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In vitro bioconversion of chitin to pyruvate with thermophilic enzymes

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Chitin is the second most abundant organic compound on the planet and thus has been regarded as an alternative resource to petroleum feedstocks. One of the key challenges in the biological conversion of biomass-derived polysaccharides, such as cellulose and chitin, is to close the gap between optimum temperatures for enzymatic saccharification and microbial fermentation and to implement them in a single bioreactor. To address this issue, in the present study, we aimed to perform an *in vitro*, one-pot bioconversion of chitin to pyruvate, which is a precursor of a wide range of useful metabolites. Twelve thermophilic enzymes, including that for NAD^+ regeneration, were heterologously produced in *Escherichia coli* and semi-purified by heat treatment of the crude extract of recombinant cells. When the experimentally decided concentrations of enzymes were incubated with 0.5 mg mL^{-1} colloidal chitin (equivalent to 2.5 mM *N*-acetylglucosamine unit) and an adequate set of cofactors at 70°C, 0.62 mM pyruvate was produced in 5 h. Despite the use of a cofactor-balanced pathway, determination of the pool sizes of cofactors showed a rapid decrease in ATP concentration, most probably due to the thermally stable ATP-degrading enzyme(s) derived from the host cell. Integration of an additional enzyme set of thermophilic adenylate kinase and polyphosphate kinase led to the deceleration of ATP degradation, and the final product titer was improved to 2.1 mM.

[**Key words:** Chitin; Thermophile; Thermophilic enzyme; Pyruvate; *In vitro* bioconversion]

Chitin, a β-1,4-linked polymer of partially deacetylated *N*-acetylglucosamine, is a major structural component of fungal cell walls, insect exoskeletons, and crustacean shells. Chitin is the second most abundant organic compound on the planet (after cellulose), and its formation rate on earth has been estimated to be of the order of 10^{10}–10^{11} per year (1). Owing to its abundance, chitin has been regarded as a promising biomass-derived resource for the production of biofuels (2,3) and other functional compounds (4).

The biological conversion of biomass-derived polysaccharides, such as starch, cellulose, and chitin, to commercially valuable metabolites generally consists of the following four steps: (i) physicochemical pretreatment of biomass, (ii) chemical and/or enzymatic hydrolysis of pretreated biomass to oligo- and monosaccharides, (iii) fermentative conversion of the resulting saccharides to target compounds, and (iv) separation and purification of the products. Because increasing attention has been paid to biomass as an alternative resource to petroleum feedstocks, considerable effort has been devoted to improve the economic feasibility of biomass-processing procedures. Among them is the development of simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing, in which enzymatic hydrolysis of polysaccharide (step ii) and microbial fermentation (step iii) are simultaneously implemented in a single bioreactor (reviewed in previous literature (5,6)). One of the key challenges in these process developments is to bridge the gap between the optimum conditions of enzymatic saccharification and those of microbial fermentation. Although many saccharolytic enzymes optimally work at approximately ≥45°C, most commonly used fermentative microorganisms (e.g., *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Escherichia coli*) cannot tolerate such high temperatures (7). Therefore, natural and engineered thermophiles have been increasingly studied as platform strains for the high-temperature processing of biomass-derived polysaccharides (8–10). However, the conversion yields and product titers achieved using thermophiles are still modest, mainly due to the lack of sufficient gene-manipulation tools for the genome-wide engineering of these microorganisms. One of the possible strategies to overcome this limitation and to construct a rationally designed metabolic pathway compatible with a high temperature is to eliminate the use of living microorganisms and to use only thermophilic enzymes in the designed pathway *in vitro*. The *in vitro* construction of synthetic metabolic pathways has been emerging as a promising approach to develop a highly tunable and scalable biotransformation system without concerns of cell proliferation, complicated metabolic regulation, and byproduct formation (reviewed in previous literature (11,12)). Thermophilic enzymes are particularly suitable as biocatalytic modules for *in vitro* pathway construction because they show remarkably higher stability than their mesophilic counterparts and can be readily semi-purified by simple heat treatment of recombinant mesophiles (e.g., *E. coli*) overexpressing corresponding genes (13). In this study, to evaluate the performance of *in vitro* bioprocessing of a biomass-derived polysaccharide, we assembled chitin-degrading and glycolytic enzymes from thermophilic sources and conducted a one-pot conversion of chitin to pyruvate.

