction was conducted under conditions: 250 μL cassava β-glucosidase liquid and 250 μL 5 mM *p*NP-Glc were preheated at 40 °C for 5 min, respectively, then they were mixed for reaction at 40 °C for 30 min, 0.2 M sodium acetate (pH 5.0) buffer solution was used. The reaction was stopped by adding 500 μL 1 M sodium carbonate, and the *p*-nitrophenol released was measured by its absorbance at 405 nm. One unit of activity is defined as the amount of enzyme used to release 1 μmol of *p*-nitrophenol in 1 min.

The temperature optimum was determined by measuring *p*-nitrophenol released from *p*NP-Glc at temperatures ranging from 10 to 70 °C at 10 °C increments, and the assay was completed as described above. The pH optimum was determined by hydrolysis of *p*NP-Glc at 40 °C for 30 min in the following 0.2 M buffers: glycine–HCl, pH 2–3; sodium citrate, pH 3–4; sodium acetate, pH 4–5; MES, pH 5–6; sodium phosphate, pH 6–8; Tris–HCl, pH 8–9; and CAPS, pH 9–10.

The preliminary experiments of transglycosylation reaction were conducted under conditions: 30 mM *p*NP-Glc; 10 % (v/v) 0.2 M pH 5.0 sodium acetate (containing recombinant cassava β-glucosidase); 90 % (v/v) n-hexyl alcohol, at 40 °C for various times (from 1 to 6 h) before analysis by HPLC.

Waters 2695, Waters 2424 ELS Detector and C18 chromatographic column were used for HPLC analysis. Samples were filtered by 0.22 μm filter membrane before detected. The mobile phase was methol–water mixture (80:20, v/v). The detection was conducted under conditions: 1.0 mL/min flow rate, 30 °C for C18 column, 60 °C for drift tube, 30 psi for nitrogen pressure.

## 2.3 Results and Discussions

### 2.3.1 Construction of Recombinant Expression Vector pPICZαA-MEBGL

The recombinant plasmid pPICZαA-MEBGL (Fig. 2.1) was constructed by the products of PCR based on the method as described by Sect. 2.2.3. Three different recombinant plasmids were constructed, which contain GAP, modified AOX and

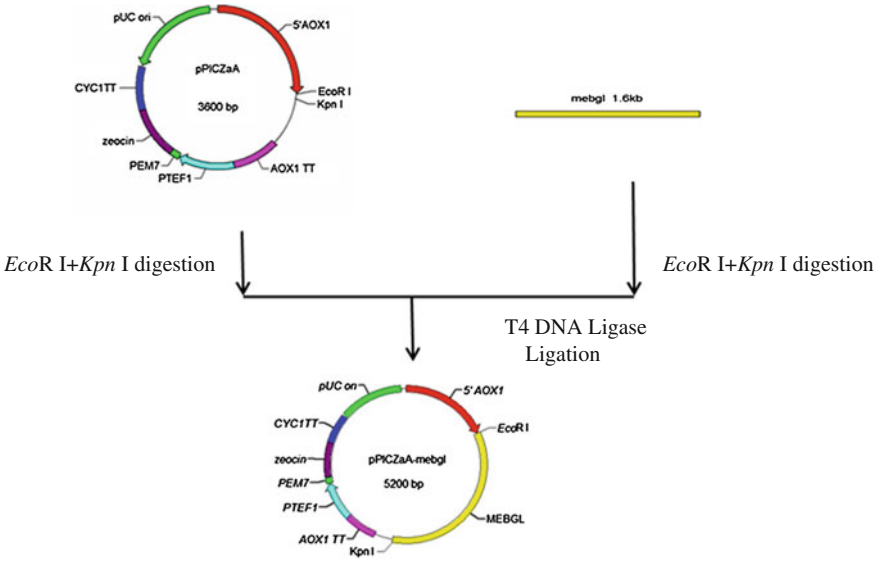


**Fig. 2.1** Sketch of construction of recombinant expression plasmid pPICZαA-MEBGL

5′AOX promoter, respectively. The recombinant plasmids were confirmed by restriction digestions (Fig. 2.2a) and DNA sequencing. The targeted band was between 1,500 and 2,250 bp.

### *2.3.2 Identification of Recombinant Pichia pastoris GS115/pPICZαA-MEBGL*

Single colonies of *P. pastoris* GS115 were grown in YPDZ medium, and then confirmed by colony PCR (Fig. 2.2b). The results showed that most single colonies were positive clone. The positive clones were used for further screening.

### *2.3.3 The Screening of Recombinant Pichia pastoris GS115*

The recombinant and control strains were cultured at the conditions as described by Sect. 2.2.5. There were no difference between their growth situations, but the recombinant strains show better hydrolytic activities, 60 U/L after been induced for 144 h (Fig. 2.3). The hydrolysis activities of recombinant strains were increased along with the induction time, but the control strains were decreased. And among GAP, modified AOX and 5′AOX, 5′AOX promoter was the best one for expression.

