Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Characterization of a recombinant thermostable β-glucosidase from *Putranjiva roxburghii* expressed in *Saccharomyces cerevisiae* and its use for efficient biomass conversion

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ARTICLE INFO

Keywords:
Putranjiva roxburghii
β-glucosidase
Saccharomyces cerevisiae
Thermostable
Cellobiose

ABSTRACT

A β-glucosidase gene from *Putranjiva roxburghii* (PRGH1) was heterologously expressed in *Saccharomyces cerevisiae* to enable growth on cellobiose. The recombinant enzyme was secreted to the culture medium, purified and biochemically characterized. The enzyme is a glycoprotein with a molecular weight of ~68 kDa and exhibited enzymatic activity with β-linked aryl substrates like *p*NP-Fuc, *p*NP-Glc, *p*NP-Gal and *p*NP-Cel with catalytic efficiency in that order. Significant enzyme activity was observed for cellobiose, however the enzyme activity was decreased with increase in chain length of glycan substrates. Using cellobiose as substrate, the enzyme showed optimal activity at pH 5.0 and 65 °C. The enzyme was thermostable up to 75 °C for 60 min. The enzyme showed significant resistance towards both glucose and ethanol induced inhibition. The recombinant *S. cerevisiae* strain showed advantages in cell growth, glucose and bio-ethanol production over the native strain with cellobiose as sole carbon source. In simultaneous saccharification and fermentation (SSF) experiments, the recombinant strain was used for bio-ethanol production from two different cellulosic biomass sources. At the end of the SSF, we obtained 9.47 g L^{-1} and 14.32 g L^{-1} of bio-ethanol by using carboxymethyl cellulose and pretreated rice straw respectively. This is first report where a β-glucosidase gene from plant origin has been expressed in *S. cerevisiae* and used in SSF.

1. Introduction

Cellulose is the most abundant polysaccharide on the earth and virtually an unlimited source of renewable bioenergy [1]. The production of fuel ethanol from this cellulosic biomass remains a highly attractive option in terms of environmental, commercial and social sustainability and is gaining increasing attention [1,2]. Sugars for the fermentation process are accessed from cellulosic material through chemical or enzymatic hydrolysis. The enzymatic hydrolysis of cellulosic biomass involves conversion of biomass to reducing sugars, and the subsequent conversion of the reducing sugars to ethanol. However, this process is very costly owing to the recalcitrance of cellulose, resulting in low yield and high cost of the enzymatic hydrolysis process [3]. Microbial cellulolytic enzyme complexes consist of three basic types of enzymes, including cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidases (EC 3.1.2.21), which work synergistically to degrade cellulose to glucose. Cellobiohydrolases and endoglucanases synergistically degrade native cellulose to generate cellobiose, which is a strong product inhibitor of both the enzymes [4]. β-glucosidases cleave the β-1, 4-glycosidic linkage of cellobiose to generate D-glucose. Therefore, β-glucosidases not only catalyze the final step of cellulose degradation, but also allow the cellulolytic enzymes to function more efficiently by relieving the cellobiose mediated inhibition [4,5]. However, cellobiose is consistently accumulated owing to the weak β-glucosidases activity of most microbial cellulases making cellobiose hydrolyzation a rate-limiting step during this enzymatic hydrolysis process of the cellulosic biomass [6].

One-step conversion of cellulosic biomass to bio-ethanol with an organism capable of cellulose degradation and efficient fermentation (consolidated bioprocessing-CBP) may offer cost reductions of bio-ethanol production [3,7]. One of the most effective ethanol-producing yeasts, *Saccharomyces cerevisiae*, has several advantages including (i) natural robustness in industrial process (ii) simplicity in genetic manipulation (iii) larger cell size, which simplify their separation from the culture broth (iv) resistance to viral infection and (v) generally regarded as safe (GRAS) status due to its long association with the food and beverage industries [8]. Despite of these advantages *S. cerevisiae* has a major drawback due to its inability to degrade cellobiose and

