Journal of Biotechnology 263 (2017) 30–35

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Research paper

DNA-guided assembly of a five-component enzyme cascade for enhanced conversion of cellulose to gluconic acid and H_2O_2

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ARTICLE INFO

Keywords:
Enzyme fuel cell
HaloTag
Cellulosome
Substrate channeling
Enzyme cascade

ABSTRACT

Enzymatic fuel cells have received considerable attention because of their potential for direct conversion of abundant raw materials such as cellulose to electricity. The use of multi-enzyme cascades is particularly attractive as they offer the possibility of achieving a series of complex reactions at higher efficiencies. Here we reported the use of a DNA-guided approach to assemble a five-component enzyme cascade for direct conversion of cellulose to gluconic acid and H_2O_2. Site-specific co-localization of β-glucosidase and glucose oxidase resulted in over 11-fold improvement in H_2O_2 production from cellobiose, highlighting the benefit of substrate channeling. Although a more modest 1.5-fold improvement in H_2O_2 production was observed using a five-enzyme cascade, due to H_2O_2 inhibition on enzyme activity, these results demonstrated the possibility to enhance the production of gluconic acid and H_2O_2 directly from cellulose by DNA-guided enzyme assembly.

1. Introduction

Fuel cells are potential alternative to thermo-mechanical power generation processes by directly converting chemical fuels into electricity. Even though the technology has been around for decades, fuel cells are still not economically competitive because expensive and non-renewable noble metals are typically used as catalysts. In addition, metallic catalysts must be used at high temperatures and can be deactivated by trace amounts of impurities such as CO and sulfur in the fuels (Kim et al., 2006). On the other hand, biofuel cells (Minteer et al., 2007; Ramanavicius et al., 2008) that utilize enzymes can effectively catalyze redox reactions of abundant raw materials (e.g. glucose) to electrical energy under ambient conditions and neutral pH. In contrast to noble metals, enzymes are renewable and the cost of production can be very low, as enzymes can be economically produced easily by large-scale fermentation.

Past efforts on enzyme fuel cells have been focusing on the use of an individual enzyme to oxidize the substrate (Moehlenbrock et al., 2010). Unfortunately, the use of a single enzyme often limits the number of released electrons due to incomplete fuel oxidation. For instance, the conversion of methanol to formaldehyde by alcohol dehydrogenase releases only two of the six available electrons, while the complete oxidation of methanol to CO_2 using a sequential three-enzyme oxidation system generates six electrons for fuel-cell applications (Addo et al., 2010). In an effort to address this shortcoming, many researchers have incorporated multi-enzyme cascades for more complete oxidization of fuels that are both abundant and renewable (Liu et al., 2013; Siu et al., 2015; Sokic-Lazic et al., 2010).

Cellulose, one of the most abundant natural resources on earth, has been the focus of considerable interest as a renewable energy source (Liao et al., 2016). Although extensive research efforts have been made toward the development of glucose oxidase (GOX)-based fuel cells using glucose as the fuel (du Toit and Di Lorenzo, 2014; Korkut and Kilic, 2016; Willner et al., 1996), progress toward the use of cellulose as a substrate has been lagging. Recently, cellulose has been used as the fuel in a microbial fuel cell (MFC) for the direct electricity generation (Ren et al., 2007; Rezaei et al., 2007) using both cellulolytic and exoelectrogenic microorganisms. More importantly, it has been shown that addition of cellulases to increase the hydrolysis of cellulose can substantially improve the overall power output to a level achieved using the same amount of glucose (Rezaei et al., 2008). This result clearly indicates the importance of cellulose hydrolysis on the overall fuel cell performance.

Cellulosomes are multi-enzyme systems found in many anaerobic bacteria for efficient degradation of cellulose to glucose (Bayer et al., 2004). Endoglucanases, excoglucanases, and β-glucosidases are organized in close proximity on a scaffold via the high-affinity cohesin-dockerin interaction for synergistic digestion of cellulose (Bayer et al., 2008). Inspired by cellulosome systems, synthetic protein scaffolds based on cohesin-dockerin interaction have been developed for efficient

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http://dx.doi.org/10.1016/j.jbiotec.2017.10.006
Received 29 June 2017; Received in revised form 25 September 2017; Accepted 9 October 2017
Available online 10 October 2017
0168-1656/

enzymatic reaction (Sun and Chen, 2016; Sun et al., 2014; Tsai et al., 2009). However, truncation of protein scaffold becomes significant when scaffold sizes increase, which limits the complexity of synthetic enzymatic systems (Morais et al., 2012).

