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Identification, purification and characterization of furfural transforming enzymes from *Clostridium beijerinckii* NCIMB 8052

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ABSTRACT

Generation of microbial inhibitory compounds such as furfural and 5-hydroxymethylfurfural (HMF) is a formidable roadblock to fermentation of lignocellulose-derived sugars to butanol. Bioabatement offers a cost effective strategy to circumvent this challenge. Although *Clostridium beijerinckii* NCIMB 8052 can transform 2–3 g/L of furfural and HMF to their less toxic alcohols, higher concentrations present in biomass hydrolysates are intractable to microbial transformation. To delineate the mechanism by which *C. beijerinckii* detoxifies furfural and HMF, an aldo/keto reductase (AKR) and a short-chain dehydrogenase/reductase (SDR) found to be over-expressed in furfural-challenged cultures of *C. beijerinckii* were cloned and over-expressed in *Escherichia coli* Rosetta-gami™ B(DE3)pLysS, and purified by histidine tag-assisted immobilized metal affinity chromatography. Protein gel analysis showed that the molecular weights of purified AKR and SDR are close to the predicted values of 37 kDa and 27 kDa, respectively. While AKR has apparent K_m and V_{max} values of 32.4 mM and 254.2 mM s^{-1} respectively, using furfural as substrate, SDR showed lower K_m (26.4 mM) and V_{max} (22.6 mM s^{-1}) values on the same substrate. However, AKR showed 7.1-fold higher specific activity on furfural than SDR. Further, both AKR and SDR were found to be active on HMF, benzaldehyde, and butyraldehyde. Both enzymes require NADPH as a cofactor for aldehydes reduction. Based on these results, it is proposed that AKR and SDR are involved in the biotransformation of furfural and HMF by *C. beijerinckii*.

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1. Introduction

Efficient bioconversion of lignocellulose-derived sugars to biofuels and other microbially-sourced industrial base chemicals is stifled by the generation of lignocellulose-derived microbial inhibitory compounds (LDMICs), including aldehydes, phenolic compounds and organic acids, during biomass pretreatment and hydrolysis [1]. Notably, furanic aldehydes such as furfural and 5-hyroxymethylfurfural (HMF), are particularly deleterious to fermenting microorganisms owing to their multifaceted toxicity, which includes damage to nucleic acids, cell membrane, inhibition of key metabolic enzymes, and induction of oxidative stress [2–4]. Nonetheless, the solventogenic *Clostridium* species, *Clostridium beijerinckii* NCIMB 8052 and *Clostridium acetobutylicum* ATCC 824 (hereafter referred to as *C. beijerinckii* and *C. acetobutylicum*, respectively) have been shown to tolerate low concentrations (up to 3 g/L) of furfural and HMF [2,5–7]. Conversely, higher concentrations (>3 g/L), similar to the levels found in lignocellulosic biomass hydrolysates, severely inhibit cell growth, sugar utilization, and product formation during acetone–butanol–ethanol (ABE) fermentation [2,5–8].

Metabolic engineering of solventogenic clostridia to detoxify >3 g/L furanic aldehydes without significantly impairing ABE production offers an inexpensive rational approach for bioconversion of biomass to bio-butanol. However, this requires an in-depth understanding of the mechanisms employed by butanol-producing *Clostridium* species in the detoxification of furanic aldehydes, the enzymes involved, and their patterns of regulation. These are better described in the ethanologenic microorganisms *Saccharomyces cerevisiae*, *Escherichia coli* and *Zymomonas mobilis* in which aldo/keto reductases (AKRs), alcohol dehydrogenases (ADs), and short chain dehydrogenase/reductase (SDR) have been demonstrated to

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catalyze NAD(P)H-dependent one-step reduction of furfural and HMF to their less toxic alcohols, furfuryl alcohol and HMF alcohol, respectively [9–12]. To date, no enzyme involved in furfural detoxification by solventogenic *Clostridium* species has been characterized. However, previous studies by our group demonstrated that furan tolerance by solventogenic clostridia also entails reduction of furfural and HMF to their less toxic alcohols [5–8]. Further, transcriptomic analysis of furfural-challenged *C. beijerinckii* showed significant up-regulation of AKR, AD, and SDR genes [6], which indicates that *C. beijerinckii* and most likely *C. acetobutylicum* recruit similar machinery as *S. cerevisiae*, *E. coli* and *Z. mobilis* in combating furans. *C. beijerinckii*, however, exhibits a high degree of gene duplicity [13,14]. In fact, there are 6 AKR, 10 SDR, and 19 AD genes in the genome of *C. beijerinckii*. It is plausible that the products of these genes are deployed against various toxicants, and biomass hydrolysates are replete with numerous LDMICs [15].

Therefore, elucidating the functional characteristics of the proteins encoded by these genes should enable a holistic design of a metabolic engineering approach to target robust detoxification of biomass hydrolysates and concomitant ABE production. For instance, coupling and over-expressing a battery of AKRs and SDRs, and/or ADs that have high affinities for various furan and phenolic toxicants in *C. beijerinckii* should enhance simultaneous detoxification of various LDMICs in biomass hydrolysates, when compared to over-expressing genes encoding enzymes that specifically target only a few furan or phenolic compounds. In this study, we report for the first time, heterologous cloning and over-expression in *E. coli* of an AKR (Cbei_3974) and an SDR (Cbei_3904), which were significantly up-regulated in furfural-challenged *C. beijerinckii* in a previous study [6]. Both enzymes were purified and characterized.

