High-Yield Cellulosic Hydrogen Production by Cell-Free Synthetic Cascade Enzymes: Minimal Bacterial Cellulase Cocktail and Thermophilic Polyphosphate Glucokinase

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ABSTRACT

Hydrogen production from abundant renewable biomass would decrease reliance on crude oils, achieve nearly zero net greenhouse gas emissions, create more jobs, and enhance national energy security. Cell-free synthetic pathway biotransformation (SyPaB) is the implementation of complicated chemical reaction by the *in vitro* assembly of numerous enzymes and coenzymes. Two of the biggest challenges for its commercialization are: effective release of fermentable sugars from pretreated biomass, and preparations of thermostable enzymes with low-cost.

The hydrolysis performance of 21 reconstituted bacterial cellulase mixtures containing the glycoside hydrolase family 5 *Bacillus subtilis* endoglucanase, family 9 *Clostridium phytofermentans* processive endoglucanase, and family 48 *Clostridium phytofermentans* cellobiohydrolase was investigated on microcrystalline cellulose (Avicel) and regenerated amorphous cellulose (RAC). The optimal ratios for maximum cellulose digestibility were dynamic for Avicel but nearly fixed for RAC. Processive endoglucanase CpCel9 was most important for high cellulose digestibility regardless of substrate type. These results suggested that the hydrolysis performance of reconstituted cellulase cocktail strongly depended on experimental conditions.

Thermobifida fusca YX was hypothesized to have a thermophilic polyphosphate glucokinase. *T. fusca* YX ORF Tfu_1811 encoding a putative PPGK was cloned and the recombinant protein fused with a family 3 cellulose-binding module (CBM-PPGK) was over expressed in *Escherichia coli*. By a simple one-step immobilization, the half-life time increased to 2 h, at 50 ℃. These results suggest that this enzyme was the most thermostable PPGK reported.

My studies would provide important information for the on-going project: high-yield hydrogen production from cellulose by cell-free synthetic enzymatic pathway.

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1. Introduction

1.1 Biofuels

Biofuels are referred to as any solid, liquid, or gaseous fuels that are predominantly produced from biomass or made from biological approaches. In recent years, increasing attention and interests have been paid to the production of transportation biofuels, because an overwhelming reliance on nonrenewable fossil fuels has raised many concerns, such as soaring oil prices, energy security concerns, long-run supply, climate change, and impacts on human health (Hill 2009; Lynd et al. 2003). A switch from nonrenewable fossil fuels to renewable resources would not only significantly decrease the reliance on crude oil and increase national energy security, but it would also decrease net greenhouse emissions and promote rural economy (Demain et al. 2005; Lynd and Weimer 2002; Zhang 2009a).

Renewable biofuels can be classified as first generation biofuels, second generation biofuels, third generation biofuels and fourth generation biofuels, based on their production technologies (Demirbas 2009). First generation biofuels refer to biofuels produced from feedstock like sugar, starch, vegetable oils, or animal fats, using conventional technology. Examples include vegetable oil, biodiesel, biosyngas, and biogas. First generation biofuels produced from food crops such as grains, sugar beet, and oil seeds have their own limitations and have a long way to go in order to achieve targets such as serving as oil-product substitution, mitigating climate change, and promoting economic development. Second generation biofuels are those produced from lignocellulosic materials including cereal straw, forest residues, bagasse, and purpose-grown

energy crops like switchgrasses and short rotation forests. Examples include bioalcohols, bio-oil, bio-DMF, biohydrogen, bio-Fischer-Tropsch diesel and wood diesel. Made from agricultural residues or non-food crop feedstock, the second generation biofuels could avoid many of the concerns facing the first generation biofuels like food and energy conflicts (Hill 2009). Third generation biofuels, also called algal fuels, are made from algae. Together with second generation biofuels, third generation biofuels are called advanced biofuels. Fourth generation biofuels are created using revolutionary processes that defies any other category of biofuels (Demirbas 2009).

A number of biocatalytic or catalytic approaches have been proposed and investigated for the production of various biofuels (Zhang et al. 2010c), such as ethanol (Shaw et al. 2008), high-chain alcohols (Atsumi et al. 2008), alkanes (Huber et al. 2005), fatty acids or their esters (Kalscheuer et al. 2006; Lu et al. 2008), hydrogen (Ye et al. 2009; Zhang et al. 2007; Zhang et al. 2008), methane (Kobayashi et al. 2004), and hydrocarbons (Bond et al. 2010). Among them, cell-free synthetic enzymatic pathway biotransformation exhibits advantages in more than one aspect, and is expected to be an important platform for low-cost biofuels production. It is the implementation of complicated biochemical reactions by in vitro assembling a number of enzymes and coenzymes. Compared to the microbial fermentations, the cell-free synthetic pathway biotransformation can be conducted under broader reaction conditions because most enzymes can be engineered to tolerate relatively severe reaction conditions, such as high temperature, low pH, high organic solvent concentration, etc. In addition, it can be controlled more easily than microbial fermentation because it has a short feedback control loop and a clear control mechanism. What's more, high yield of products can be achieved by circumventing

undesired pathways (Ye et al. 2009; Zhang et al. 2007; Zhang et al. 2010c; Zhang et al. 2008). One of the biggest challenges facing us is the high cost and instability of enzymes and cofactors. To overcome these obstacles, intensive work have been done on developing low-cost enzyme purification technologies (Zhang and Zhang 2010a), recombinant thermophilic enzyme production (Myung et al. 2010; Wang and Zhang 2010; Ye et al. 2010; Zhang 2009b), and enzyme immobilization (Myung et al. 2011).

Although numerous advantages of biofuels exist, there are still some barriers for their development and commercialization. These barriers could result from technology, economy, supply, storage, safety and policy. Even at high oil process, biofuels may not become fully commercial for the several years, without significant additional government support. Considerably more investment in research and development, demonstration and publicity is needed (Demirbas 2009).

1.2 Enzymatic cellulose hydrolysis and cellulase engineering

Cellulose is a polysaccharide consisting of a linear chain of D-glucose units linked by β-1,4-glycosidic bonds, with a degree of polymerization (DP) from 100 to 20,000 . It is the most abundant renewable natural biological resource (Zhang et al. 2006a; Zhang et al. 2006b; Zhang and Lynd 2005; Zhang and Lynd 2006). Production of biobased products and bioenergy from less costly renewable cellulosic materials are significant for the sustainable development of our society.

Enzymatic conversion for biomass utilization is key to economic success of engineered systems, because it can be performed under a moderate reaction conditions, and nearly theoretical yields of sugars are possible. Nevertheless, the enzymatic cellulose hydrolysis is a very slow and complicated biological process. Extensive research has been done to generate an in-depth understanding of the mechanism of enzymatic cellulose hydrolysis process, and a number of mathematical models have been developed (Jalak and Valjamae 2010; Levine et al. 2010; Lu et al. 2006; Wood et al. 1989; Zhang and Lynd 2004; Zhang and Lynd 2006). In general, the enzymatic saccharification of cellulose requires the simultaneous presence and coordinated activity of a number of cellulases and accessory proteins, which is implemented by two different cellulase systems: a non-complexed cellulase system and a complexed cellulase system (cellulosome). Three essential categories of cellulolytic enzymes are: endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Specifically, as shown in Figure 1-1, endoglucanase randomly cuts the accessible intramolecular β-1,4-glucosidic bonds of solid cellulose chains to produce new chain ends; exoglucanases cleaves cellulose chains from reducing and non-reducing ends respectively, to release soluble cellobiose or glucose; and β-glucosidases hydrolyze cellobiose to glucose, eliminating cellobiose inhibition. The primary depolymerization of long solid cellulose chains to short soluble cellodextrins is the rate-limiting step for the overall hydrolysis process (Zhang and Lynd 2004; Zhang and Lynd 2006).

Cellulases are expensive because they must be produced by living systems. As a result, the cost of enzymes for cellulose hydrolysis is a major impediment for an economical and viable bio-based products industry. Extensive research on enzyme optimization, aimed at reducing the cost,

has been actively pursued in private industry, academia, and government laboratories (Banerjee et al. 2010c; Zhang et al. 2006b; Zhang and Lynd 2004). Biotechnology companies, like Novozymes Biotech and Genencor International, have developed technologies that could reduce the cost of cellulase for the cellulose-to-ethanol process from US$5.40 per gallon ethanol to approximately 20 cents (Moreira 2005). Banerjee and coworkers have recently optimized a multi-component synthetic enzyme mixture for improved cellulose hydrolysis performance (Banerjee et al. 2010a; Banerjee et al. 2010b). The two main strategies they took were: (1) an economical improvement in production of cellulase by strain enhancement or process optimization, e.g., introducing alternative inducer system, or using cheaper medium from lactose to glucose; (2) an improvement in cellulase performance by individual component improvement or by reconstituting cellulase cocktails (Knauf and Moniruzzaman 2004; Zhang et al. 2006b).

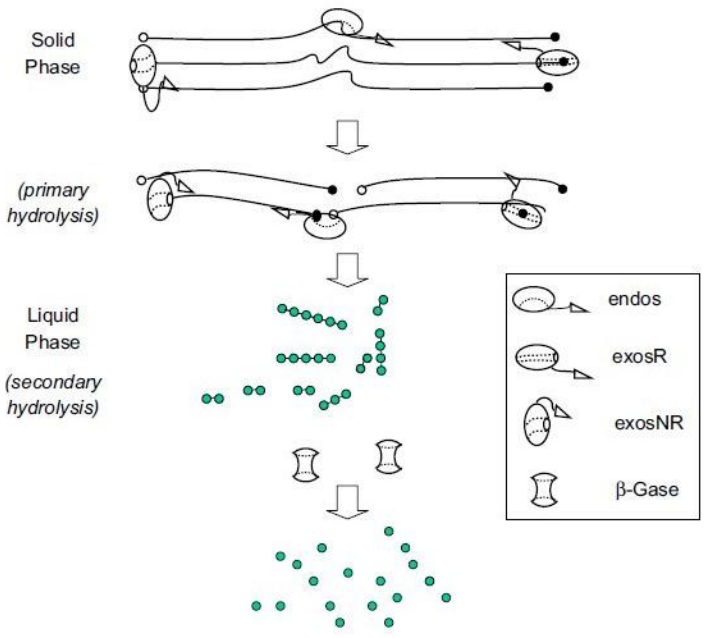

Figure 1-1. Mechanistic scheme of enzymatic cellulose hydrolysis by *Trichoderma* non-complexed cellulase system (Zhang et al. 2006b).

It is important to engineer cellulase enzymes to fit the biorefinery needs, such as higher catalytic efficiency on insoluble cellulosic substrates, increased stability at an elevated temperature or at a broad pH range, and higher tolerance to end-product inhibition. To this end, research endeavors are focusing on mainly three directions as indicated in Figure1-2: (1) improve individual cellulase performance by rational design, based on a deep understanding of cellulase structure as well as catalytic mechanism (Wilson 2004; Zhang et al. 2006b); (2) improve individual cellulase performance by directed evolution, in which the enzymes with improved performance or new features were selected or screened after random mutagenesis and/or molecular recombination (Arnold et al. 2001; Cherry and Fidantsef 2003; Hilbbert et al. 2005; Shoemaker et al. 2003; Tao and Cornish 2002; Zhang et al. 2006b); (3) develop enzyme cocktails with an improved hydrolysis rate or higher cellulose digestibility (Baker et al. 1998; Banerjee et al. 2010a; Banerjee et al. 2010b; Berger et al. 2007; Kleman-leyer et al. 1996; Riedel et al. 1997; Selig et al. 2008; Watson et al. 2002; Zhang et al. 2006b; Zhang and Lynd 2004; Zhou and Ingram 2000).

1.3 Enzyme immobilization

Enzymes are remarkable catalysts capable of performing the most complex chemical processes under relatively benign conditions, with high activity, selectivity and specificity (Schmid et al. 2001). As a result, it is promising to engineer enzymes with desired properties, for industrial applications (Sheldon 2007). As industrial biocatalysts, the enzyme requires, in many instances, its recovery and reuse to make an economically feasible process. Immobilization of enzymes has been revealed as a powerful tool to optimize the operational performance of an enzyme, if

properly designed (Mateo et al. 2007). Immobilized enzymes can be handled more conveniently. For example, it makes it possible to stop the reaction rapidly by removing the solid immobilized enzyme from the reaction solution. Besides, it provides feasibility to enzyme-product separation, which can minimize the protein contamination of the product, and make the enzyme possible to be used in continuous reactors. This technology is especially useful in the food and pharmaceutical industries. A further benefit of enzyme immobilization is the enhanced stability, under both storage and operational conditions (Sheldon 2007). To conclude, improved enzyme performance by immobilization is reflected in enhanced stability, and decreased enzyme cost (Sheldon 2007).