DNA is a promising scaffold for immobilization of enzymes based on its programmable hybridization rule and predictable hybridized structures (Pinheiro et al., 2011). More importantly, DNA scaffolds provide the unique benefit of site-specific enzyme organization, which could have a significant effect when the diffusion of intermediates is the determining factor for cascade enzymatic reactions (Han et al., 2012; Piperberg et al., 2009; Sun and Chen, 2016). Recently, a DNA-guided approach for enzyme fuel cell applications has been reported in which the mediator ferrocene and glucose oxidase (GOX) were coupled to a gold electrode based on DNA hybridization (Müller and Niemeyer, 2008; Piperberg et al., 2009). By simply switching the positioning of ferrocene on the DNA scaffold, a strong effect on the rate of glucose oxidation was observed. This example demonstrates the importance of using DNA to control the order of assembly for enzyme fuel cell applications.

Motivated by these examples, our goal is to demonstrate the possibility of using site-specific organization of cellulases and GOX onto a synthetic DNA template for the efficient conversion of cellulose to gluconic acid and H_2O_2 (Fig. 1). In our design, three cellulosomal components, CelA (endoglucanase), CelE (exoglucanase), and CBM (cellulose binding module), were used to first convert cellulose to the disaccharide, cellobiose, which was subsequently converted to glucose by the last cellulosomal component, BglA (β-glucosidase) and finally to gluconic acid and H_2O_2 by glucose oxidase (GOX). Although the synergistic effect of synthetic cellulosome on cellulose hydrolysis has been well documented (Tsai et al., 2013a; Tsai et al., 2013b), it was not clear whether a similar beneficial effect could be obtained by channeling glucose between BglA and GOX by DNA-guided assembly. As the current generated by GOX-based fuel cells depends on how fast H_2O_2 is generated (Piperberg et al., 2009), we focused our investigation on the effect of enzyme assembly on enhancing H_2O_2 generation.

2. Materials and methods

2.1. Protein expression and conjugation with DNA linkers

The four cellulosomal components (CelA-ELP-Halo, CBM-ELP-Halo, CelE-ELP-Halo, BglA-ELP-Halo) were constructed as reported. (Sun and Chen, 2016) All four proteins were expressed in *E. coli* BLR in TB medium supplemented with 50 μg/ml kanamycin at 37 °C. The cultures were transferred to 25 °C shaker for overnight leaky expression when the OD_{600} reached 1. Cells were harvested and resuspended in PBS for sonication. The cell debris was removed by centrifugation for 10 min at 4 °C. Two cycles of ELP purification were conducted to purify the proteins from cell lysis. 1 M Na_2SO_4 was added to protein samples to induce inverse-phase transition of ELP. The proteins samples were incubated at 37 °C for 10 min and centrifuged for 10 min in 37 °C. The supernatants were removed and the pellets containing ELP proteins were resuspended in PBS and incubated on ice for resolubilization. Centrifugation at 4 °C was used to remove insoluble proteins in the pellet and the supernatant containing ELP proteins were collected for DNA conjugation.

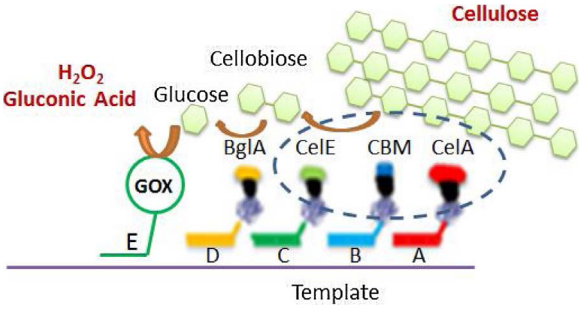

Fig. 1. Schematic of a DNA-guided five-enzyme cascade for direct conversion of cellulose to gluconic acid and H_2O_2.

