



***In vitro* conversion of glycerol to lactate by bacterial thermophilic enzymes**

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Abstract

In vitro reconstitution of an artificial metabolic pathway has emerged as an alternative approach to conventional *in vivo* fermentation-based bioproduction. Particularly, employment of thermophilic and hyperthermophilic enzymes enables us a simple preparation of highly stable and selective biocatalytic modules and the construction of *in vitro* metabolic pathways with an excellent operational stability. In this study, we designed and constructed an artificial *in vitro* metabolic pathway consisting of 9 (hyper)thermophilic enzymes and applied it to the conversion of glycerol to lactate. The *in vitro* artificial pathway was designed to balance the intrapathway consumption and regeneration of energy and redox cofactors. The enzyme stability was assessed by measuring the remaining activity of enzymes after the incubation at 60 °C. Most enzymes could retain more than 80% of their initial activity for 8 h, except that PK_{T1} lost 35% of the activity after the incubation for the same time period. We also investigated the effect of methanol, which is the primary impurity contained in crude glycerol. All enzymes show stability was not markedly affected by the co-existing of up to 100 mM methanol. When the reaction was carried out at 60 °C in different buffers, the highest lactate production rate of 0.036 μmol ml⁻¹ min⁻¹ was observed in HEPES-NaOH (pH 7). Our approach would be widely applicable to the rational optimization of artificial metabolic pathways as well as to the *in vitro* production of value-added biomolecules.

Keywords: *in vitro* metabolic engineering; thermophilic enzymes; glycerol; methanol; lactate

Introduction

Integration of diverse biocatalytic modules to construct an advanced microbial cell factory has emerged as a powerful approach for the production of industrially important metabolites (Rabinovitch-Deere et al. 2013). Bioprospecting efforts for exploring novel biocatalytic molecules with unique properties have inspired the design and construction of a wider variety of artificial metabolic pathways (Bond-Watts et al. 2011). However, installation of an artificially engineered metabolic pathway in living organisms often leads to competition with natural metabolic pathways for intermediates and cofactors, resulting in insufficient yield of desired metabolites. A possible solution to this problem is to avoid the use of living microorganisms and to construct an *in vitro* artificial metabolic pathway in which only a limited number of enzymes are involved. Until now, a variety of *in vitro* synthetic pathways have been designed and constructed for the production of alcohols (Guterl et al. 2012; Krutsakorn et al. 2013), organic acids (Ye et al. 2012, 2013), carbohydrates (You et al. 2013), hydrogen (Woodward et al. 2000; Zhang et al. 2007), bioplastic (Opgenorth et al. 2014), and even electricity (Zhu et al. 2014). Particularly, employment of enzymes derived from thermophiles and hyperthermophiles enables the simple preparation of catalytic modules with excellent selectivity and thermal stability (Ye et al. 2012; Ninh et al. 2014). Concerns about

the global warming and depletion of fossil fuel reserves have led to the rapid increase of biodiesel production. Generally, 10 kg of crude glycerol, which is the primary byproduct of the biodiesel industry, is released for every 100 kg of biodiesel and the growing production of biodiesel has resulted in a worldwide surplus of crude glycerol (Nguyen et al; 2013). Although many studies have been conducted to use crude glycerol as a starting material for the fermentation-based production of industrially valuable chemicals, these attempts often suffer from the inhibitory effects of impurities contained in crude glycerol on the growth and biocatalytic activity of living organisms (Venkataramanan et al. 2012; Yang et al. 2012). Particularly, methanol, which is the most abundant impurity in crude glycerol, accounts for up to 70% (w/w) of a raw glycerol obtained through a biodiesel production process (Asad-ur-Rehman et al. 2008). Lactic acid is naturally presented in many foodstuffs. It is formed by natural fermentation in products such as cheese, yogurt, soy sauce, sourdough, meat products and pickled vegetables and also used in a wide range of food applications such as bakery products, beverages, meat products, confectionery, dairy products, salads and dressings (Xu and Xu 2014). Lactic acid in food products usually serves as either as a pH regulator or as a preservative (Xu and Xu 2014). Lactic acid is not only use in food but also **pharmaceutical, biodegradable plastics**, detergents and animal feed. Therefore, lactic acid reagents are an important industrial material with numerous potential applications (Zhou et al. 2014). In this study, we focused on the high operational stabilities of thermophilic enzymes and employed them as modules to construct an *in vitro* synthetic pathway for the conversion of glycerol to lactate.