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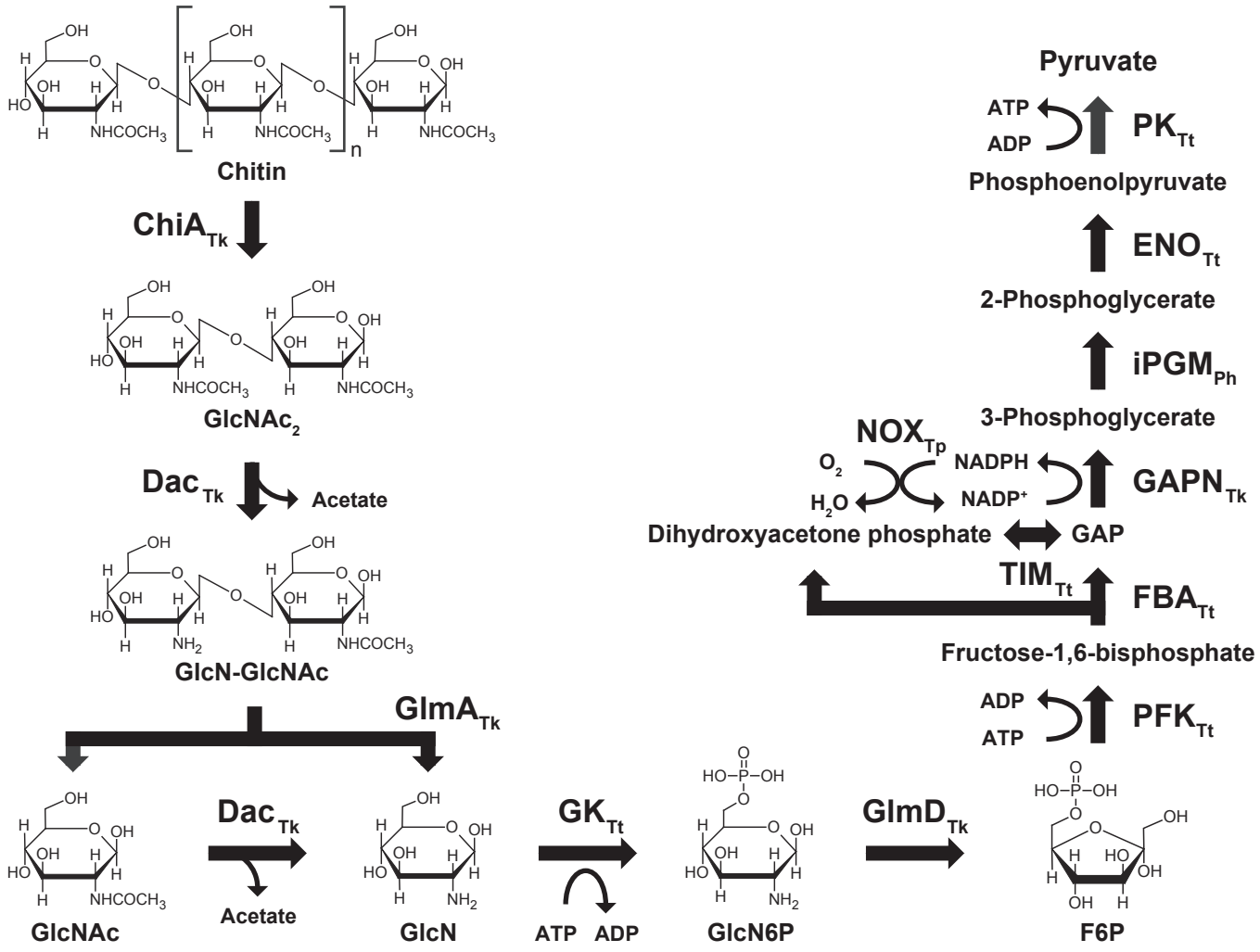

FIG. 1. Schematic illustration of the *in vitro* synthetic pathway constructed in this study.

MATERIALS AND METHODS

Reagents Chitin was purchased from Wako Pure Chemical (Osaka, Japan). *N*-Acetylglucosamine (GlcNAc), glucosamine (GlcN), and glucosamine-6-phosphate (GlcN6P) were products of Sigma–Aldrich Japan (Tokyo, Japan). ATP, ADP, $NADP^+$, and NADPH were obtained from Oriental Yeast (Osaka, Japan). Colloidal chitin was prepared from chitin powder as described by Tanaka et al. (14). Diacetyl chitobiose ($GlcNAc_2$) was enzymatically prepared from colloidal chitin using the chitinase from *Thermococcus kodakarensis* ($ChiA_{Tk}$, TK1765) (14,15). Colloidal chitin (4.0 mg mL^{-1}) was incubated with an appropriate amount of the enzyme at 70°C in 50 mM HEPES-NaOH buffer (pH 7.0). After the enzymatic digestion, the mixture was centrifuged (12,000 ×*g*, 10 min, 4°C) to remove unreacted chitin. Enzymes were removed by ultrafiltration and $GlcNAc_2$ in the filtrate was quantified by a modified version of the 3,5-dinitrosalicylic acid (DNS) method (16). The disaccharide of GlcN and GlcNAc (GlcN-GlcNAc) was prepared from $GlcNAc_2$ using *T. kodakarensis* deacetylase (Dac_{Tk}, TK1764) (17). $GlcNAc_2$ (10 mM) was enzymatically deacetylated at 70°C and pH 7.0, and the product was separated from the enzyme by ultrafiltration. The concentration of GlcN-GlcNAc in the filtrate was estimated by determining the concentration of liberated acetate by high-performance liquid chromatography (see HPLC analysis below). Polyphosphate was chemically synthesized as described elsewhere (18) and had an average phosphate-chain length of 10 or higher.