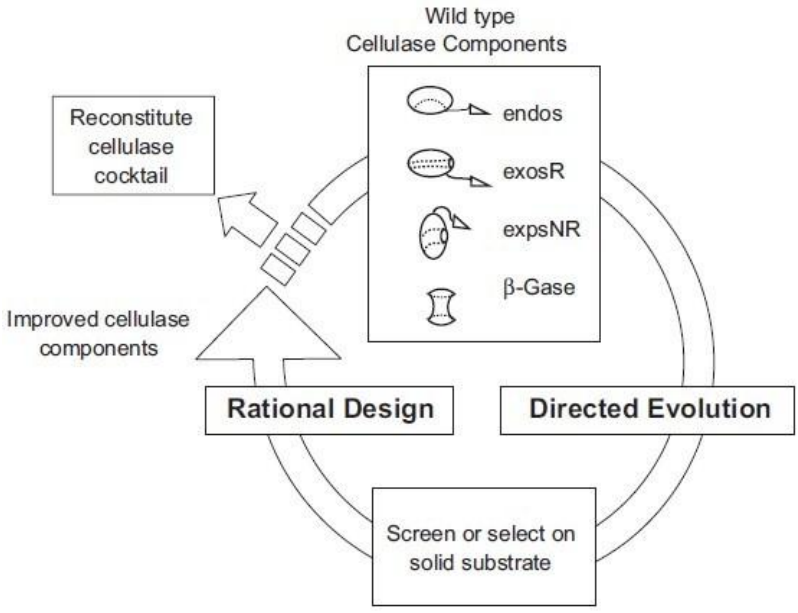

Figure 1-2. Scheme of cellulase engineering for non-complexed cellulases. Endos, endoglucanses; exosR, exoglucanases acting on reducing ends; exosNR, exoglucanases acting on non-reducing ends; β-Gase, β-glucosidase (Zhang et al. 2006b) .

Basically, enzyme immobilization can be achieved by three methods: binding to a support (carrier), entrapment (encapsulation) and cross-linking (Sheldon 2007).

Support binding could be physical (hydrophobic and van der Waals interactions), or chemical (ionic or covalent bonding). Physical adsorption is simple, cheap, and has little or no conformational change of the enzyme or destruction of its active center. However, it is generally too weak to keep the enzyme fixed to the carrier, especially when used in industrial conditions of high reactant and product concentrations and high ionic strength. Ionic or covalent bonding is usually much stronger, which can minimize the enzyme leakage from the carrier. However, ionic or covalent bonding may cause a significant enzyme activity loss, due to exposure of the enzyme to harsh environments or toxic reagents, and if the enzyme is irreversibly deactivated, both the costly enzyme and carrier are unusable (Sheldon 2007).

Entrapment is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. It requires the synthesis of the polymeric network in the presence of the enzyme. Otherwise, the enzyme would probably not be entrapped. Besides, the physical restraints are generally too weak to prevent the enzyme from leaking completely. As a result, additional covalent attachment is often needed (Sheldon 2007).

Cross-linking is based on the formation of covalent bonds between enzyme molecules, using a bi- or multi- functional reagent, leading to three dimensional cross-linked aggregates. The obvious advantages include high stability and low production costs owing to the exclusion of an

additional expensive carrier. However, cross-linking may cause significant changes in the active site of enzymes, and may lead to a significant loss of activity. It is best used in conjunction with one of the other methods, as a means of stabilizing adsorbed enzymes and also for preventing leakage (Sheldon 2007).

1.4 Goals and significance

This study contains two parts:

(1) To investigate the hydrolysis performance of reconstituted ternary cellulase mixtures on microcrystalline cellulose and amorphous cellulose, respectively. The ternary cellulase mixtures are composed of *Bacillus subtilis* endoglucanase Cel5, *Clostridium phytofermentans* processive endoglucanase Cel9, and *Cellobiohydrolase* Cel48. The aim of this project is to develop the optimal ratios of the three enzymes that can achieve maximum glucan digestibility (Chapter 2);

This study would provide instructive information for developing better bacterial enzyme mixtures on pretreated cellulosic materials.

(2) To purify a recombinant polyphosphate glucokinase from *Thermobifida fusca* YX, to characterize its properties and increase its thermostability through immobilization (Chapter 3).

The recombinant polyphosphate glucokinase from *Thermobifida fusca* YX is prepared with an increased thermostability for its potential application in hydrogen production through an in vitro cell-free synthetic enzymatic pathway.

Both studies would provide valuable information for an improvement for the project: High-yield hydrogen production by cell-free synthetic enzymatic pathway.

2. Selection of a minimal set of bacterial cellulases for an expression in a host for consolidated bioprocessing of lignocellulose

2.1 Abstract

The hydrolysis performance of 21 bacterial cellulase mixtures containing the glycoside hydrolase family 5 *Bacillus subtilis* endoglucanase (BsCel5), family 9 *Clostridium phytofermentans* processive endoglucanase (CpCel9), and family 48 *C. phytofermentans* cellobiohydrolase (CpCel48) was studied on partially ordered microcrystalline cellulose (Avicel) and disordered regenerated amorphous cellulose (RAC). Faster hydrolysis rates and higher rates of digestion were obtained on RAC than on Avicel. The optimal ratios for maximum cellulose digestibility were dynamic for Avicel but nearly fixed for RAC. Processive endoglucanase CpCel9 was the most important for high cellulose digestibility regardless of substrate type. These results suggested that the hydrolysis performance of reconstituted bacterial cellulase cocktail with different action mode, strongly depended on experimental conditions, such as substrates type and reaction time. It also provides important information for the construction of a minimal set bacterial cellulases for the consolidated bioprocessing of lignocellulose.

2.2 Introduction

Cellulosic material is one of the most abundant renewable bioresource. Cellulose biodegradation mediated by cellulases and/or cellulolytic microorganisms represents one of the largest material flows in the global carbon cycle (Zhang 2008). The utilization of a small fraction of cellulosic

materials (e.g., 5-10%) for the production of biofuels and value-added chemicals would greatly decrease reliance on crude oil, promote rural economy, decrease net greenhouse emissions, and increase national energy security (Himmel et al. 2007; Lynd and Weimer 2002; Zhang 2008). Cost-effective release of fermentable sugars from non-food biomass through biomass pretreatment/enzymatic hydrolysis is still the largest obstacle to second generation biorefineries (Lynd et al. 2008; Zhang 2008; Zhu et al. 2009a).

Although cellulose $(C_6H_{10}O_5)_n$ is a linear polysaccharide of several hundred or over ten thousand beta-1,4-glucosidic bond linked anhydroglucose units, its enzymatic hydrolysis requires three different action mode cellulase components -- endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase to work together . In general, endoglucanases (EC 3.2.1.4) randomly cut accessible beta-glucosidic-1,4-bonds of cellulose chains, generating new ends for cellobiohydrolases. Two different types of cellobiohydrolases (EC 3.2.1.91) processively act on reducing and non-reducing ends, releasing cellobiose (mainly) from solid cellulose to the aqueous phase. β-glucosidase is responsible for cleaving cellobiose to glucose for eliminating cellobiose inhibition to endoglucanase and cellobiohydrolase.

High cellulase cost remains to be one of the cost-limiting factors for biomass refineries because it is estimated to consume nearly 100-200 grams of cellulase per gallon of ethanol produced (Taylor et al. 2008; Zhu et al. 2009b). To decrease cellulase consumption per gallon of cellulosic ethanol production, intensive cellulase engineering efforts have been made by enhancing individual components using rational design or directed evolution (Lantz et al. 2010; Li et al. 2010; Zhang and Zhang 2011) and reconstitution of different action-mode cellulase components

(cocktail) on cellulosic substrates (Baker et al. 1998; Banerjee et al. 2010c; Irwin et al. 1993; Kim et al. 1998; Rosgaard et al. 2007; Zhang et al. 1999). Novozymes and Genencor have worked on fungal enzyme cocktails involving more than ten cellulase components for improved mass-specific activity on diluted acid pretreated biomass. Recently, Walton and his coworkers reconstituted the fungal cellulase cocktail containing core hydrolases including cellobiohydrolase, endoglucanase, β-glucosidase, endoxylanase, and β-glucosidase, along with accessory enzymes including esterases, proteases, nonhydrolytic proteins, and glycosyl hydrolases, on AFEX-pretreated biomass (Banerjee et al. 2010c). On the other hand, a study of minimal fungal cellulase mixture suggested that some pretreated lignocellulosic biomass was hydrolyzed well by a careful combination of several enzymes (Rosgaard et al. 2007). However, the studies on minimal sets of bacterial cellulases are relatively sparse and the bacterial cellulase cocktail with the performance on Avicel close to that of an integrated Clostridial cellulosome (Bayer et al. 1998a; Bayer et al. 1998b) has not been determined yet in spite of the attempts of constructing an artificial aggregated system (Fierobe et al. 2005). It was found that two noncellulosomal cellulases of Clostridium thermocellum, Cel9I and Cel48Y, hydrolyse crystalline cellulose synergistically and a 2.1 times synergism was determined at 17 – fold excess (ca. 95:5) of noncellulosomal Cel48 over processive Cel9 (Berger et al. 2007).

Microcrystalline cellulose (Avicel) and regenerated amorphous cellulose (RAC) are two model cellulosic materials (Zhang et al. 2006b). Avicel is made from wood pulp by acid hydrolysis that can remove most amorphous cellulose and all hemicellulose, but it still contains a significant fraction of amorphous cellulose (Sathitsuksanoh et al. 2011). RAC is prepared from Avicel through a series of steps: cellulose slurrying in water, cellulose dissolution in concentrated

phosphoric acid, and regeneration in water (Zhang et al. 2006a). As a result, RAC, a completely disordered insoluble substrate, has much larger cellulose accessibility than of Avicel (Hong et al. 2008b) but has the same degree of polymerization when ice-cold concentrated phosphoric acid is used (Zhang and Lynd 2005). Different hydrolysis patterns have been observed on these two substrates by using fungal cellulase (Zhang et al. 2006a). Since most types of pretreated biomass containing amorphous and crystalline cellulose have substrate accessibility ranges falling between the ranges of Avicel and RAC (Table 2-1) (Rollin et al. 2011), the information pertaining to minimal bacterial cellulases performance on Avicel and RAC would be useful for developing better bacterial enzyme mixtures expressed by consolidated bioprocessing bacteria on pretreated cellulosic materials.

In this study, we investigated the synergistic action of a ternary bacterial cellulase component mixture – glycoside hydrolase family 5 *Bacillus subtilis* endoglucanase (BsCel5) (Zhang and Zhang 2011), family 9 *Clostridium phytofermentans* processive endoglucanase (CpCel9) (Zhang et al. 2010a), and family 48 *C. phytofermentans* cellobiohydrolase (CpCel48) (Zhang et al. 2010b) on Avicel and RAC. As was demonstrated, the two noncellulosomal cellulases of *Clostridium thermocellum*, of the families Cel9 and Cel48 have provided efficient hydrolysis of a crystalline cellulose acting in synergism (Berger et al. 2007; Vazana et al. 2010). CpCel9 is validated to be a critical component for microbial cellulose hydrolysis (Tolonen et al. 2009); CpCel48 is among the most active family 48 enzymes, possibly due to its low processivity (Zhang et al. 2010b). The BsCel5, CpCel9, and CpCel48 were thought to have respective functions of fungal *Trichoderma reesei* EG, CBH II, and CBH I (Table 2-2), where CBH I (TrCel7A) and CBH II (TrCel6A) hydrolyze glucosidic bonds close to reducing and non-

reducing ends, respectively. This study of minimal bacterial cellulase cocktails on crystalline cellulose and amorphous cellulose would help develop better enzyme mixtures for pretreated cellulosic materials and create recombinant cellulolytic consolidated bioprocessing microorganisms that can express different cellulase components with proper ratios (Lynd and Weimer 2002; Zhang and Zhang 2010a).

2.3 Materials and Methods

2.3.1 Chemicals. All chemicals were reagent grade or higher, purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Avicel PH105, microcrystalline cellulose, was purchased from FMC (Philadelphia, PA). Regenerated amorphous cellulose (RAC) was made from Avicel as described elsewhere (Zhang et al. 2006a). The *Trichoderma* cellulase (Novozyme® 50013, 84 filter paper units (FPU)/mL) and β-glucosidase (Novozyme® 50010, 270 β-glucosidase U/mL) were gifts from Novozymes North American (Franklinton, NC). Cellodextrins were prepared by hydrolysis of a mixture of concentrated HCl/H_2SO_4 and separated by chromatography (Zhang and Lynd 2003). A broad range protein marker (2-212 kDa) in SDS-PAGE was purchased from New England Biolabs (Ipswich, MA).

2.3.2 Strains and media. *Escherichia coli* DH5α was used as a host cell for DNA manipulation, and *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) were used as the host for recombinant protein expression. The Luria-Bertani (LB) medium was used for *E. coli* cell growth and recombinant protein expression. Ampicillin (100 μg/mL) was added in the *E. coli* media.