The DNA linkers were ordered with a 5′ amine group. They were first modified with a chlorohexane (CH) ligand (Promega P675A) for HaloTag attachment by mixing the CH ligand and DNA linkers at a molar ratio of 30:1. The mixture was incubated at room temperature for 4 h. To purify CH-conjugated DNA and remove excessive CH ligands, a 3000 DA ultrafiltration column (Vivaspin 500, Sartorius Stedim Biotech) was used for purification. The purified DNA linkers were then mixed with ELP purified cellulase components for conjugation via HaloTag at a molar ratio of 3:1 overnight at 4 °C. The excessive DNA linkers were removed by ELP purification. 10% SDS-PAGE was used to check the conjugation efficiency.

Glucose oxidase (GOX) from *Aspergillus niger* was purchase from Sigma-Aldrich (G2133). The DNA linker for GOX was modified with a 5′ thiol group. Sulfo-EMCS (Sigma-Aldrich 803235) was used as the cross linker for conjugation. Thiolated DNA linkers at 25 μM were incubated with 25 mM DTT for 2 h at room temperature to reduce possible disulfide linkage between DNA linkers. A 3000 DA ultrafiltration column was used to purify DNA linkers. GOX at 50 μM was mixed with 5 mM sulfo-EMCS in PBS buffer (pH 7.4) at room temperature for 6 h.

A 50 kDa centrifugal column (EMD Millipore, Amicon Ultra-UFC505096) was used to remove the excessive sulfo-EMCS. The treated DNA linkers and purified GOx were mixed at 1:1 molar ratio for 2 h at room temperature. The conjugation efficiency of DNA linker to GOx was evaluated by 8% native gel running at 100 V for 1 h.

2.2. Proteins assembly onto the DNA template

The sequences of DNA template and linkers are list in Table 1 with the corresponding regions in same color coding.

To assemble DNA-conjugated proteins onto the DNA template, the proteins and DNA template were mixed at 1:1 molar ratio for 1 h at room temperature. Binding was detected by electromobility shift assays by running the samples on a 0.8% agarose gel at 90 V for 30 min.

2.3. Measurements of enzyme activities

To evaluate the H_2O_2 production rate from BglA and GOX, 50 nM enzymes were incubated with 20 mM cellobiose (equivalent to 40 mM glucose) and the H_2O_2 produced was detected kinetically at OD570 nm using the glucose oxidase activity assay kit (Sigma MAK097).

To evaluate the glucose production rate from the four cellulosomal components, 1 μM enzymes were incubated with 8 g/l phosphoric acid swollen cellulose (PASC, equivalent to 40 mM glucose). PASC was prepared from Avicel PH101 (Sigma) as previously described (Tsai et al., 2009). The glucose concentration was measured using a glucose (HK) assay kit (Sigma GAHK20) at various time points. The pH of the reaction buffer was adjusted using 100 mM citric acid-sodium citrate buffer.

To measure H_2O_2 production rate from the 5-enzyme system, 1 μM enzymes were incubated with 8 g/l PASC. The H_2O_2 concentration samples were collected periodically measured using the glucose oxidase activity assay kit.

3. Results and discussion

3.1. Protein conjugation with DNA linkers

We have previously demonstrated that CelA (endoglucanase from *Clostridium Thermocellum*), CBM (the carbohydrate binding module from *Clostridium Thermocellum*), CelE (exoglucanase from *Clostridium* Cellulolyticum), and BglA (β-glucosidase from *Clostridium*

Table 1
List of DNA sequences used in this study.