Plasmid construction Expression vectors for $ChiA_{Tk}$, Dac_{Tk}, exo-β-D-glucosaminidase ($GlmA_{Tk}$, TK1754) (19), and GlcN6P deaminase ($GlmD_{Tk}$, TK1755) (20) of *T. kodakarensis* were kind gifts from Dr. H. Atomi. Genes encoding phosphofructokinase (PFK_{Tt}, TTHA1962), fructose-1,6-bisphosphate aldolase (FBA_{Tt}, TTHA1773), triose phosphate isomerase (TIM_{Tt}, TTHA0947), enolase (ENO_{Tt}, TTHA0002), and pyruvate kinase (PK_{Tt}, TTHA0003) of *Thermus thermophilus*, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase of *T. kodakarensis* ($GAPN_{Tk}$, TK0705), and cofactor-independent phosphoglycerate mutase of *Pyrococcus horikoshii* ($iPGM_{Ph}$, PH0037) were assembled in an artificial operon using the OGAB method (21), as described previously (13). Briefly, codon-optimized genes, which were flanked by *Bsp*QI restriction sites at both 5′- and 3′-terminals, were synthesized by BIOMATIK (Wilmington, DE, USA). After digestion with *Bsp*QI, genes were introduced at the corresponding sites of the series of pUC destination vectors. The resulting plasmids, namely, pUC19V-1st-$GAPN_{Tk}$, pUC19V-2nd-FBA_{Tt}, pUC19V-3rd-ENO_{Tt}, pUC19V-4th-$iPGM_{Ph}$, pUC19V-5th-PFK_{Tt}, pUC19V-6th-PK_{Tt}, and pUC19V-7th-TIM_{Tt}, were digested by *Dra*III to generate unique three-base cohesive ends. Digested fragments were purified from an agarose gel and mixed at equimolar concentrations (2 fmol/ml each). They were ligated to the *Sfi*I restriction site of pGETS118 (22) and introduced into *Bacillus subtilis*. The plasmid with assembled genes was isolated from the resulting transformant and introduced into *E. coli* DH5α harboring pRCI (13). Expression vectors for the glucokinase (GK_{Tt}, TTHA0299), glucose-6-phosphate isomerase (PGI_{Tt}, TTHA0277), adenylate kinase (ADK_{Tt}, TTHA1671), and lactate dehydrogenase (LDH_{Tt}, TTHA1113) from *T. thermophilus* were obtained from the Riken *T. thermophilus* HB8 expression plasmid set (23). The expression plasmid for the *T. thermophilus* polyphosphate kinase (PPK_{Tt}, TT_C0637) was obtained as described elsewhere (24). The gene encoding NAD(P)H oxidase from *Thermococcus profundus* (Nox_{Tp}) was cloned and expressed in *E. coli* as described by Ninh et al. (13).

Enzyme preparation *E. coli* Rosetta 2 (DE3) (Novagen, Madison, WI, USA) was used for the expression of genes introduced under the control of the T7 promoter ($ChiA_{Tk}$, Dac_{Tk}, $GlmA_{Tk}$, $GlmD_{Tk}$, GK_{Tt}, PGI_{Tt}, ADK_{Tt}, PPK_{Tt}, Nox_{Tp}, and LDH_{Tt}). The other genes were expressed using the temperature-inducible Pr/CI857 system (25) in *E. coli* DH5α. The recombinant cells were aerobically cultivated in Luria–Bertani medium at 37°C supplemented with adequate antibiotics (100 μg mL^{-1} ampicillin, 30 μg mL^{-1} chloramphenicol, and/or 10 μg mL^{-1} tetracycline). Gene expression from the T7 promoter was induced by adding 0.2 mM isopropylthiogalactoside (IPTG) to the bacterial culture, whereas that from the Pr promoter was induced by shifting the cultivation temperature to 42°C at

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