2.3.3 Purification of cellulase components. The cellulase cocktails were composed of BsCel5, CpCel9, and CpCel48. The strains *E. coli* BL21 (pET20b-Bscel5), *E. coli* BL21(pET20b-Cpcel9), and *E. coli* BL21(pET20b-Cpcel48) were cultivated in the LB media supplemented with 1.2% glycerol at 37 ºC for expression. 50 μM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the cultures when A_{600} reached ~2.0, and the temperature of the cultures was decreased to 18 ℃ for 16 h. The recombinant BsCel5 and CpCel9 were purified through a simple affinity adsorption on RAC followed by desorption by ethyl glycol (Hong et al. 2008b). The recombinant CpCel48 was purified using nickel-resin column and PD-10 desalting column as described elsewhere (Zhang et al. 2010b).

Table 2-1. A comparison of characteristics of different cellulosic materials.

Substrates	Crl	DP	CAC (m^2/g)	References
Avicel	0.5-0.6	150-500	2.38 ± 0.10	(Hong et al. 2007; Rollin et al. 2011; Zhang et al. 2006b)
BMCC	0.8~0.95	600-2000	33.5 ± 1.5	
Cotton	0.8~0.95	1000-3000	N. A.	
Pretreated cellulosic substrates	0.1-0.7	400-1000	5-30	
RAC	~0	30-200	41.9 ± 2.2	

2.3.4 Hydrolysis of cellulose. Enzymatic hydrolysis of cellulose was carried out at 37 ℃ in 50 mM 2-N-morpholino-ethanesulfonic acid (MES) buffer (pH 7.5) containing 1 mM $CaCl_2$ and 5 g/L RAC (RAC was prepared as 10 g/L sludge, and 500 μL of 10 g/L RAC sludge was added in 1 mL reaction system to reach a final concentration of 5 g/L) or Avicel PH105. Initial reaction

rates for individual enzymes at 10 μg/mL were measured on 1% (w/v) of the substrate (Avicel and RAC) after 20 min hydrolysis. The reactions were terminated by boiling for 5 min. After centrifugation, aliquots of the supernatants were assayed for the release of the reducing sugars as described elsewhere (Dubois et al. 1956; Zhang et al. 2010b; Zhang and Lynd 2005). For the cocktail experiments, total enzyme loadings were 50 mg cellulase mixture/L (i.e., cellulose/cellulase (wt./wt.) = 100) for both bacterial and fungal cellulases. The specific activity of the fungal enzyme was approximately one filter paper unit per mg of protein. *β*-glucosidase was supplemented for eliminating cellobiose inhibition. *β*-glucosidase was supplemented for eliminating cellobiose inhibition. The final concentration of β-glucosidase added to the reaction is 30 U/g glucan, which is sufficient to convert all the soluble oligomers into glucose, according to our preliminary test by HPLC for the hydrolysis products (Sathitsuksanoh et al. 2010). Enzymatic hydrolysis experiments were conducted in a miniaturized digestion apparatus holding 2.0-mL Wheaton glass sample vials. Each cellulase composition was tested in duplicate. The vials were placed in a rack and then the rack was fixed with screws in a rotary shaker. The reactions were then continuously mixed with the shaker at 250 rpm at 37 ℃. At hours 2, 12, and 72, 150 μL aliquots were withdrawn. The samples were boiled for 5 min to stop the reactions. After centrifugation, the soluble sugar concentration in the supernatants was measured by the phenol-sulfuric acid method because it accurately measure primary hydrolysis products regardless of sugar chain lengths. All hydrolysis experiments were conducted in triplicate. Contour plots based on average experimental data with standard deviation of less than 5% were generated by using Origin Pro 8.1 (Northampton, MA) with a ternary contour option. The contour lines in Fig. 2-2 &2-3 represent iso-surfaces fitted to the experimental results.

2.3.5 Other assays. Protein mass concentration was measured by the Bradford assay (Bradford 1976). The purity of protein samples was examined by 10% SDS-PAGE. The soluble cellodextrins released from cellulosic materials were measured by using a Beckman HPLC equipped with a Bio-Rad HPX-42A column (Richmond, CA) at a flow rate of 0.4 mL/min water (Zhang and Lynd 2003).

Table 2-2. Comparison of key cellulase components in fungal and bacterial cellulase systems.

	EC #	Modular structure	Function	Molecular weight (KDa)	Optimum pH	Optimum Temp. (℃)	Reference
Fungal cellulase							
T. reesei cellobiohydrolase I (TrCel7A)	3.2.1.91	GH7-CBM1	Reducing-end acting CBH	54.1	4	60	(Voutilainen et al. 2007)
T. reesei cellobiohydrolase II (TrCel6A)	3.2.1.91	CBM1-GH6	Non-reducing end acting CBH	49.6	5	44	(Wohlfahrt et al. 2003)
T. reesei endoglucanase I (TrCel7B)	3.2.1.4	GH7-CBM1	Random β-bond cleaving EG	48.2	5	60	(Busto et al. 1998)
Bacterial cellulase components							
C. phytofermentans Cel48 (CpCel48S)	3.2.1.91	GH48-Ig-CBM3-His6	Reducing-end acting CBH	98.5	5-6	60	(Zhang et al. 2010b)
C. phytofermentanas Cel9 (CpCel9)	3.2.1.4	GH9-CBM3	Non-reducing end acting processive endoglucanase	104.8	6.5	65	(Zhang et al. 2010a)
B. subtilis Cel5 (BsCel5)	3.2.1.4	GH5 -CBM3	Random β-bond cleaving EG	52.4	6	60	(Zhang and Zhang 2010b)

2.4 Results and discussion

2.4.1 Production and purification of bacterial cellulase components

The recombinant bacterial enzymes -- BsCel5, CpCel9, and CpCel48 -- were produced in *E. coli* BL21 (DE3) harboring the respective plasmid. *E. coli* BL21 (DE3) (pET20b-Bscel5) produced *ca.* 310 mg of soluble BsCel5 per liter of the cell culture and approximately 100 mg BsCel5 was purified with a purification yield of ~ 36%. Approximately 61 and 19 mg of CpCel9 and CpCel48 were purified from per liter of the cell culture of *E. coli* BL21 (DE3) (pET20b-Cpcel9) and BL21 (DE3) (pET20b-Cpcel48), respectively, with similar purification yields of 32%. The purified bacterial cellulases appeared to be homogeneous, examined by SDS-PAGE (Fig. 2-1a). BsCel5, CpCel9 and CpCel48 had apparent molecular weights of 50, 102 and 96 kD, consistent with their estimated molecular weights (52.4, 104.8, and 98.5 kD) based on their deduced amino acid sequences. The specific activities of BsCel5, CpCel9 and CpCel48 were 42.9 ± 0.1, 36.3 ± 1.3, and 4.6 ± 0.1 U/μmole of enzyme on Avicel, respectively. Clearly, all the enzymes exhibited higher activities on RAC than on Avicel (Table 2-3). On RAC, primary hydrolysis products of BsCel5, CpCel9 and CpCel48 were examined by HPLC (Fig.2-1b). Endoglucanase BsCel5 produced cellobiose and cellotriose as two sole major products. Processive endoglucanase CpCel9 was able to produce glucose, cellobiose, cellotriose, and cellotetraose, where cellotetraose was the most dominant product. Cellobiohydrolase CpCel48 produced cellobiose as a major product plus small amounts of cellotriose and cellotetraose. Since primary hydrolysis mediated by endoglucanases and cellobiohydrolases is a rate-limiting step , excess beta-glucosidase was added in the following experiments for eliminating possible influences due to cellobiose inhibition to the cellulase mixtures.

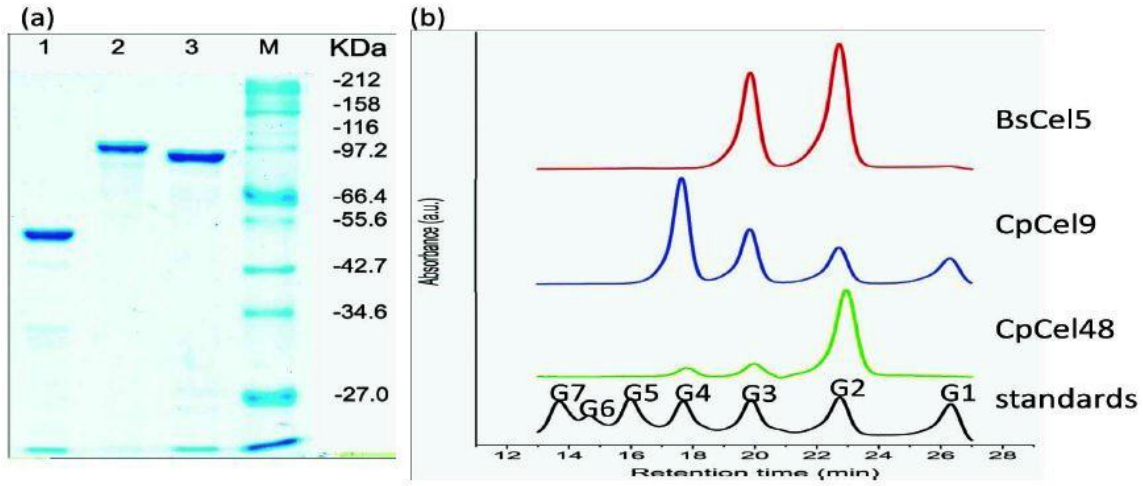

Figure 2-1. SDS-PAGE analysis of purified bacterial enzymes (a): Lane 1, BsCel5; Lane 2, CpCel9; Lane 3, CpCel48; and Lane M, protein makers; and HPLC spectra of the hydrolysis products by BsCel5, CpCel9 and CpCel48 (b).

2.4.2 Hydrolysis with ternary bacterial cellulase mixtures

Ternary mixtures of three cellulases (i.e., CpCel48, CpCel9 and BsCel5) were reconstituted into 21 different compositions. Each corner of the triangle represented the purified cellulase only (100%) without the other two cellulase components; each side represented a mixture of the binary cellulases with a step length of 20% (i.e., six compositions were tested for each binary mixture at the weight ratios of : 100:0; 80:20; 60:40, 40:60, 20:80; 0:100), and the points inside contained a ternary cellulase mixture, where the composition was defined by its triangle coordinates. In a total of 21 compositions, 3 compositions were individual cellulase only at the corners, 12 compositions for binary mixtures in three sides, and 6 compositions for ternary mixture inside the triangle. The total cellulase mass concentration was 50 mg/L, i.e., substrate/enzyme = 100:1, which is close to practical enzyme loadings in cellulosic ethanol biorefineries (Taylor et al. 2008; Zhu et al. 2009b).

The hydrolysis performance of the cellulase cocktails was examined on Avicel (Fig. 2-2) at hour 2, 12 and 72. For this partially ordered microcrystalline cellulose, the contour line patterns changed greatly over time, suggesting that optimal enzyme ratios for maximum cellulose digestibility were dynamic but not fixed, depending on several aspects. This observation was in agreement with the prediction of a functionally-based mathematic model for endoglucanase and exoglucanase (Zhang and Lynd 2006). Regardless of reaction time, the highest digestibility contour lines were observed along the CpCel9 line (binary mixtures between CpCel9 and CpCel48) and the lowest digestibilities were obtained along the CpCel48 side (binary mixtures between BsCel5 and CpCel48). Both results suggested that the family 9 processive endoglucanase was most important in microcrystalline cellulose hydrolysis, in good agreement with the previous *in vivo* and *in vitro* experimental reports (Tolonen et al. 2009; Zhang et al. 2010a). The second most important cellulase component was family 48 cellobiohydrolase CpCel48 at the beginning. Not surprisingly, it was long thought that family 48 exoglucanase would play a central role in crystalline cellulose hydrolysis because these enzymes are the dominant components in microbial cellulase systems (Zhang et al. 2010b) and their expression levels were enhanced when crystalline cellulose was the growth substrate as compared to soluble cellobiose (Raman et al. 2009). The least important cellulase component was BsCel5, although it had the highest specific activity on Avicel (Table 2-3). At hour 72, a second highest rate of digestion zone was observed along the BsCel5 side (Fig. 2-2), suggesting that the key role of CpCel48 might be replaced by BsCel5. This relatively unimportant role of family 48 cellobiohydrolase in crystalline cellulose hydrolysis was partially supported by an *in vivo* Cel48S knock-out experiment for *C. thermocellum* (Olson et al. 2010).

Table 2-3. Enzyme Specific Activity on Avicel PH105 and RAC. (PH=7.5, T=37°C)

Enzyme	Substrate	Specific activity (U/μmole)[a]
BsCel5	Avicel PH105	42.9 ± 0.1
	RAC	105 ± 2.5
CpCel9	Avicel PH105	36.3 ± 1.3
	RAC	95.7 ± 4.3
CpCel48	Avicel PH105	4.6 ± 0.1
	RAC	10.6 ± 0.6

[a)] One unit of enzyme was defined as one μmol of reducing ends generated per min.