Template	GAGAGTCAGTCAGGAATTTTTAAAGGAGGGAGGGGAATTTTTACAGC GAGCGTCTACATTTTTACACCAGCCAGCCAACTTTTTGATTGACTGCT ACGTATTTTT
LinkerA	AAAAATTCCTGACTGACTCTC
LinkerB	AAAAATTCCCCTCCCTCCTTT
LinkerC	AAAAATGTAGACGCTCGCTGT
LinkerD	AAAAAGTTGGCTGGCTGGTGT
LinkerE	AAAAATACGTAGCAGTCAATC
BlockerA	GAGAGTCAGTCAGGAATTTTT
BlockerB	AAAGGAGGGAGGGGAATTTTT
BlockerC	ACAGCGAGCGTCTACATTTTT
BlockerD	ACACCAGCCAGCCAACTTTTT
BlockerE	GATTGACTGCTACGTATTTTT

Thermocellum) can be assembled on the same DNA template via their corresponding conjugated DNA linkers (Fig. 1) to achieve enhanced hydrolysis of cellulose (Sun and Chen, 2016). In all cases, a self-labeling HaloTag (Los et al., 2008) was used for covalent attachment of chlorohexane (CH)-modified DNA linkers in order to minimize enzyme deactivation. Based on protein sizes (Alzari et al., 1996; Hecht et al., 1993), DNA linkers of 21 base pairs in length were used to assemble the cellulosomal components and GOX onto a 105 bp template.

As reported previously, the four cellulosomal components were fused to an elastin like polypeptides (ELP) tag at the N-terminus for easy purification, and a HaloTag at the C-terminus for specific conjugation to DNA linkers (Fig. 2A) (Blackstock and Chen, 2014; Sun and Chen, 2016). The presence of ELP enabled the easy purification of proteins and protein-DNA conjugates using two cycles of thermal precipitation and resolubilization (Kim and Chen, 2016). After expression and purification of the cellulosomal components via ELP purification (Fig. 2B), 3-fold molar excess of chlorohexane-modified DNA linkers were incubated with the corresponding purified proteins. Two more cycles of thermal precipitation and resolubilization were used to remove excessive DNA linkers. Based on SDS-PAGE analysis (Fig. 2C), the conjugation efficiency was close to 100% in all cases as indicated by the slower migrating DNA-protein conjugates as compared to unconjugated proteins.

Since GOX from *Aspergillus niger* cannot be produced in large quantities in *E. coli* without refolding (Witt et al., 1998), commercial GOX was conjugated with a DNA linker via the well-known sulfo-EMCS chemistry (Xin et al., 2013; You et al., 2011) (Fig. 2D). Size-exclusion centrifugal column was used to remove excessive DNA linkers after conjugation. Again, successful conjugation was confirmed by native gel and the conjugation efficiency was about 100% (Fig. 2E).

3.2. Enhanced H_2O_2 production by assembled BglA and GOX

Since the synergistic effect of cellulosome on cellulose hydrolysis has been demonstrated before (Tsai et al., 2010; Tsai et al., 2009), we first investigated the impact of enzyme assembly on the conversion of cellobiose to gluconic acid and H_2O_2 by BglA and GOX. In this cascade reaction, cellobiose is first converted to glucose, which is sequentially converted to gluconic acid and H_2O_2 by GOX (Fig. 3A). Since the reported K_m for GOX is 10 mM (Karmali et al., 2004), this very low