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http://dx.doi.org/10.1016/j.procbio.2017.08.005
Received 24 April 2017; Received in revised form 25 July 2017; Accepted 7 August 2017

longer chain cello-oligosaccharides efficiently and these are the dominant soluble by-products of cellulose hydrolysis. Therefore, construction of β-glucosidases overproducing strain is an important strategy to enhance the efficient utilization of cellobiose. Great efforts have been made to genetically engineer *S. cerevisiae* to grow on cellobiose by expressing heterologous β-glucosidases genes. Mostly, β-glucosidase from bacterial and fungal origin have been transferred to *S. cerevisiae* enabling the growth on cellobiose [9–11], but no effort has been made with efficient thermostable plant β-glucosidases till date. Enzyme thermostability is crucial during the saccharification step because steam is always used to make the substrates more appropriate for enzymatic hydrolysis process without a pre-cooling process thus reducing the processing time, chance of contamination and enhancing fermentation qualities and yield [12]. To get efficient and thermostable β-glucosidases has become an important objective in industrial research.

Our previous studies focused on the identification, cloning and characterization of a thermostable family 1 glycosyl hydrolase enzyme (PRGH1) which was sourced from a *Putranjiva roxburghii* plant [13,14]. *P. roxburghii* is a medicinal plant that belongs to the Euphorbiaceae family. In this study, the β-glucosidase gene (*prgh1*) was overexpressed under the control of the *PGK1* promoter and terminator in *S. cerevisiae*. The enzymatic properties of the purified recombinant PRGH1 were characterized. The ability to sustain the growth of the recombinant strain on cellobiose as sole carbon source was studied. In addition, we have carried out simultaneous saccharification and fermentation of different cellulosic biomasses by using the recombinant strain. To our knowledge, it is the first report where a plant β-glucosidase gene was used to engineer *S. cerevisiae* for efficient utilization of biomass.

2. Materials and methods

2.1. Microorganism strains and culture conditions

Escherichia coli strain XL1-Blue (Stratagene Inc., La Jolla, Calif.) was used as host strain for maintenance and amplification of constructs. Luria-Bertani medium was used to cultivate the bacterial strain, and 100 μg/ml of ampicilin was added for selecting transformants. *S. cerevisiae* Y294 (Genotype: *α leu2-3, 112ura3-52 his3 trp1-289*, Source of reference: ATCC 201160) was cultured in YPD medium at 30 °C. *S. cerevisiae* transformants were cultured in synthetic complete (SC^{-Ura}) medium supplemented with appropriate amino acids [9]. After *FUR1* gene disruption the autoselective *S. cerevisiae* transformants were maintained and cultivated in $SC^{-Ura-Leu}$ or SC medium.

2.2. Recombinant vector construction

The enzymes for amplification, restriction digestion and ligation were purchased from NEB. The yXYNSEC vector, pDF1 plasmid and *S. cerevisiae* Y294 strain were kindly provided by Prof. Willem Heber van Zyl, Stellenbosch University, South Africa. Previously, we cloned the 1617 bp β-glucosidase gene (*prgh1*), into the pGEM-T vector and the gene sequence was submitted in the NCBI GenBank with the accession no KF006311 [14]. The gene sequence analysis showed that the gene product belongs to glycosyl hydrolase family 1. The ORF of the PRGH1 encoding gene product without the sequence for the native signal was amplified by PCR from the recombinant plasmid pGEM-T-PRGH1 by using a forward primer, PRGH1-Fwd (5′-GAGCTCGCGAAATTCCTTCA-ACAGAAGTG-3′) and a reverse primer, PRGH1-Rev (5′-CGGAAGATC-TTAAGCGGCTGCTGATCTAATAG-3′) having overhanging regions encoding *Nru*I and *Bgl*II restriction sites respectively. The purified PCR product was digested with *Nru*I and *Bgl*II and was ligated to the *Nru*I/*Bgl*II site of the yXYNSEC vector, which enabled the fusion of the *prgh1* gene to the *xyn2* sequence for the secretion signal from *Trichoderma reesei*. The details of this multi-copy yeast expression vector yXYNSEC were described by Van Rooyen et al. [9]. Correct construction was confirmed by restriction digestion and subsequent DNA sequencing. The resulting construct was designated as yXYNSEC-PRGH1.