For high surface accessibility regenerated amorphous cellulose (RAC), the hydrolysis contour plots of the cellulase cocktails are shown in Fig. 2-3. The contour lines for RAC did not change their patterns significantly, which was different from those of Avicel (Fig. 2-2). The highest digestibility zones were obtained along the BsCel5 side and inside area along this side, suggesting that the best enzyme cocktails contained both BsCel5 and CpCel9 plus an optional CpCel48. The lowest digestibility zones appeared on the CpCel48 corner, suggesting the relatively unimportant role of Cel48 in amorphous cellulose hydrolysis. This result seemed consistent with the fact that Cel48 expression was repressed when amorphous cellulose was used as the carbon source compared to Avicel (Raman et al. 2009). The regions of highest digestibility on RAC were ore than three times larger than those on Avicel PH105, suggesting that it was easier to find out the nearly constant optimized enzyme cocktails for amorphous cellulose than for Avicel.

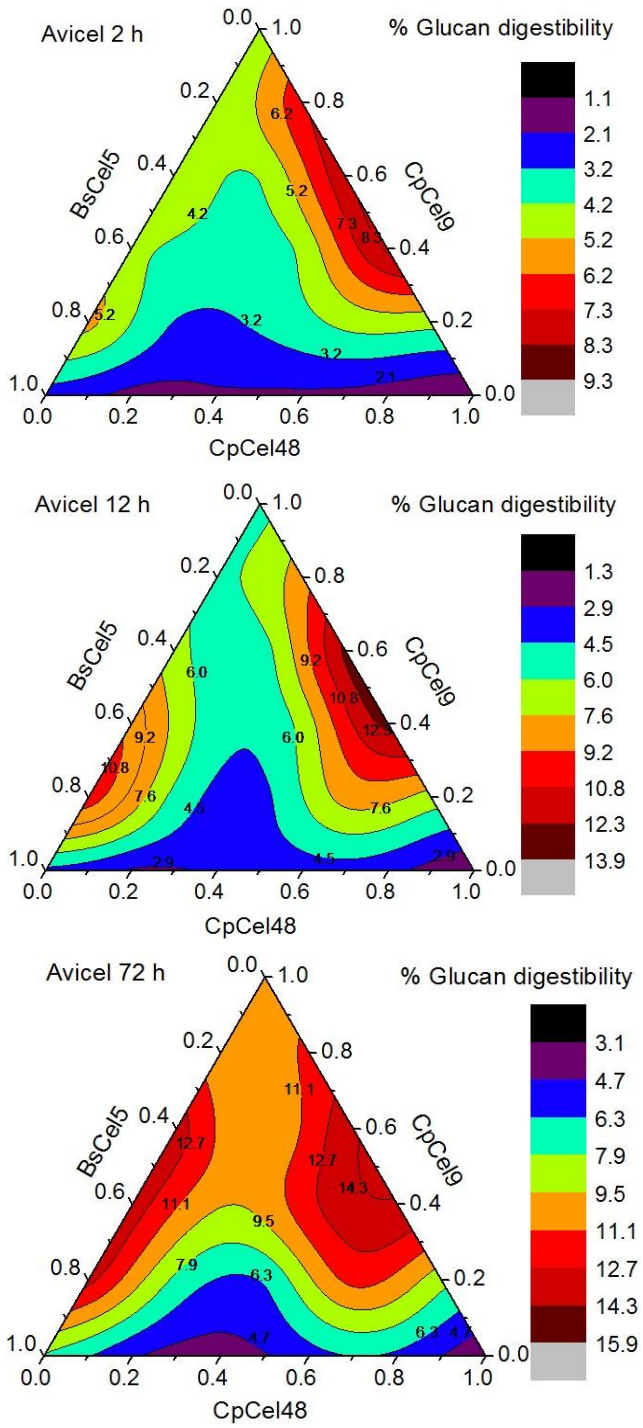

Figure 2-2. Contour plots of enzymatic hydrolysis by ternary bacterial cellulase mixtures (BsCel5, CpCel9 and CpCel48) on Avicel at different times (hour 2, A; hour 12, B; and hour 72, C).

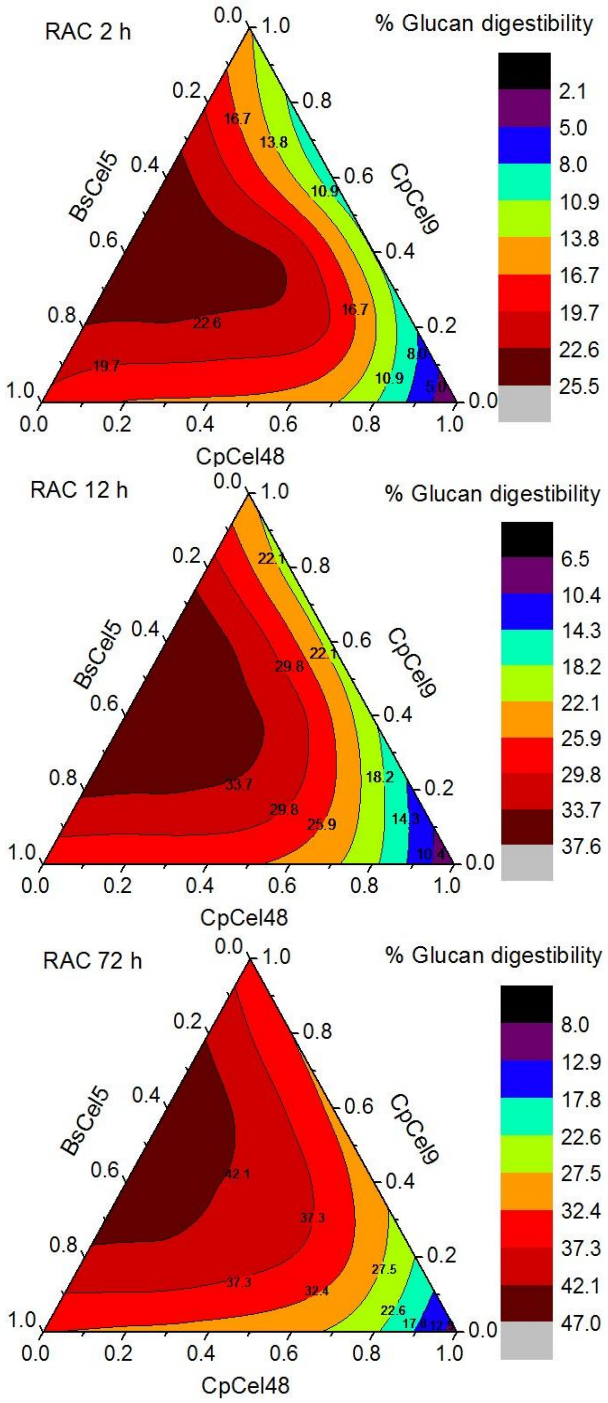

Figure 2-3. Contour plots of enzymatic hydrolysis by ternary bacterial cellulase mixtures (BsCel5, CpCel9 and CpCel48) on RAC at different times (hour 2, A; hour 12, B; and hour 72, C).

2.4.3 Comparison of bacterial cellulase mixtures and a commercial fungal cellulase

Nearly all commercial cellulases are produced by fungi because aerobic fungal fermentation can produce secretory protein levels of more than 100 gram of protein per liter (Zhang et al. 2006b). These cellulase expenditures could range from 20-100 cents per gallon of ethanol produced . But the enzyme expenditures in starch ethanol industry are as low as 2-5 cents per gallon of ethanol, where starch-hydrolyzing enzymes are produced by bacterial *Bacillus* spp. This large difference has raised a question – is it possible to hydrolyze pretreated cellulosic biomass using bacterial cellulase cocktails? Therefore, we compared the hydrolysis performance of the best ratio bacterial enzyme mixture (Cel48:Cel9 = 40:60) (Table 2-4) and a commercial fungal cellulase mixture on Avicel (Fig. 2-2). At the same protein mass concentration, a Cel48 and Cel9 mixture exhibited faster hydrolysis rates and better rate of digestion than any individual cellulase component, indicating the well-known endo/exo synergistic effect. At hour 2, the bacterial enzyme mixture reached a digestibility of 8.4%, slightly higher than that of the fungal cellulase (7.3%). The bacterial cellulase reached a digestibility of 13.9% at hour 12 and leveled off later. In contrast, the fungal cellulase retained most of its hydrolysis ability, and the digestibility was 40.4% at hour 72. The lower digestibility for the bacterial cellulase mixture was not attributed to their denaturation (data not shown).

The best bacterial cellulase mixture (Cel9:Cel5 = 60:40, Table 2-5) was then compared with the fungal cellulase for RAC hydrolysis (Fig. 2-3). Similarly to the Avicel case, the fungal cellulase exhibited faster hydrolysis rates and higher rate of digestion than the bacterial cellulase mixture. The highest digestibility was 95.8% for the fungal cellulase at hour 72, about two times of that of bacterial cellulase (47%). The superiority ratio of the fungal to bacterial cellulase on Avicel (2.6)

was larger than that on RAC, suggesting that cellulase performance evaluation was strongly associated with the substrates tested, as predicted by the functionally-based mathematic model (Zhang and Lynd 2006).

These reconstitution experiments of the minimal bacterial cellulases (Fig. 2-2 and 2-3) clearly suggested that the optimal ratios of cellulase mixtures really depend on the substrates and reaction times. Regardless of substrate type, family 9 processive endoglucanase was the most important because this enzyme was a bifunctional enzyme with endo- and exo-glucanase activities (Zhang et al. 2010a). On partially ordered microcrystalline cellulose, family 48 cellulase was important at the beginning but its importance decreased with an increase in conversion. The changed role of family 48 cellulase in the enzyme cocktails may be explained by a great change in substrate reactivity of Avicel over conversion. For high accessibility amorphous cellulose, family 48 was the least important.

Developing an optimal enzyme mixture depends greatly on substrate characteristics and reaction time. Several commonly-used biomass pretreatments, such as dilute acid and steam explosion, aim to remove hemicellulose in order to break down the linkage among cellulose, hemicellulose and lignin; ammonia-based pretreatments attempt to remove lignin and hemicellulose to achieve a similar disruption (Mosier et al. 2005; Rollin et al. 2011; Zhu et al. 2009a). Although they can increase substrate accessibility to cellulase, post-pretreatment fiber structures and relatively high crystallinity index suggested that common pretreatments cannot break highly-ordered hydrogen bonds in cellulose fibers (Mosier et al. 2005; Sathitsuksanoh et al. 2011). High heterogeneity of pretreated biomass resulted in great challenges in identifying the best ratio of cellulase mixtures

(Banerjee et al. 2010c). In contrast, cellulose solvent-based biomass pretreatment was a recently-developed technology that aimed to dissolve cellulose fibers in cellulose solvents so that the regenerated amorphous cellulose had a much higher substrate accessibility than substrates by common pretreatments (Rollin et al. 2011; Zhu et al. 2009a). High accessibility pretreated biomass had higher digestibilities and faster hydrolysis rates (Rollin et al. 2011; Zhu et al. 2009a), consumed less enzyme use , and may simplify enzyme cocktail formulation (Fig. 2-3).

Table 2-4. Different cellulase compositions for obtaining the top three highest Avicel digestibilities at hour 2, 12 and 72.

Time (h)	Ternary cellulase system (Cel48: Cel9: Cel5)	Glucan digestibility (%)
2	60:40:0	9.3 ± 0.2
	40:60:0	8.4 ± 0.1
	20:80:0	7.5 ± 0.1
12	40:60:0	13.9 ± 0.3
	60:40:0	13.7 ± 0.1
	0:20:80	13.6 ± 0.6
72	0:60:40	15.9 ± 0.6
	0:20:80	15.7 ± 0.1
	40:60:0	15.5 ± 0.5

Comparison of the hydrolysis ability of fungal cellulase with that of bacterial cellulase (Fig. 2-3&2-5) clearly indicated that fungal cellulase exhibited much faster hydrolysis rates than bacterial cellulase at the same temperature and protein mass concentration. But this observation

seemed to conflict with another observation that cellulolytic bacteria can hydrolyze crystalline cellulose faster than fungi (Lynd and Weimer 2002). Such conflicting results may be explained by that the following: (1) some bacterial cellulase components can form complexed cellulase (cellulosome), which increases mass-specific cellulase activity by several-fold (Wei et al. 2009); (2) bacterial cellulase displayed on the surface of microorganisms can exhibited several-fold higher activity compared to non-cellulose-associated enzymes (Lu et al. 2006; Vazana et al. 2010); and (3) some bacterial cellulases can work at much higher

temperatures than commercial fungal cellulase (Lynd and Weimer 2002). To increase mass-specific bacterial cellulase activity, future research directions would be to engineer individual cellulase performance by rational design or directed evolution as well as displaying the linked cellulase complex on the surface of microorganisms (Lynd and Weimer 2002; Zhang and Zhang 2010a). A combination of cellulase engineering and metabolic engineering would help develop industrially-important cellulolytic microorganisms that can produce cellulase, hydrolyze cellulose and ferment desired product in a single step (Lynd and Weimer 2002; Zhang and Zhang 2010a).

Table 2-5. Different cellulase compositions for obtaining the top three highest RAC digestibilities at hour 2, 12 and 72.