2. Methodology

2.1. Bacterial strains, plasmids and culture conditions

C. beijerinckii NCIMB 8052 (ATCC 51743) was obtained from the American Type Culture Collection (Manassas, VA). *E. coli* DH5α from New England Biolabs (Ipswich, MA) was used for cloning and maintenance of recombinant plasmids. Competent cells of *E. coli* strain Rosetta-gami™ B(DE3)pLysS was purchased from EMD Biosciences (San Diego, CA), and used as a host for recombinant protein expression. The plasmid pET-15b was purchased from EMD Millipore (Billerica, MA). Recombinant plasmids pET-15b_3974 and pET-15b_3904 were generated by cloning Cbei_3974 (AKR) and Cbei_3904 (SDR) coding regions respectively into pET-15b.

Laboratory stocks of *C. beijerinckii* were routinely maintained as spore suspensions in sterile double distilled water at 4 °C. To revive *C. beijerinckii* spores, 200 μl was heat-shocked for 10 min at 75 °C followed by cooling on ice. The heat-shocked spore suspension was inoculated into 10 ml anoxic pre-sterilized tryptone–glucose–yeast extract (TGY) medium and incubated in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI) as previously described [16]. *E. coli* DH5α was grown in Luria broth (LB), and Rosetta-gami™ B(DE3)pLysS was grown in LB medium supplemented with chloramphenicol (34 μg/ml) to maintain the plasmid, pRARE. *E. coli* strains (DH5α and Rosetta-gami™ B[DE3]pLysS) with recombinant plasmids pET-15b_3974 or pET-15b_3904 were grown in LB medium with addition of ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml).

2.2. Cloning of AKR and SDR genes

Genomic DNA of *C. beijerinckii* was prepared as previously described [17]. The AKR and SDR gene fragments were amplified by PCR using *C. beijerinckii* genomic DNA as template, and Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The PCR primers (Eurofins MWG Operon) were designed by the use of Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA) to target the respective entire open reading frames (ORFs) for AKR and SDR (Table 1). The PCR reaction conditions were: 98 °C for 30 s (initial denaturation), 98 °C for 10 s (denaturation), 59 °C for 30 s (annealing), and 72 °C for 30 s (extension) for 35 cycles and an additional 72 °C for 5 min (final extension).

The PCR product was digested with *Nde*I and *Cla*I for AKR, and *Bam*HI and *Cla*I for SDR, whose recognition sites were included in the respective PCR primers (Table 1). All restriction enzymes were purchased from New England Biolabs. The vector, pET-15b, was isolated from *E. coli* using the GenCatch plasmid DNA miniprep kit (Epoch Life Science, Sugar Land, TX). The vector was digested with appropriate restriction enzymes (*Nde*I and *Cla*I for Cbei_3974 [AKR]; *Bam*HI and *Cla*I for Cbei_3904 [SDR]). The digested vectors and PCR products were purified by agarose gel electrophoresis using the GenCatch advanced PCR extraction kit (Epoch Life Science, Sugar Land, TX). Ligation of PCR products and vector was performed at 4 °C overnight with T4 DNA ligase (New England Biolabs, Ipswich, MA) with an insert: vector ratio of 10:1.

The resulting ligation mixture was purified using the GenCatch advanced PCR extraction kit (Epoch Life Science, Sugar Land, TX). *E. coli* DH5α were transformed with recombinant DNA by electroporation in a 1 mm cuvette using Bio-Rad Gene Pulser Xcell™ (Bio-Rad, Hercules, CA) set at a voltage of 1.8 KV with a 25 μF and 200Ω. The duration of the electroporation time ranged from 4.3 to 4.4 ms. Following electroporation, cells were diluted in 1 ml SOC medium (2% w/v bacto-tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose) and incubated for 1 h at 37 °C with shaking at 250 rpm. The recovered cells were plated on LB agar with ampicillin (50 μg/ml), after which discrete colonies were picked and assessed for the presence of recombinant plasmids by single colony PCR and agarose gel electrophoresis. The recombinant plasmids, pET-15b_3974 and pET-15b_3904, were isolated and purified from *E. coli* DH5α transformants grown in LB medium supplemented with ampicillin using the GenCatch plus plasmid DNA miniprep kit (Epoch Life Science, Sugar Land, TX). Glycerol stocks of transformants were stored at −80 °C. The sequences of Cbei_3974 and Cbei_3904 were verified by DNA sequencing performed by the Plant–Microbe Genome Facility at The Ohio State University, Columbus, OH.

2.3. Expression of AKR and SDR genes in Rosetta-gami™ B(DE3) pLysS

For the expression of the cloned AKR and SDR, the recombinant plasmids, pET-15b_3974 and pET-15b_3904 were transformed into Rosetta-gami™ B(DE3)pLysS competent cells by electroporation according to the manufacturer's instructions. Transformants were selected on LB plates supplemented with chloramphenicol (34 μg/ml) and ampicillin (50 μg/ml), and grown at 37 °C overnight. A single colony for each clone was picked from the plate and sub-cultured into LB medium (5 ml) with addition of the same antibiotics as above, and grown overnight at 37 °C. Cultures were then inoculated into 250 ml of fresh LB medium followed by incubation in a rotary shaker at 250 rpm and 37 °C. When the cultures reached OD_{600} of 0.6 as measured by a DU800 spectrophotometer (Beckman Coulter Inc., Brea, CA), IPTG was added to a final concentration of 400 μM followed by incubation at 20 °C for 6 h to induce the expression of the recombinant proteins AKR and SDR. In addition, control cultures (*E. coli* Rosetta-gami™ B(DE3)pLysS carrying the plasmid vector, pET-15b) with and without IPTG induction, plus *E. coli* Rosetta-gami™ B(DE3)pLysS carrying the recombinant plasmids, pET-15b_3974 and pET-15b_3904 without IPTG induction

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