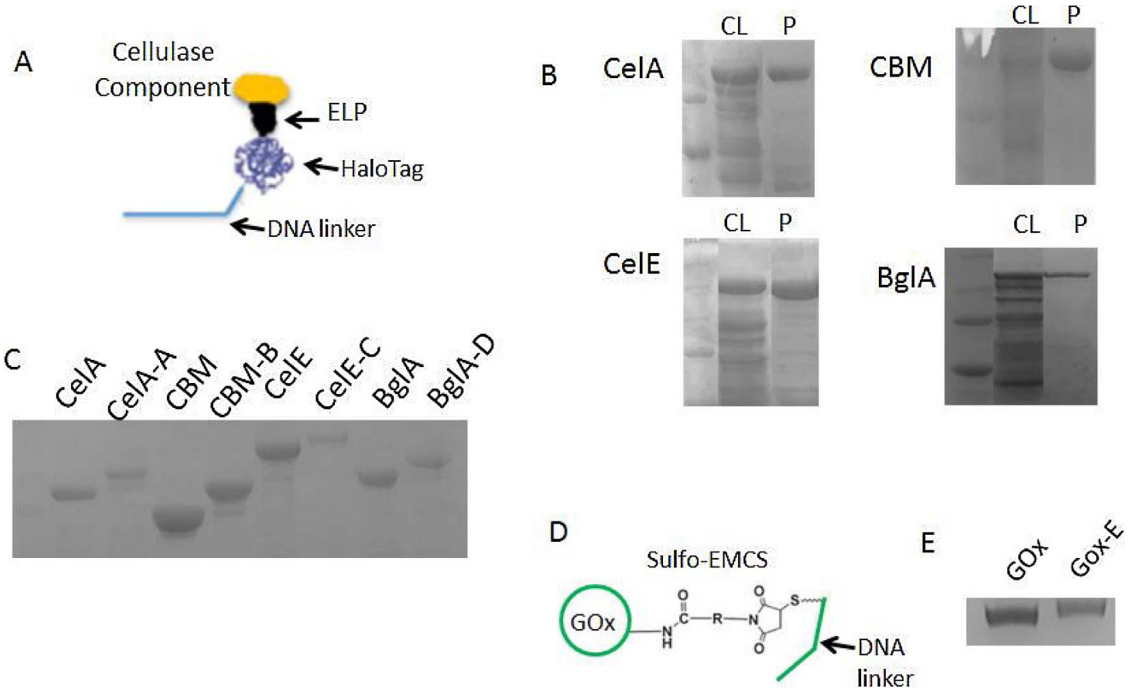

Fig. 2. Protein conjugation with DNA linkers. (A) Conjugation of a DNA linker onto each cellulosomal component using the N-terminus HaloTag. (B) Expression and purification of each cellulosomal component. CL: cell lysate, P: purified protein. (C) Confirmation of DNA conjugation onto the cellulosomal components by 10% SDS-PAGE. Presence of a slower mobility band confirmed the successful conjugation. (D) Conjugation of a DNA linker to GOX using the well-known EDC chemistry. (E) Confirmation of GOX conjugation with a DNA linker by 10% native acrylamide gel.