2.3. Yeast transformation and FUR1 gene disruption

S. cerevisiae Y294 was transformed with the yXYNSEC-PRGH1 plasmid by the dimethyl sulfoxide-lithium acetate method described by Hill et al. [15]. Transformants were screened and maintained on SC^{-Ura} medium. Further confirmation of the transformation was done with PCR. To ensure autoselection of the *URA3*-bearing yXYNSEC-PRGH1 plasmid in non-selective medium, disruption of the uracil phosphoribosyltransferase (*FUR1*) encoding gene in the *S. cerevisiae* Y294 transformants was performed by using the pDF1 plasmid [16]. *fur1*:*leu2* autoselective transformants were screened on $SC^{-Ura-Leu}$ medium.

2.4. Purification of recombinant PRGH1

The autoselective *S. cerevisiae* Y294 transformants were inoculated onto $SC^{-Ura-Leu}$ plates with cellobiose as the sole carbon source. The plate was incubated at 30 ° C for 2 days and then overlaid with 0.8% agar containing 5 mM 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc). After that, the plate was incubated at 50 °C for 15 min and the colonies with β-glucosidase activity were monitored under UV light (365 nm). For protein purification, autoselective *S. cerevisiae* Y294 cells with strong β-glucosidase activity were grown in 1 L of SC medium with cellobiose as the sole carbon source at 30 °C for 48 h. The supernatant of the culture was separated from the cellular mass by centrifugation for 5 min at 5000 X g. The supernatant was utilized as crude enzyme for the purification procedure. The supernatant was percolated through filter paper and concentrated up to 50 mL using a 10 kDa cutoff Amicon Ultra-15 concentrator (Millipore, Bedford, Massachusetts, USA) as recommended by the manufacturer. The concentrated supernatant was precipitated by the addition of solid ammonium sulfate up to 30% saturation. The resultant mixture was centrifuged at 12,500 X g for 30 min at 4 °C and the supernatant was brought to 60% saturation. Subsequently, the resulting precipitates containing most of the enzyme were collected by centrifugation (12,500 X g, 30 min), dissolved in distilled water and dialyzed against the binding buffer (50 mM Tris-HCl; pH 8.0, 25 mM NaCl) and loaded on a DEAE-sepharose column pre-equilibrated with the same buffer. The column was washed extensively with binding buffer to remove unbound proteins and bound proteins were eluted with a NaCl step gradient from 0 to 0.3 M. Fractions having recombinant enzyme activity were pooled together and dialyzed against buffer containing 20 mM potassium phosphate; pH 7.4, 100 mM NaCl and loaded onto a concanavalin A-agarose column. The bound proteins were eluted using 500 mM of α-D-mannopyranoside. Homogeneity of the each fraction was analyzed by 12% SDS-PAGE. The pure and active fractions were pooled, dialyzed and concentrated by using an Amicon Ultra concentrator with a cut off value of 10 kDa (Millipore, Bedford, Massachusettes, USA).

2.5. SDS-PAGE and zymogram analysis

SDS-PAGE was conducted with a 12% polyacrylamide gel using the method described by Laemmli [17]. The β-glucosidase activity of the purified enzyme was observed by the zymogram assay with 4-methylumbelliferyl β-D-glucopyranoside (MUGlc)/4-methylumbelliferyl β-D-galactopyranoside (MUGal) as substrate by using 6% native PAGE. After electrophoresis, the gel was repeatedly rinsed with distilled water and 50 mM citrate phosphate buffer; pH 5.0 before being overlaid with 0.5 mM MUGlc/MUGal in same buffer, and the gel was incubated at 50 °C for 10 min. The fluorescence product was visualised under UV light at 365 nm, and subsequently the gel was stained with Coomassie Brilliant Blue R-250.