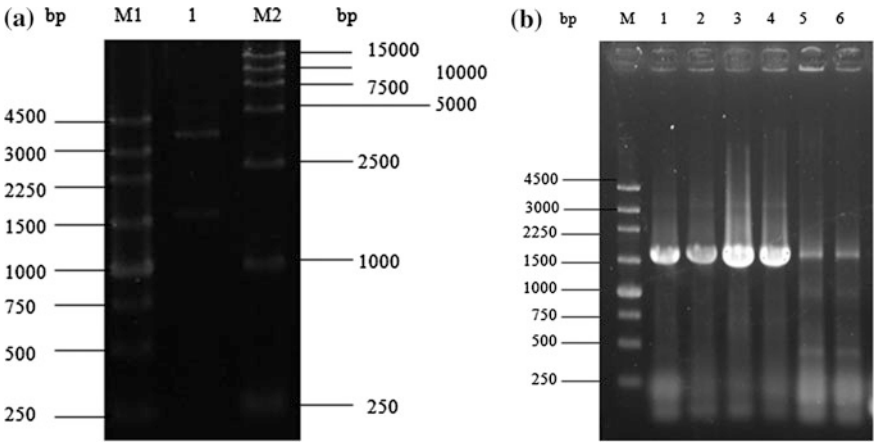


**Fig. 2.2** **a** Identification of recombinant plasmid pPICZαA-MEBGL. *M1* 250 bp DNA marker; *1* Double restriction of pPICZαA-MEBGL by *Eco*RI and *Kpn*I; *M2* DL15000 DNA marker. **b** Identification of recombinant *Pichia pastoris* GS115/pPICZαA-MEBGL. *M1* 250 bp DNA marker; *1*, *2*, *3*, *4*, *5*, *6* recombinant *P. pastoris* GS115

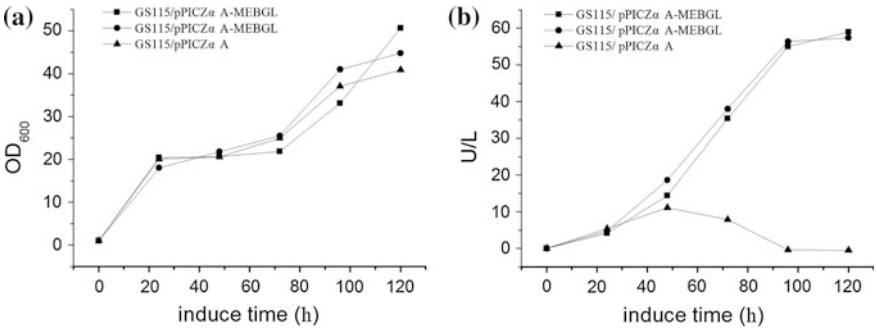


**Fig. 2.3** The culture of recombinant *Pichia pastoris* GS12. **a** $OD_{600}$ of recombinant *P. pastoris* GS115/pPICZαA-MEBGL (GS115/pPICZαA as control); **b** supernatant hydrolytic activities of recombinant *P. pastoris* GS115/pPICZαA-MEBGL (GS115/pPICZαA as control)

### *2.3.4 Analysis of SDS-PAGE*

The supernatant of the recombinant and control strains were applied for SDS-PAGE analysis. The molecular mass of the recombinant monomer protein was estimated to be 70 kDa (Fig. 2.4). This is consistent with literature reports that the recombinant protein was 71 kDa [6].

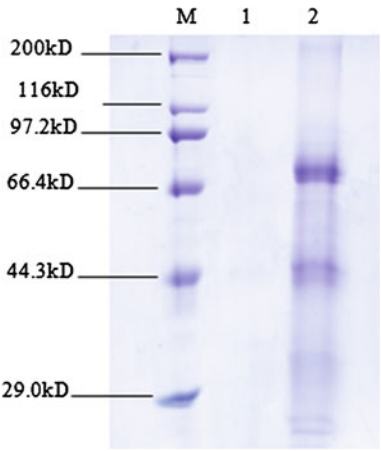


**Fig. 2.4** SDS-PAGE analysis of the recombinant cassava β-glucosidase expressed in *Pichia pastoris* GS115 (GS115/pPICZαA as control); *M* protein marker; *1* supernatant of GS115/pPICZαA; *2* supernatant of *P. pastoris* GS115/pPICZαA-MEBGL

### 2.3.5 Optimization of Temperature and pH for Hydrolytic Activity of Recombinant Cassava β-Glucosidase

The recombinant cassava β-glucosidase was sensitive to temperature and pH values. As shown in Fig. 2.5a, when the temperature increased from 10 to 35 °C, the activity increased gradually. The maximum activity was achieved at 35 °C. If the temperature continued to rise, the activity decreased seriously. At 60 °C, the activity only retained 14.0 % of maximal activity.