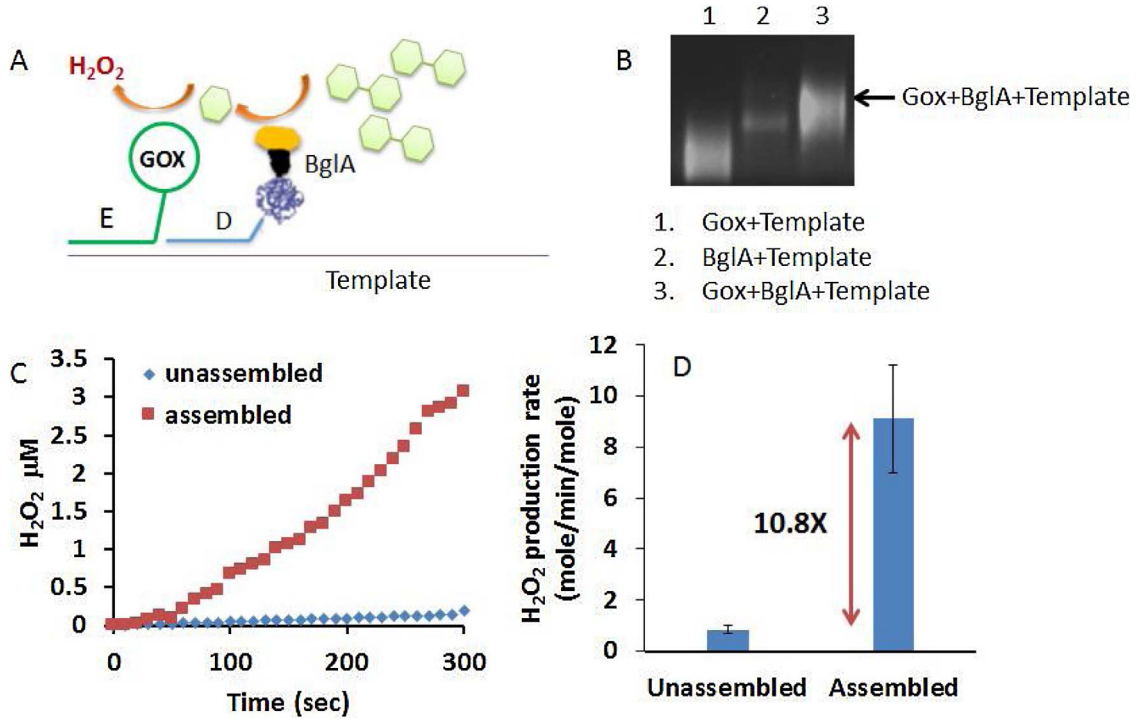

Fig. 3. (A) Production of gluconic acid and H_2O_2 by BglA and GOX. (B) Confirmation of BglA and GOX assembly on agarose gel. (C) H_2O_2 production for either assembled or unassembled BglA and GOX. (D) The rate of H_2O_2 production for either assembled or unassembled BglA and GOX.

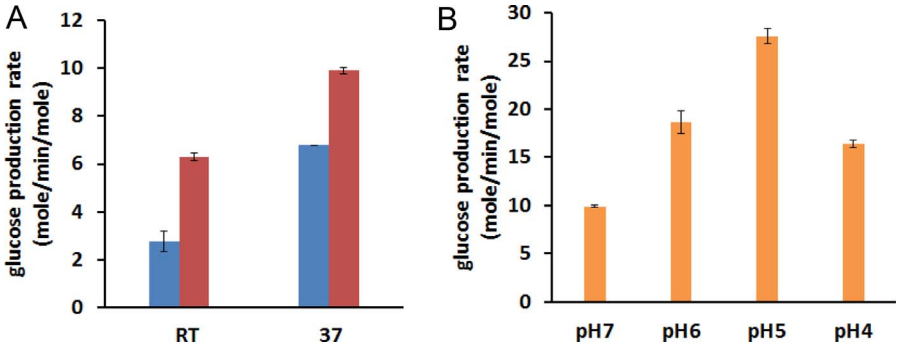

Fig. 4. Effect of temperature (A) and pH (B) on glucose production by either unassembled (blue) or assembled (red) CelA, CBM, CelE and BglA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

affinity toward glucose may significantly limit the cascade reaction due to the very low local concentration of glucose. It is possible that the direct channeling of glucose between BglA and GOX may partially overcome this limitation.

To assess this possibility, the rate of H_2O_2 production was monitored for both assembled and unassembled BglA and GOX. The assembly of DNA-conjugated GOX and BglA onto the same DNA template were first evaluated by agarose gel (Fig. 3B). Due to the co-assembly of BglA and GOX, the resulting complex migrated slower than the single-assembly complexes formed by hybridizing with either BglA or GOX alone. For the control experiment where BglA and GOx were not assembled on the same DNA template, a DNA blocker that is complimentary to the corresponding DNA linker was added to prevent unspecific association of the two proteins and to avoid any potential stabilization effect by hybridization. The rate of H_2O_2 production from cellobiose was monitored using a glucose oxidase activity assay kit (Fig. 3C). Under this condition, barely any H_2O_2 production was detected for the unassembled enzyme mixture, while the production rate was almost 11-fold higher for the assembled BglA/GOx complex (Fig. 3D). This observation highlights the importance of site-specific assembly in controlling enzyme proximity and in enhancing glucose transfer between BglA and GOX for better H_2O_2 production.

3.3. Conversion of cellulose to H_2O_2 by the five-enzyme assembly

After confirming the benefit of enzyme proximity on the conversion of cellobiose, we further investigated the effect of enzyme assembly on the overall conversion of cellulose to gluconic acid and H_2O_2. The optimum reaction conditions for the five-enzyme system were first explored. GOX has a reported reaction rate that is 1000-fold faster than the cellulases used with an optimum reaction condition at pH 5 and 37 °C (Meng et al., 2014; Wong et al., 2008). As GOX is not the limiting step in converting cellulose to H_2O_2, the reaction conditions of the cellulosomal components were compared at room temperature and 37 °C and at pH values from 4 to 7.