Methodology

Microorganisms and plasmid

Escherichia coli JM109 was used for general cloning purpose. *E. coli* Rosetta 2 (DE3) was used for gene expression. Recombinant *E. coli* was aerobically cultivated at 37 °C in Luria-Bertani medium supplemented with 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol. Gene expression was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside at the late log phase. The expression vector encoding the glycerol kinase of *Thermococcus kodakarensis* (GK_{Tk}, gi|3986088) was donated by Dr. Y. Koga, Osaka University (Koga et al. 2001). Sources of expression vectors for triose phosphate isomerase (TIM_{Tt}, gi|3169211), enolase (ENO_{Tt}, gi|55979971), pyruvate kinase (PK_{Tt}, gi|55979972), lactate dehydrogenase (LDH_{Tt}, gi|55981082) of *Thermus thermophilus*, non-phosphorylating GAP dehydrogenase of *T. kodakarensis* (GAPN_{Tk}, gi|57640640), and cofactor-independent phosphoglycerate mutase of *Pyrococcus horikoshii* (iPGM_{Ph}, gi|14589995) were described previously (Ye et al. 2012). The expression vector for G3P dehydrogenase of *T. thermophilus* (G3PDH_{Tt}, gi|55981709) was obtained from the Riken *T. thermophilus* HB8 expression plasmid set (Yokoyama et al. 2000). Gene encoding NADH oxidase of *Thermococcus profundus* (NOX_{Tp}, gi|187453160) was cloned and expressed in *E. coli* as described elsewhere (Ninh et al. 2014).

Enzyme assay

E. coli cells were collected by centrifugation, resuspended in 50 mM HEPES-NaOH (pH 7), and then disrupted by ultrasonicator (Kubota UD-201, Kubota Corp., Osaka, Japan). After the removal of cell debris by centrifugation, the cell-free extract was incubated at 70 °C for 30 min. The heat-precipitated proteins were removed by centrifugation and the resulting supernatant was used as an enzyme solution. One unit of an enzyme was defined as the amount consuming 1 µmol of the substrate per min under the below-mentioned standard assay conditions. Protein concentration was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) using bovine serum albumin as the

standard. Enzyme activities were spectrophotometrically determined at 60 °C by monitoring consumption or generation of NADH at 340 nm. When necessary, NADH generation was coupled with the reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and detected at 438 nm. GK_{Tk} activity was determined by coupling with G3PDH_{Tt}. The standard assay mixture for GK_{Tk} was composed of 50 mM HEPES-NaOH (pH 7), 0.2 mM glycerol, 0.2 mM ATP, 1 mM NAD⁺, 5 mM MgCl₂, 0.5 mM MnCl₂, 0.15 mM WST-1, 6 μM 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS), an excess amount of G3PDH_{Tt}, and an appropriate amount of GK_{Tk}. The mixture without glycerol was pre-incubated at 60 °C for 2 min and the reaction was initiated by the addition of the substrate. Enzyme reaction was monitored through the reduction of WST-1 to the corresponding formazan dye at 438 nm using a UV-2450 spectrophotometer (Shimadzu Corp., Kyoto, Japan). G3PDH_{Tt} assay was performed in the same manner except that 0.2 mM G3P was used as a substrate. TIM_{Tt} was assayed in a mixture containing 50 mM HEPES-NaOH (pH 7), 0.2 mM DHAP, 1 mM NAD⁺, 5 mM MgCl₂, 0.5 mM MnCl₂, 1 mM glucose-1-phosphate (G1P), an excess amount of GAPN_{Tk}, and an appropriate amount of the enzyme. After a pre-incubation at 60 °C for 2 min, the substrate was added to the mixture and the reduction of NAD⁺ was monitored at 340 nm. For the determination of GAPN_{Tk} activity, 0.2 mM GAP was used instead of DHAP. Similarly, iPGM_{Ph} activity was assessed by coupling with ENO_{Tt}, PK_{Tt}, and LDH_{Tt}. The enzyme was assayed in a mixture containing 50 mM HEPES-NaOH (pH 7), 0.2 mM 3-PG, 0.2 mM ADP, 0.2 mM NADH, 5 mM MgCl₂, 0.5 mM MnCl₂, and excess amounts of ENO_{Tt}, PK_{Tt}, and LDH_{Tt}. The reaction rate was determined by monitoring the concomitant decrease of NADH at 340 nm. Assays for ENO_{Tt}, PK_{Tt}, and LDH_{Tt} were performed in the same mixture using 0.2 mM each of 2-PG, PEP, and pyruvate, respectively. NOX_{Tp} activity was determined by monitoring the oxidation of NADH under an air atmosphere. A reaction mixture comprising 50 mM HEPES-NaOH (pH 7), 5 mM MgCl₂, 0.5 mM MnCl₂, 0.02 mM flavin adenine dinucleotide (FAD), and 0.2 mM NADH was preincubated at 60 °C for 2 min and then the reaction was started initiated by adding an appropriate amount of enzyme.