Time (h)	Ternary cellulase system (Cel48: Cel9: Cel5)	Glucan digestibility (%)
2	40:40:20	25.5 ± 0.1
	20:20:60	24.7 ± 1.4
	0:40:60	23.7 ± 0.4
12	20:20:60	38.3 ± 0.8
	0:60:40	36.7 ± 0.2
	0:20:80	35.7 ± 0.7
72	0:60:40	47.0 ± 2.5
	0:20:80	44.6 ± 3.2
	0:80:20	43.1 ± 3.6

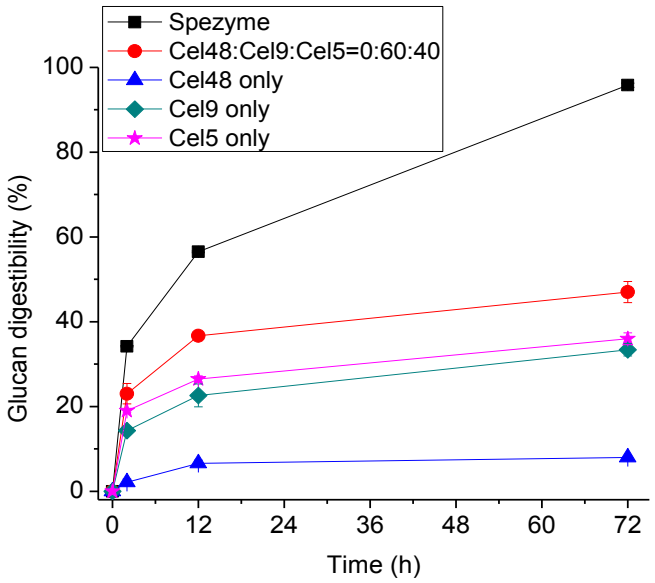

Figure 2-4. Hydrolysis profiles of an optimal bacterial cellulase mixture (CpCel48:CpCel9:BsCel5 = 40:60:0), three individual cellulases, and a commercial fungal mixture on Avicel. All substrate/cellulase weight ratios were 100:1.

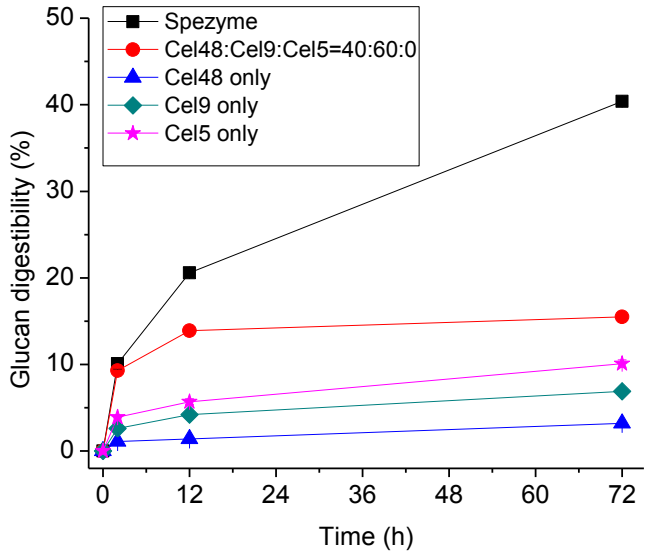

Figure 2-5. Hydrolysis profiles of an optimal bacterial cellulase mixture (CpCel48:CpCel9:BsCel5 = 0:60:40), three individual cellulases, and a commercial fungal mixture on RAC. All substrate/cellulase weight ratios were 100:1.

3. Thermophilic Polyphosphate Glucokinase (PPGK) from *Thermobifida fusca* YX: purification, characterization and immobilization

3.1 Abstract

Discovery of highly active and thermostable polyphosphate glucokinase (PPGK, EC 2.7.1.63) would be vital for cell-free synthetic enzymatic biotransformation, for example, high-yield enzymatic hydrogen production. *Thermobifida fusca* YX was hypothesized to have a thermophilic PPGK with high activity and broad pH range. The *T. fusca* YX ORF Tfu_1811 encoding a putative PPGK was cloned and the recombinant protein fused with a family 3 cellulose-binding module (CBM-PPGK) was over expressed in *Escherichia coli*. Mg^{2+} is an indispensible activator, and the enzyme exhibits highest activity with 4 mM Mg^{2+}, at 55℃, pH 9.0. The k_{cat} and K_m of CBM-PPGK on glucose at pH 9.0, were 128.6 s^{-1} and 0.85 mM, respectively. The half-life time of CBM-PPGK was 15min. By a simple one-step immobilization on a high-capacity regenerated amorphous cellulose (RAC), to form iCBM-PPGK, the half-life time increased to 2h, at 50 ℃. Cross-linking of iCBM-PPGK using 1, 4-benzoquinone further increased the thermostability at the sacrifice of enzyme activity. These results suggest that while this enzyme was the most thermostable PPGK reported, further research work is needed to engineer the enzyme with enhanced thermostability, before becoming an efficient buiding block for in vitro synthetic biology projects (e.g. high-yield cellulosic hydrogen production by cell-free synthetic enzymatic pathway biotransformation).

3.2 Introduction

Poly$(P_i)_n$ is a linear polymer of orthophosphate (P_i) with a degree of polymerization of tens or up to thousands. Poly$(P_i)_n$ is readily formed by dehydration of P_i, and it can be found in abundance in volcanic condensates and deep-oceanic steam vents. Hence, ancient organisms may have utilized poly$(P_i)_n$ instead of ATP in their metabolic reactions without the participation of the ATP-ADP system (Hsieh et al. 1996a; Mukai et al. 2003). Currently, low-cost poly$(P_i)_n$ can be produced by dehydration of phosphoric acid at an elevated temperature or through biological conversion by polyphosphate-accumulating microorganisms in wastewater treatment processes. Being highly stable and cost effective, polyphosphate is considered as an attractive phosphoryl donor for ATP generation, which is aimed to improve the yields of ATP-dependent enzymatic reactions for preparative-scale organic synthesis and biocatalysis (Hoffman et al. 1988; Iwanoto et al. 2007; Resnick and Zehander 2000).

Polyphosphate glucokinase (E.C.2.7.1.63) is responsible for transferring a terminal phosphate group from inorganic polyphosphate (poly$(P_i)_n$) to glucose for yielding glucose 6-phosphate [Eq. (3-1)] (Hsieh et al. 1996a):

$$glu\cos e + poly(P_i)_n \xrightarrow{PPGK} glu\cos e - 6 - phosphate + poly(P_i)_{n-1} \qquad (3\text{-}1)$$

Several polyphosphate glucokinases are reported from different microorganisms. This enzyme was firstly discovered in *Mycobacterium phlei* by Szymona in 1957 (Szymona 1957). Later the enzymes were found in a variety of bacteria, such as *Mycobacterial species (Szymona 1978)*, *Nocardia minima* (Szymona 1979), *Corynebacterium diphtheria* (Szymona 1961), *Propionibacterium shermanii* (Pepin and Wood 1986), *Mycococcus coralloudes* (Gonzales et al.

1990), *Microlunatus phosphovorus (Tanaka et al. 2003), Arthrobacter sp.* (Mukai et al. 2003) and so on (Phillips et al. 1999). However, enzymes produced from the above mentioned sources are limited in pH range, or temperature stability, which restrict them from possible wide-ranging applications in industry (Table 3-3). For example, polyphosphate glucokinase from *Mycobacterium phlei* has very little activity below pH 6.0 and above pH 8.4 (Szymona and Ostrowski 1964); Enzyme from *M. phosphovorus* has an optimum temperature of only 30 ℃ (Tanaka et al. 2003); Enzyme preparations from *Arthrobacter sp.* lost 50% of its activity in as short as 5 minutes, at 40 ℃, pH 7.5 (Mukai et al. 2003).

Hyperthermophiles and thermophiles are great resources for isolation and discovery of thermostable enzymes for potential industrial applications (Adams and Kelly 1998; Myung et al. 2010; Vieille and Zeikus 2001; Zhang 2010a). *Thermobifida fusca* YX is a rod - shaped, aerobic, moderate thermophilic, filamentous, gram-positive soil bacterium that grows optimally at 55 ℃ and has a broad pH range of ~4-10. It degrades plant cell walls in heated organic materials such as compost heaps, rotting hay, manure piles or mushroom growth medium. Its extracellular enzymes, including cellulases and xylanases, have been extensively studied because of their thermostability, broad pH range, and high activity (McGrath and Wilson 2006; Wilson 2004). The complete genomic sequence of *T. fusca* YX has been determined by the Joint Genome Institute of the Department of Energy and has been available since 2007 (Lykidis et al. 2007). The open reading frame (ORF) Tfu_1811 is annotated to encode a putative polyphosphate glucokinase (PPGK). It is expected that the enzyme produced for *T. fusca* YX would have an enhanced activity, stability and a broad pH range.

In this study, the ORF Tfu_1811 putatively encoding *ppgk* from *T. fusca* YX has been cloned, and the recombinant protein was expressed in *E. coli* BL21 Star (DE3), purified, and characterized. To enhance its expression levels and facilitate its immobilization, a family 9 cellulose-binding module (CBM3) was added on its N-terminal. As a result, the recombinant protein was purified as a fusion protein, through a simple affinity adsorption on regenerated amorphous cellulose (RAC). The immobilized PPGK through one-step purification and immobilization exhibited enhanced stability as compared to the free enzyme. This enzyme could have great potential in cell-free systems replacing costly ATP-generating substrates.

3.3 Materials and methods

3.3.1 Chemicals and strains. All chemicals were reagent grade, and purchased from Sigma (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. Restriction enzymes, Taq DNA polymerase, T4 ligase, and a broad range protein marker (2-212 kDa) were purchased from New England Biolabs (Ipswich, MA). Microcrystalline cellulose – Avicel PH105 – was purchased from FMC (Philadelphia, PA, USA). Regenerated amorphous cellulose (RAC) with a high external binding capacity was prepared from slurrying in distilled water, dissolution in H_3PO_4, and followed by regeneration in water as described elsewhere. *Thermobifida fusca* YX genomic DNA was received as a gifted from Dr. David Wilson at Cornell University (Ithaca, NY). *E.coli* DH5α was used as a host cell for DNA manipulation; *E.coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) was used as a host strain for recombinant protein expression. The Luria-Bertani (LB) medium with 100 μg/mL ampicillin was used for *E. coli* cell growth and recombinant protein expression.

3.3.2 Plasmids construction. Plasmids pC-ppgk, pCI-ppgk expressing the fusion protein containing cellulose binding module (CBM)-PPGK, and (CBM)-intein-PPGK were constructed by replacing the *gfp* gene fragment in plasmids pCG, and pCIG with the *T. fusca ppgk* gene (ORF Tfu1811), respectively(Figure 3-1). The *T. fusca ppgk* DNA fragment was amplified by PCR using a forward primer (5'-GCATC CTCGAG ATGGCATCTC GGGGACGGGT -3', XhoI site underlined) and a reverse primer (5'-GCTAT GGATCC TCAGGCAGAG ACCCGGTCAC, BamHI site underlined) based on the *T. fusca* genomic DNA. The PCR product after double digestion by XhoI/BamHI was ligated with the XhoI/BamHI-digested pCG, pCIG vectors for plasmids pC-ppgk, pCI-ppgk, respectively. The plasmids sequence was validated by DNA sequencing (VBI, Virginia Tech).

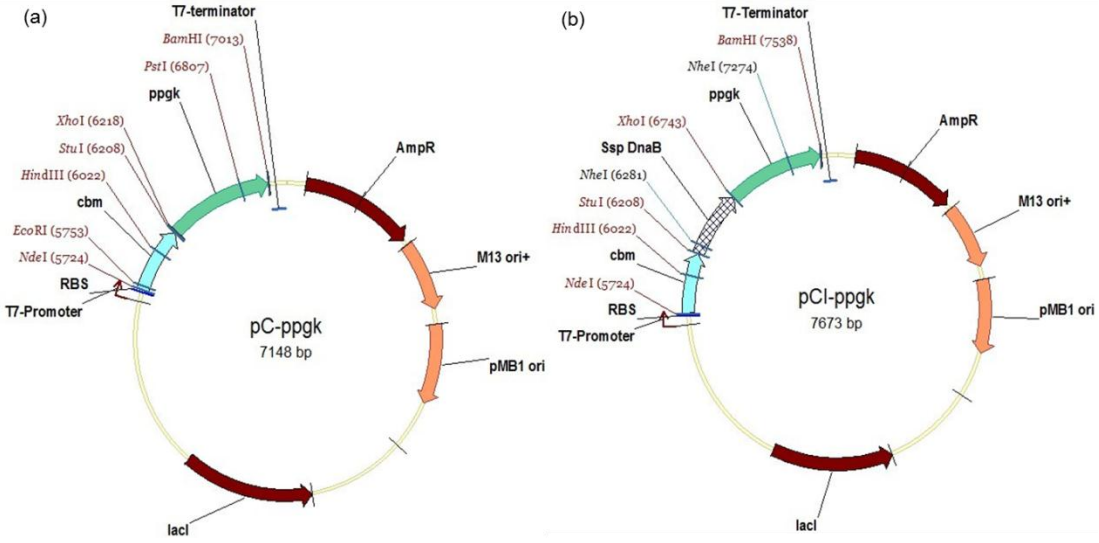

Figure 3-1. Construction of the protein expression plasmid (a) pC-ppgk; (b) pCI-ppgk.