Cellulose was supplied as the substrate and the glucose concentration was measured periodically to determine the glucose production rate. While a 2-fold faster glucose production rate was observed for the assembled enzymes at room temperature (Fig. 4A), a result consistent with that reported previously[20], the enhancement was only 1.5-fold at 37 °C. This result is not surprising as the benefit of substrate channeling is the more prominent at lower temperatures with less random diffusion (Wheeldon et al., 2016). The effect of pH was further investigated with the assembled enzymes and the optimum condition was identified at pH 5 (Fig. 4B).

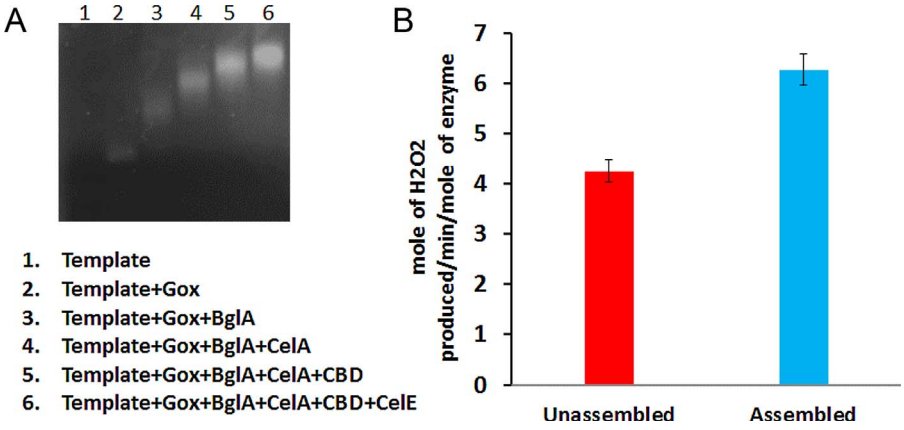

Fig. 5. Enhanced H_2O_2 production from cellulose using an assembled five-enzyme cascade. (A). Confirmation of five-enzyme assembly by agarose gel. (B) Comparison of H_2O_2 production using either the assembled or unassembled five-enzyme system.

Using the optimized conditions, we proceeded to evaluate the conversion of cellulose to gluconic acid and H_2O_2 using the five-enzyme assembly. Assembly of the five proteins onto a single DNA template was evaluated by agarose gel and as expected, the complex migrated much slower when more proteins were assembled on the DNA template (Fig. 5A). After incubating with cellulose, the H_2O_2 concentrations were measured periodically. Consistent with the increase in glucose production, the overall H_2O_2 production rate was improved by 1.5-fold with the assembled enzymes. This improvement is significantly lower than the 11-fold enhancement observed using cellobiose as the substrate for the BglA and GOX bi-enzyme system. One plausible reason is the well-known H_2O_2 inhibition on enzyme activity, which can play a role in reducing the overall reaction rate and activity enhancement by enzyme assembly (Bao et al., 2003). This inhibition effect is further supported by a significantly lower level of glucose produced in 3 h using the five-enzyme cascade (58.4 μM) vs the four-enzyme cascade (233.3 μM). Nevertheless, our results demonstrated the possibility of enhanced production of gluconic acid and H_2O_2 directly from cellulose using a five-enzyme cascade assembled onto a single DNA template. Further optimization is necessary to improve the overall enhancement for more practical applications.

4. Conclusion

In conclusion, we demonstrated the direct conversion of cellulose to gluconic acid and H_2O_2 using a five-enzyme cascade. Site-specific co-localization of BglA and GOX using DNA-guided assembly resulted in over 11-fold improvement in H_2O_2 production, highlighting the benefit of substrate channeling. However, the level of enhancement is substantially less using the five-enzyme cascade, likely the result of activity imbalance and H_2O_2 inhibition. It is expected that the removal of H_2O_2 can reduce the toxic effect on enzyme activities, which can be accomplished by incorporating both an anode (GOX producing electron) and a cathode in the cellulosic fuel cell system (Calabrese Barton et al., 2004; Rasmussen et al., 2016). To match the higher reaction rate of GOX, longer DNA templates can be generated to varying the ratio of cellulases and GOX in order to further fine-tune the overall reaction rate (Sun and Chen, 2016).

Acknowledgement

We would like to acknowledge the funding support from NSF (CBET1264719, CBET1263818, and CBET1604925).

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