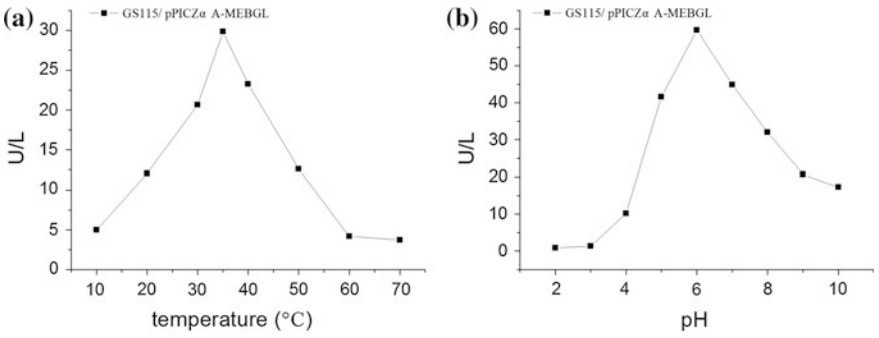


**Fig. 2.5** Optimization of temperature and pH for hydrolytic activity of recombinant cassava β-glucosidase. **a** Optimization of temperature for hydrolytic activity of recombinant cassava β-glucosidase. **b** Optimization of pH for hydrolytic activity of recombinant cassava β-glucosidase

As shown in Fig. 2.5b, the recombinant β-glucosidase had an optimal pH of 6. At pH 5.0 and pH 7.0 the activity kept about 60–70 % of the maximum activity. There were only 17.1 and 29.0 % of maximal activity, when the pH values were 4.0 and 10.0.

### 2.3.6 Purification of Recombinant Cassava β-Glucosidase

The fermentation liquor was desalted through desalting crude, then the supernatant was used for Ni-chelating affinity chromatography. The results indicated that most of the proteins could not bind to nickel column, a few proteins were eluted by 10 mM imidazole, and no proteins were eluted by other concentrations of imidazole. The supernatants that containing unbound proteins were concentrated through 10 kDa filter membrane bag, so were the eluents. Both of the concentrated solutions were used for SDS-PAGE analysis and transglycosylation reactions. The results of SDS-PAGE indicated that most of recombinant cassava β-glucosidase could not bind to nickel column. The concentrated cassava β-glucosidase was used for the synthesis of alkyl polyglycoside.

### 2.3.7 The Preliminary Experiment: Transglycosylation of Recombinant Cassava β-Glucosidase

A certain amount of cassava β-glucosidase, the hydrolysis activity was 0.1 U, was used for transglycosylation reaction. The preliminary experiment of transglycosylation reaction of cassava β-glucosidase showed great alkyl polyglycoside synthesis ability (Fig. 2.6). This was consistent with literature reports that cassava β-glucosidase showed better alkyl polyglycoside synthesis ability than Thai rosewood and

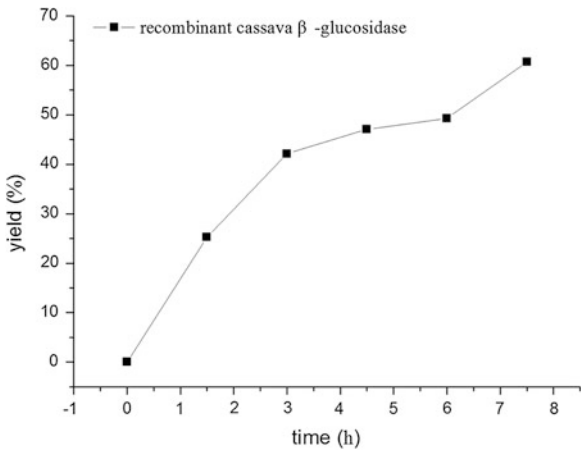


**Fig. 2.6** The synthesis of *n*-hexyl β-D-glucopyranoside through transglycosylation, while *p*NP-Glc was used as the substrate

almond β-glucosidases [7]. The cassava β-glucosidase could synthesize tertiary alkyl polyglycosides, while the other two β-glucosidases could not. Cassava β-glucosidase was important to the enzymatic synthesis of alkyl polyglycoside for its high synthesis ability.

## 2.4 Conclusion

This study realized the expression of β-glucosidase gene *(mebgl)* from cassava in *P. pastoris* GS115. SDS/PAGE analysis showed that the molecular mass of the recombinant monomer protein was around 70 kDa. The optimal temperature of this enzyme was about 35 °C, and the optimal pH was about 6.0, the maximum supernatant hydrolytic activity was 60 U/L, while *p*NP-Glc was used as the substrate. Study had shown that cassava β-glucosidase shows greater ability to transfer glucose from *p*NP-Glc to primary and secondary alcohol acceptors than Thai rosewood and almond β-glucosidases. And it is the only one β-glucosidase that is able to synthesize tertiary alkyl β-glucosides. This β-glucosidase is expected to promote the industrialization process of alkyl glucoside enzymatic synthesis.

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