3.3.3 Recombinant protein expression and purification. The plasmids pC-ppgk, and pCI-ppgk were transformed into *E. coli* BL21 (DE3), yielding *E. coli* BL21 (pC-ppgk), and *E. coli* BL21

(pCI-ppgk), respectively. The strains *E. coli* BL21 (pC-ppgk) or *E. coli* BL21 (pCI-ppgk) were cultivated in 200 mL of Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin at 37 ℃, with a rotary shaking rate of 250 rpm. When A_{600} reached ~0.8, 100 μM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the cultures, and the temperature of the cultures was decreased to 18 ℃ for an additional 16 h.

For purification of CBM-PPGK, the cells were harvested by centrifugation at 4 ℃, washed twice with 50 mM Tris-HCl buffer (pH 7.5), and resuspended in 20 mL of 50 mM Tris-HCl buffer (pH 8.5). The cells pellets were lysed by Fisher Scientific Sonic Dismembrator Model 500 (3-s pulse, total 300s, at 50% amplitude) in an ice bath. After centrifugation, the supernatant of cell lysate was used for purification through affinity adsorption on RAC followed by ethylene glycol elusion. Then, 5 mL of 10 g/L RAC was mixed with 20 mL of the cell lysate at room temperature for 15 min followed by centrifugation. The RAC pellet was suspended in 20 mL of 50 mM Tris-HCl buffer (pH 8.5) to remove contaminating proteins in the RAC matrix. After centrifugation, the RAC pellet containing the adsorbed CBM-PPGK was suspended in 4 RAC pellet volumes of 100% ethylene glycol , (i.e., the final EG concentration was ~ 80% v/v). After centrifugation, the purified CBM-PPGK was collected from the supernatant.

For purification of CBM-intein-PPGK, the cells were harvested by centrifugation at 4 ℃, washed twice by 50 mM Tris-HCl buffer (pH 7.5), and resuspended in 20 mL of 50 mM Tris-HCl buffer (pH 8.5). The cells were lysed by Fisher Scientific Sonic Dismembrator Model 500 (3-s pulse, total 300s, at 50% amplitude) in an ice bath. After centrifugation, the supernatant of cell lysate was used for purification through affinity adsorption on RAC followed by intein self-

cleavage. Then, 5 mL of 10 g/L RAC were mixed with 20 mL of the cell lysate at room temperature for 15 min, and followed by centrifugation. The RAC pellet was suspended in 20 mL of 50 mM Tris-HCl buffer (pH 8.5) to remove contaminating proteins in the RAC matrix. After centrifugation, the RAC pellet containing the adsorbed CBM-intein-PPGK was washed with 20 mL of 50 mM HEPES buffer (pH 6.5). After centrifugation, the RAC pellet containing the adsorbed CBM-intein-PPGK was suspended with 5 mL of 50 mM HEPES buffer (pH 6.5) at room temperature overnight for intein self-cleavage. After centrifugation, the cleaved CBM-free PPGK was collected from the supernatant.

3.3.4 Enzyme immobilization. Two immobilization methods were tested: physical adsorption and chemical cross-linking between support and enzyme. Regenerated amorphous cellulose (RAC) was used as a support. For preparation of RAC-bonded CBM-PPGK (iCBM-PPGK), the cell lysate was mixed with RAC at a ratio of ~300 mg of CBM-PPGK per gram of RAC added, followed by 15-min incubation at room temperature. The pellets were washed twice using 50 mM HEPES buffer (pH 7.5) followed by centrifugation. The washed pellets were suspended in 50 mM HEPES buffer (pH 7.5) containing 4 mM Mg^{2+}, producing the iCBM-PPGK slurry. For preparation of cross-linked iCBM-PPGK (crCBM-PPGK), 5mM 1,4-Benzoquinone was added to the iCBM-PPGK slurry followed by an 1-h incubation at room temperature. The pellets were washed twice in 50 mM HEPES buffer (pH 7.5) followed by centrifugation. The washed pellets were suspended in 50 mM HEPES buffer (pH 7.5) containing 4 mM Mg^{2+}, producing crCBM-PPGK slurry.

3.3.5 Activity assays. The enzyme activities were measured by using a discontinuous approach since thermophilic glucose-6-dehydrogenase was not available. The initial reaction rates of PPGK were measured based on the generation of G6P from the substrate (Eq. 1). The formation of G6P was followed indirectly by monitoring the amount of NADH formed ($\varepsilon 340 = 6220\ M^{-1}\ cm^{-1}$), at 340 nm spectrophotometrically (Eq. 3-2).

$$G6P + NAD^{+} \xleftrightarrow{G6PDH} 6-phosphogluconate + NADH \qquad (3\text{-}2)$$

The PPGK activity was measured in a 50 mM HEPES buffer (pH 7.5) containing 4 mM Mg^{2+}, 5 mM D-glucose, and 1 mM polyphosphate (17 mM P_i in total) or 5 mM ATP, at 50 ℃ for 5 min. The reactions were stopped by boiling for 3 min. The G6P amount in the product was measured in 50 mM HEPES buffer, containing 1 mM NAD and 0.5 U/mL glucose-6-phosphate-dehydrogenase, at room temperature for 15 min. One unit of polyphosphate glucokinase activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of G6P/min.

3.3.6 Optimization of CBM-PPGK reaction conditions. As an activator, the concentration of magnesium ion, was optimized on CBM-PPGK activity. The effects of different pH on the enzyme activity were tested at 37 ℃, in 50 mM buffers of citric acid/sodium citrate (pH 4.0, 5.0, and 6.0), HEPES buffer (pH 7.0, 7.5, 8.0), Glycine-NaOH (pH 8.5, 9.0, 9.5, 10.0, 10.5, and 11). The temperature effects on PPGK activities were tested from 20 ℃ to 80 ℃.

3.3.7 Enzyme kinetics. The kinetics of the enzyme was determined based on the initial velocities of PPGK. The reactions were conducted in a 50 mM HEPES buffer (pH 7.5), or 50 mM Glycine-NaOH (pH 9.0), containing 4 mM Mg^{2+}, 0.5 mM polyphosphate, and varing concentration of D-glucose (0.2-5mM), at 50 ℃.

3.3.8 Thermostability. The thermostability of the purified CBM-PPGK, RAC immobilized CBM-PPGK (iCBM-PPGK), and 1,4-Benzoquinone-cross-linked iCBM-PPGK (crCBM-PPGK) was studied in 50 mM HEPES buffer (pH 7.5) containing 4mM Mg^{2+} and 1mg/ml bovine serum albumin (BSA) at 50℃. The residual PPGK activity was measured according to the PPGK activity assay as described above.

3.3.9 Other assays. Mass concentration of soluble protein was measured by the Bio-Rad modified Bradford protein kit with bovine serum albumin (BSA) as a standard protein, as described elsewhere (Zhang and Lynd 2002). The purity of the enzymes was examined by 10% SDS-PAGE in Tris-glycine buffer.

3.4 Results

3.4.1 Identification of putative PPGK by using sequence analysis

Tfu_1811 from *T.fusca* YX was designated to encode a putative polyphosphate glucokinase (PPGK). The deduced amino acid of PPGK has molecular mass of 27,800.

Homology analysis of protein sequence of this putative PPGK was performed by using BL2SEQ tool in Biology Workbench (http://workbench.sdsc.edu/) as compared with known PPGKs. The deduced 262 amino acid sequence was found to be homologous with the primary structures of the polyphosphate glucokinase from *Microlunatus phosphovorus (Tanaka et al. 2003)* (61% identity over 255 amino acids), *Arthrobacter sp.* KM (Mukai et al. 2003; Mukai et al. 2004) (59%

identity over 244 amino acids), *Propionibacterium shermanii* (Pepin and Wood 1986) (58% identity over 250 amino acids), and *Mycobacterium tuberculosis* H37Rv (Hsieh et al. 1996a; Hsieh et al. 1993; Hsieh et al. 1996b) (55% identity over 249 amino acids).

Multiple-sequence alignment of this putative *T. fusca* YX PPGK and its homologs clearly suggests that the key amino acid sequences responsible for substrate binding and catalysis are highly conserved (Figure 3-2). Five regions were suggested to be common motifs interacting within the vicinity of the ATP molecule. They are 'phosphate-1' and 'phosphate-2' motifs contacting the β- and γ-phosphates of ATP, the 'connect-1' and 'connect-2' motifs which are the two hinge-regions at the interface between the subdomains, and an 'adenosine' motif which contacts the adenine ring of ATP. 'Glucose' was suggested to be responsible for the binding of glucose. 'Phosphate-3' region was suggested to be responsible for the binding of polyphosphate. In the phosphate-3 region of the primary structure of polyphosphate glucokinase from *M. tuberculosis* H37Rv, Trp 193, Trp 198, and several charged groups around this region (Lys 188, Glu 189, Lys 190, Asp 192, Lys 197, and Lys 200) have been reported to be important for the binding of polyphosphate (Mukai et al. 2003; Phillips et al. 1999). Through alignment, high similarity was recognized in the phosphate-3 region between the primary structure of polyphosphate glucokinase from *T. fusca* YX and other homologous proteins. For example, Trp 198, whose site-directed mutagenesis resulted in complete loss of catalytic activity, was completely conserved between *T. fusca* YX, *M.* phosphovorus, *P.shermanii*, *M. tuberculosis* H37Rv.

3.4.2 Expression and purification of polyphosphate glucokinase

The fusion protein CBM-PPGK was overexpressed in *E. coli* BL21 (DE3) at 18℃ after 0.1 mM IPTG induction. The overexpressed protein in the crude extract is clearly shown in Lane 1 in Figure 3-3. Most of the protein is soluble in the supernatant as shown in Lane 2. The fusion protein was specifically bound on a high-binding-capacity cellulosic adsorbent. After elution with 80% ethyl glycol, the purified protein was obtained as a single band as shown in lane 4 of Figure 3-3. Approximately 22.8 mg of the purified CBM-PPGK was obtained per liter of fermentation broth with a yield of 24.9% (Table 3-1). The enzyme exhibits a specific activity of 64.6 U/mg on polyphosphate at 50 ℃, pH 7.5, while it has only a minor activity of approximately 3.2 U/mg, when 5 mM ATP is used as substrate instead of polyphosphate. It suggests that the CBM-PPGK is a polyphosphate-dependent enzyme.

The expression level of CBM-intein-PPGK is much lower than that of CBM-PPGK, suggesting that an insertion of ‘intein’ may be toxic to the protein, which is different from our previous work (Hong et al. 2008a; Wang and Zhang 2009).

Table 3-1. The CBM-PPGK purification from 250 mL of the *E.coli* cell culture (Measured at 50 ℃, 50 mM HEPES (pH 7.5), 4 mM Mg^{2+}).

Fraction	Vol. (mL)	Protein (mg/mL)	Total Protein (mg)	Sp. Act. (U/mg)	Total act. (U)	Yield (%)	Purif. fold
cell lysate	23	3.2	73.6	20.1	1479.4	100	1
Purified protein	5	1.14	5.7	64.6	368.2	24.9	3.2

Sp. Act., specific activity

Figure 3-2. Multiple-sequence alignment of the primary structure of PPGK from *Thermobifida fusca* (YX) and that of PPGKs from different organisms using Clustalw (1.81) tool in Biology WorkBench . Seven conserved regions, the phosphate-1, phosphate-2, phosphate-3, connect-1, connect-2, glucose and adenosine regions, are enclosed in boxes. The symbol '*' indicates single, fully conserved residue; ':' indicates conservation of strong groups; '.' indicates conservation of weak groups; ' ' indicates no consensus. Trp 193 and Trp 198 in the phosphate-3 region of PPGK from *M.tuberculosis* H37Rv are underlined. The amino acid residues aligned with Trp 193 and Trp 198 are shaded.

3.4.3 Biochemical characterization

The molecular mass of the CBM-PPGK is 45, 000 based on the deduced amino acid sequence. It is close to the estimated molecular mass of the target protein from 10% SDS-PAGE (Figure 3-3).

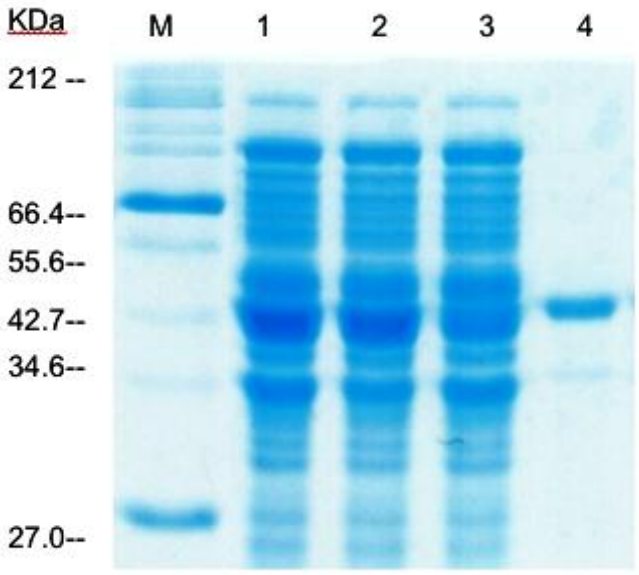

Figure 3-3. 10% SDS-PAGE analysis of of CBM-PPGK purification. M: protein markers; 1: CBM-PPGK total protein; 2: CBM-PPGK supernatant; 3: CBM-PPGK after RAC adsorption; 4: CBM-PPGK by EG elution (1 μg).

Generally, Mg^{2+} is an indispensible cofactor for the activity of CBM-PPGK. The effect of different concentrations of Mg^{2+} on the enzyme activity is shown in Figure 3-4a. Initially, the enzyme activity increases with the increase of Mg^{2+}, and the activity reaches a maximum when the Mg^{2+} concentration reaches to 4 mM. After that, an additional increase of Mg^{2+} did not further increase of the activity, instead, it inhibited the enzyme activity.

The pH profile of PPGK activity is shown in Figure 3-4b. The effects of different pH on the enzyme activity were tested at 37 ℃, in 50 mM buffers of citric acid/sodium citrate (pH 4.0, 5.0, 6.0), HEPES buffer (pH 7.0, 7.5, 8.0), Glycine-NaOH (pH 8.5, 9.0, 9.5, 10.0, 10.5, 11). The enzyme had the maximum activity at pH 8.5-9.0, and was relatively active at pH 7.0-7.5 (~ 60% activity).

The effects of temperature on PPGK activity over a range of 20-80 ℃, at pH 9.0 is shown in Figure 3-4c. The optimum temperature of PPGK was found to be around 55 ℃, which is higher than that of PPGK from other organisms ever reported.

The kinetic constants of the enzyme acting on glucose are presented in Table 3-2. The K_m and k_{cat} values of CBM-PPGK on glucose were 0.85 mM and 128.6 s^{-1} (50 ℃, pH 9.0), respectively. The iCBM-PPGK had similar kinetic characteristics of CBM-PPGK, and both of them exhibited approximately 3-fold k_{cat} values when the temperature increased from 30 to 50 ℃, at pH 7.5. When in pH 9.0, both CBM-PPGK and iCBM-PPGK showed an increase in k_{cat} values. The above results suggested that the immobilization on RAC did not change the enzyme properties greatly.

3.4.4 Thermostability

The half-life time of CBM-PPGK at 50 ℃ is independent of its mass concentration (Figure 3-5). The half-life time of thermo-inactivation was ca. 15 min, for both 0.01 g/L and 0.1 g/L enzyme solutions. The iCBM-PPGK exhibited a prolonged half-life time of ca. 2 h, for both 0.01 g/L and 0.1 g/L enzyme solution, as compared to those of free CBM-PPGK by 8-fold.

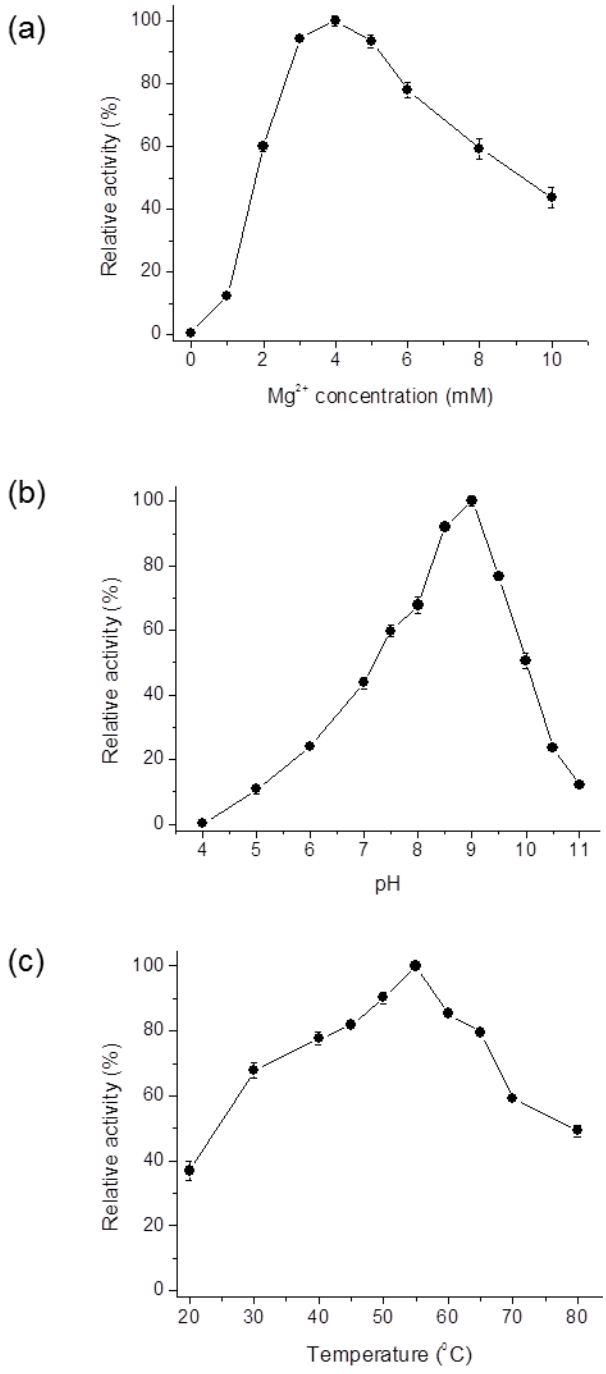

Figure 3-4. (a) Effect of Mg^{2+} concentration on the CBM-PPGK activity; (b) Effect of pH on the CBM-PPGK activity; (c) Effect of temperature on the CBM-PPGK activity.

Table 3-2. The kinetic characteristics of CBM-PPGK and iCBM-PPGK.

Enzyme	pH	Temp. (℃)	$K_{(Glucose)}$		
			K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($10^3 M^{-1} s^{-1}$)
CBM-PPGK	9.0	50	0.85 ± 0.03	128.6 ± 2	151
	7.5	50	0.77 ± 0.03	96.9 ± 6	126
	7.5	30	0.45 ± 0.01	39.7 ± 0.1	88.5
iCBM-PPGK	9.0	50	0.94 ± 0.1	135.6 ± 4	145
	7.5	50	0.80 ± 0.02	92.6 ± 1	116
	7.5	30	0.52 ± 0.01	33.6 ± 1.2	64.4

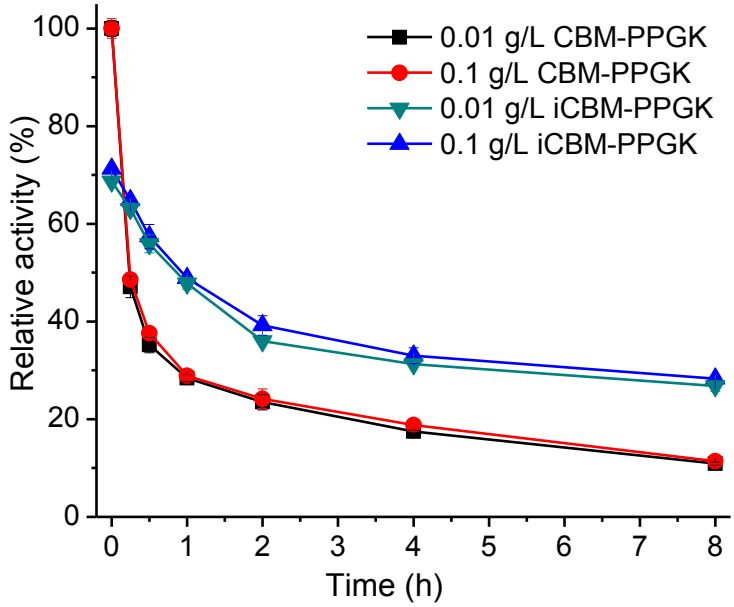

Figure 3-5. Thermostability of CBM-PPGK and iCBM-PPGK. (Enzymes are incubated at 50 mM Hepes (pH 7.5), with 4 mM Mg^{2+} added, and 1mg/mL BSA solution, at 50 ℃.)

In an attempt to further increase the enzyme thermostability, a cross linking was performed between RAC and CBM-PPGK using 1,4-benzoquinone. The effect of different concentrations of 1,4-benzoquinone (5 mM, 10 mM, 25 mM, 50 mM) on the enzyme activity was tested. It was found that with the increase of the concentration of 1,4-benzoquinone, the enzyme activity decreased significantly (Figure 3-6a). As a result, 5 mM 1,4-benzoquinone was chosen for the cross linking, to prepare crCBM-PPGK. The crCBM-PPGK prolonged the half-life time of the enzyme significantly. It was found that 50% of the enzyme activity remained after 12-h incubation at 50 ℃. However, there is a significant loss of the enzyme activity due to cross-linking (~ an additional 75% activity lost, Figure 3-6b). This result shows that a significant optimization of the conditions (e.g. cross linker concentration, immobilization temperature and time) for cross linking are needed in order to get a best result that could balance between the loss of enzyme activity and the increase of enzyme thermostability.

3.5 Discussion

A putative PPGK from mesophilic bacterium *T.fusca* YX was cloned, heterologously expressed in *E.coli* BL21 (DE3) and purified. The experimental data confirmed that the ORF Tfu_1811 encoded an enzyme that is capable of yielding glucose-6-phosphate by transferring a terminal phosphate group from inorganic polyphosphate (poly(P_i)$_n$) to glucose. The enzyme had a high specific activity of 64.6 U/mg at 50 ℃, pH 7.5, while it exhibited only a minor activity when ATP is used as substrate instead of polyphosphate. It suggested that the enzyme is a polyphosphate-preferred enzyme.

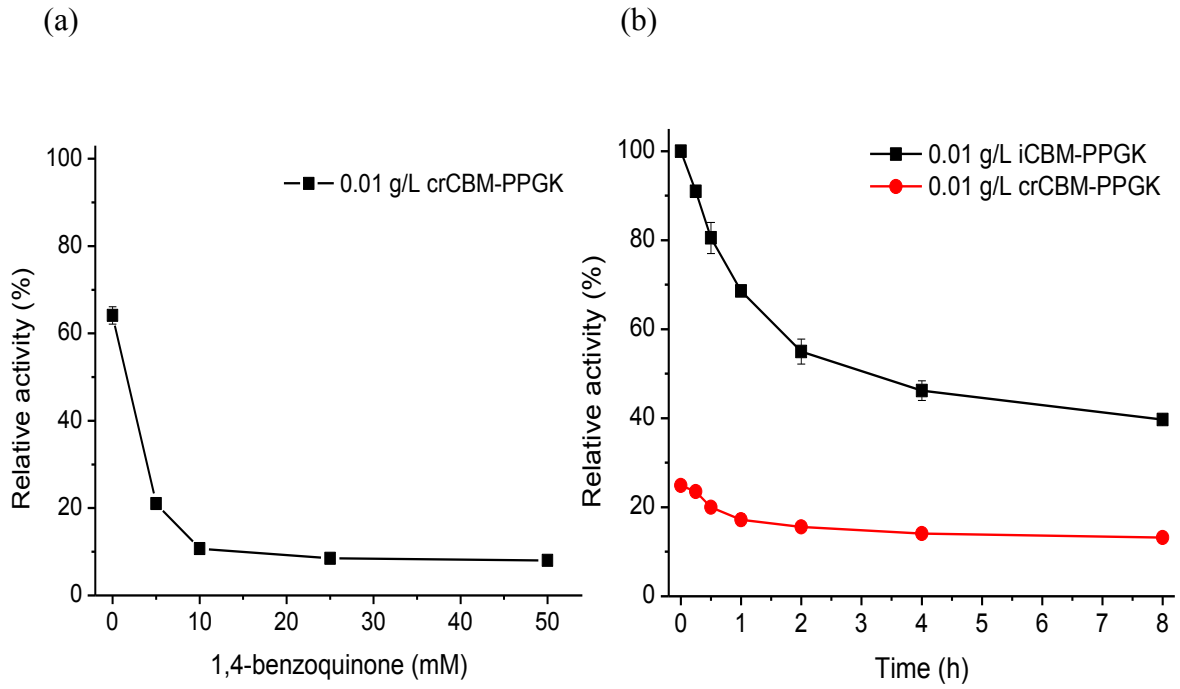

Figure 3-6. (a) Cross-linking of iCBM-PPGK by 1,4-benzoquinone and (b) thermostability of crCBM-PPGK at 50 ℃. Different concentrations of 1,4-benzoquinone were used for cross linking and the residual activity of crCBM-PPGK were tested after 2-h incubation at 50 ℃.

By a comparison of PPGKs from different organisms, the PPGK from *T. fusca* YX had the highest optimum temperature (Table 3-3). The half-life time of the enzyme increased from 15 min to 2 h, while maintaining a majority of the enzyme activity (~35% loss), by a simple one-step immobilization on the high-binding capacity adsorbent RAC (Figure 3-5). After cross linking by 1-h incubation with 5 mM 1,4-benzoquinone, the half-life time of the enzyme increased to 12 h, however, there is a significant loss of the enzyme activity (~ additional 75% loss, Figure 3-6). These results suggested that to increase the enzyme thermo-stability while minimizing the enzyme activity, a proper immobilization protocol should be developed by further optimization of immobilization conditions.

The PPGK protein was purified as a CBM-fused protein by using affinity adsorption of CBM on an ultra-large capacity adsorbent RAC. The advantages included (i) improve the soluble expression level of PPGK; (ii) introduce low-cost protein purification method by a simple adsorption on a high-binding capacity adsorbent RAC, followed with ethyl glycol elution; (iii) facilitate a one-step immobilization on the low-cost, biodegradable high-capacity RAC; (iv) ease of scale-up using simple solid/liquid unit operations (e.g. centrifugation).

Thermostable enzymes are playing an increasingly important role in industrial biocatalysis and emerging cell-free synthetic pathway biotransformation projects. Through further improvement of its thermo-stability, the PPGK from *T. fusca* YX has the potential for broad applications in industries. One potential application is to introduce this enzyme in the synthetic enzymatic pathway for high-yield hydrogen production (Ye et al. 2009; Zhang et al. 2007; Zhang et al. 2008). Once we get glucose through biomass pretreatment and hydrolysis, PPGK could be responsible for the phosphorylation of glucose to form glucose 6-phosphate, which enters the pentose phosphate pathway and leads to hydrogen production by a novel synthetic pathway (Zhang et al. 2007).

4 Conclusions and perspectives

4.1 Conclusions

The hydrolysis performance of bacterial cellulase mixtures on Avicel varied greatly over time, while it is relatively fixed on regenerated amorphous cellulose.

Processive endoglucanase CpCel9 is important for high cellulose digestibility regardless of substrate type. These results suggested that reconstitution of optimal ratios of different action mode cellulase components strongly depended on experimental conditions, such as chosen substrates and reaction time. This study provides important information for the construction of minimal sets of bacterial cellulases for the consolidated bioprocessing of lignocellulose.

The recombinant polyphosphate glucokinase purified from the thermophile *Thermobifida fusca* YX, shows an higher temperature optimum than those from other organisms. Its thermostability increased through one-step immobilization on regenerated amorphous cellulose. Cross-linking by 1, 4 - benzoquinone further increased the half-life time but decreases enzyme activity greatly.

Table 3-3. A comparison of of PPGKs from different organisms.

Organism	$T_{opt.}$ (℃)	Sp. activity (U^*/mg) (condition)	Half- life time (condition)	References
Arthrobacter sp.	45	220 (pH 7.0, 30 ℃)	5 min (pH 7.5; 40 ℃)	(Mukai et al. 2003)
Mycobacterium phlei	N. A.	N. A.	24 h (pH 3.5; 3 ℃)	(Szymona and Ostrowski 1964)
Microlunatus phosphovorus	30	N. A.	N. A.	(Tanaka et al. 2003)
Mycobacterium tuberculosis	N. A.	203 (pH 7.5; 30 ℃)	N. A.	(Hsieh et al. 1996b)Hsieh, Shenoy et al. 1996)
Propionibacterium shermanii	N. A.	15.3 (pH 7.5; 30 ℃)	N. A.	(Pepin and Wood 1986)
Thermobifida fusca	55 (free enzyme);	64.6, free enzyme; 48.1, immobilized (pH 7.5, 50 ℃)	0.25 h, free enzyme; 2 h, immobilized (pH7.5; 50 ℃)	This study

*1 Unit of enzyme activity was defined as the production of 1 μmole product per minute.

4.2 Perspectives

To further decrease cellulase costs in biofuels production, more research work is needed on minimal bacterial cellulase sets. Mass-specific activity of minimal bacterial cellulase would be increased through engineering individual cellulase performance by rational design or directed evolution, and through displaying linked cellulase complexes on the surface of microorganisms. A combination of cellulase engineering and metabolic engineering would help develop microorganisms that can produce cellulase, hydrolyze cellulose and produce desired products in a single step.

One-step PPGK purification and immobilization from *T. fusca* YX turns out to be a wonderful approach to producing low-cost stable enzyme. Further research will be done for enhancing further stability, by developing a proper immobilization protocol.

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Appendix A

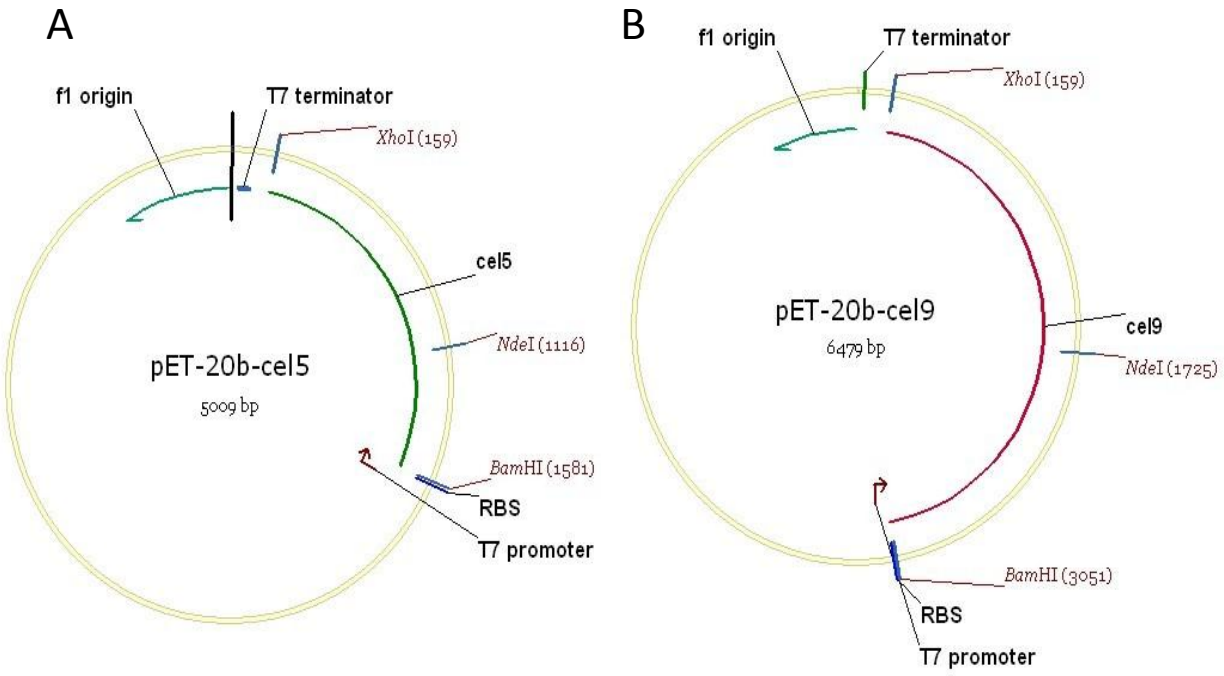

Figure A1. Plasmids construction for the expression of (A)CpCel5, and (B)CpCel9.

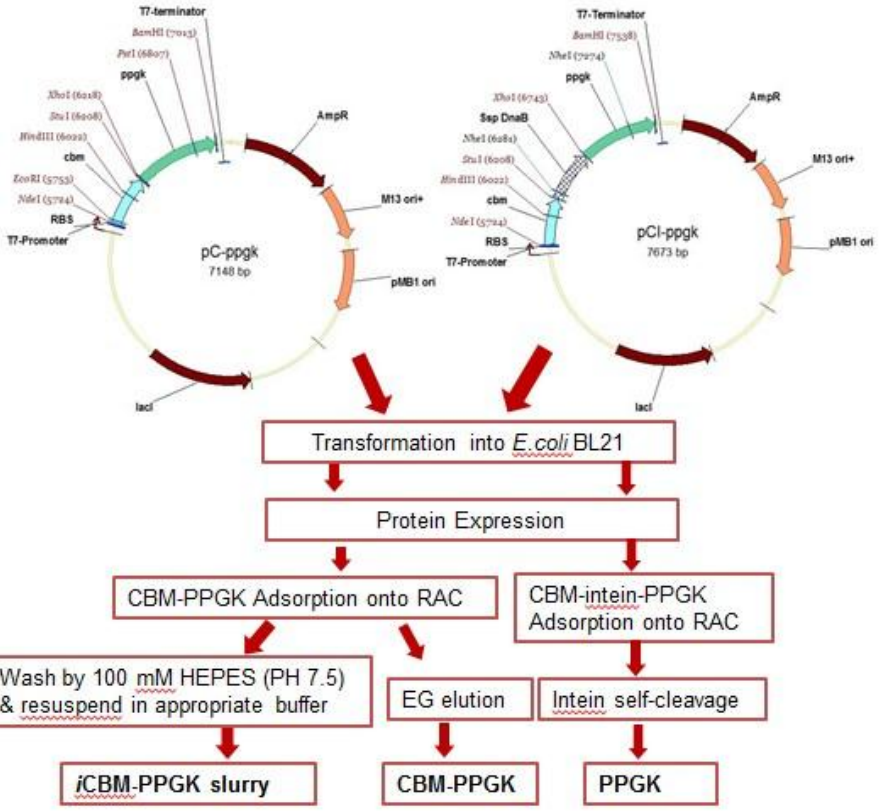

Figure A2. A Scheme of PPGK expression, purification and immobilization.

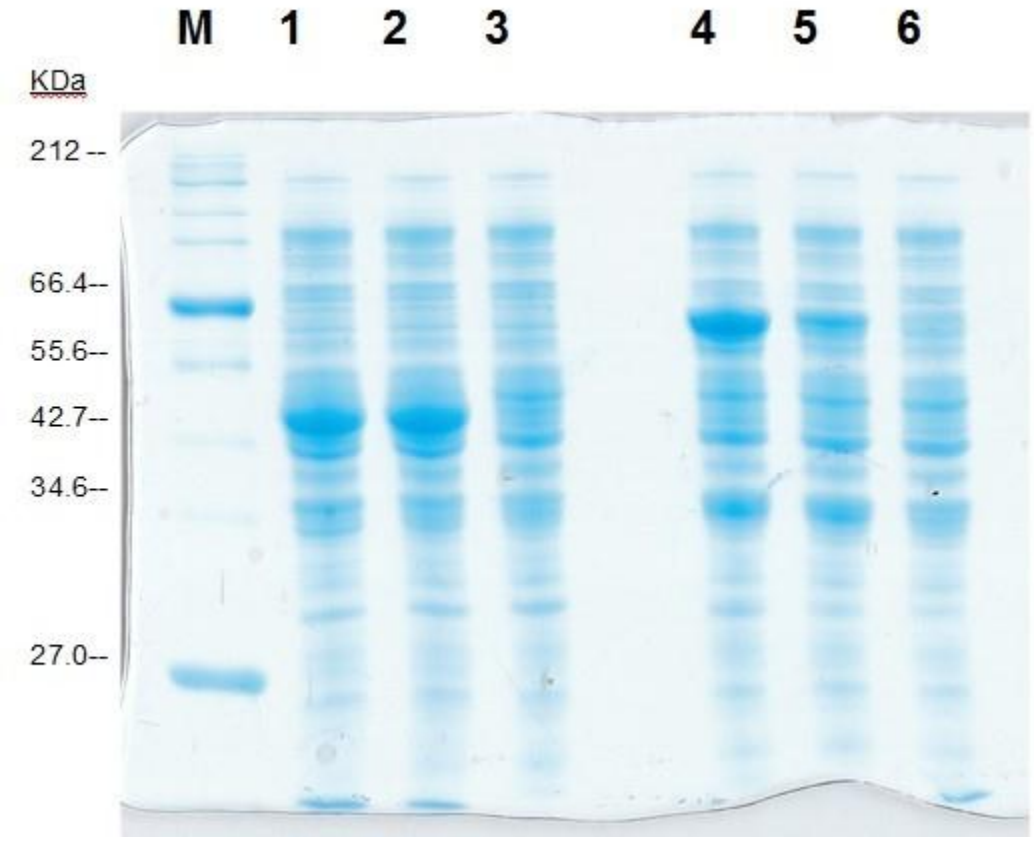

Figure A3. 10% SDS-PAGE analysis of PPGK expression. M: Protein marker p7702S; Lane 1: CBM-PPGK total protein; Lane 2: CBM-PPGK supernatant; Lane 3: CBM-PPGK after RAC adsorption; Lane 4: CBM-intein-PPGK total protein; Lane 5: CBM-intein-PPGK supernatant; Lane 6: CBM-intein-PPGK after RAC adsorption.