Analytical method

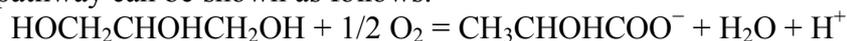
Lactate was quantified by high-performance liquid chromatography (HPLC) equipped with two tandemly connected ion exclusion columns (Shim-pack SPR-H, 250 mm × 7.8 mm, Shimadzu Corp., Kyoto, Japan). The columns were eluted at 50 °C using 4 mM *p*-toluenesulfonic acid as a mobile phase at a flow rate of 0.2 ml min⁻¹. The eluent was mixed with a pH-buffered solution (16 mM Bis-Tris, 4 mM *p*-toluenesulfonic acid, and 0.1 mM EDTA) supplied at a flow rate of 0.2 ml min⁻¹, and then analyzed for lactate using a conductivity detector (CDD-20A, Shimadzu Corp., Kyoto, Japan).

Results

Design of the *in vitro* synthetic pathway

Figure 1 illustrates the newly designed synthetic pathway for the conversion of glycerol to lactate. To construct an *in vitro* synthetic pathway, it is vital to prevent the depletion of energy and redox cofactors (ATP/ADP, and NAD⁺/NADH) by balancing their intrapathway consumption and regeneration. In a previous study, we constructed an ATP/ADP-balanced chimeric Embden-Meyerhof (EM) pathway by swapping the enzyme couple of GAP dehydrogenase and phosphoglycerate kinase in the bacterial/eukaryotic EM pathway with the non-phosphorylating GAP dehydrogenase (GAPN_{Tk}) involved in the modified EM pathway of a hyperthermophilic archaeon, *Thermococcus kodakarensis* (Ye et al. 2012). Similarly, we employed the GAPN-mediated non-ATP-forming dehydrogenation of GAP to 3-PG for

balancing the consumption and regeneration of ATP and ADP through the glycerol converting pathway. On the other hand, the conversion of one molecule of glycerol to lactate through the designed pathway was accompanied by the generation of one molecule of NADH. To re-oxidize the cofactor and to maintain the redox balance of the whole pathway, a hyperthermophilic NADH oxidase was integrated into the pathway. NADH oxidases catalyze the reduction of O₂ using NAD(P)H as a reductant and can be divided in two groups: those catalyzing two-electron reduction of O₂ to H₂O₂ and those catalyzing four-electron reduction of O₂ to H₂O. In this study, we employed the NADH oxidase from *T. profundus* (NOX_{Tp}), which preferably catalyzed four-electron reduction of O₂ (Jia et al. 2008), to eliminate the inhibitory effects of H₂O₂ on enzymes. The chemical equation of the overall reaction through the synthetic pathway can be shown as follows:



The standard Gibbs energy change (ΔG°) of the reaction was calculated to be -256.4 kJ/mol.

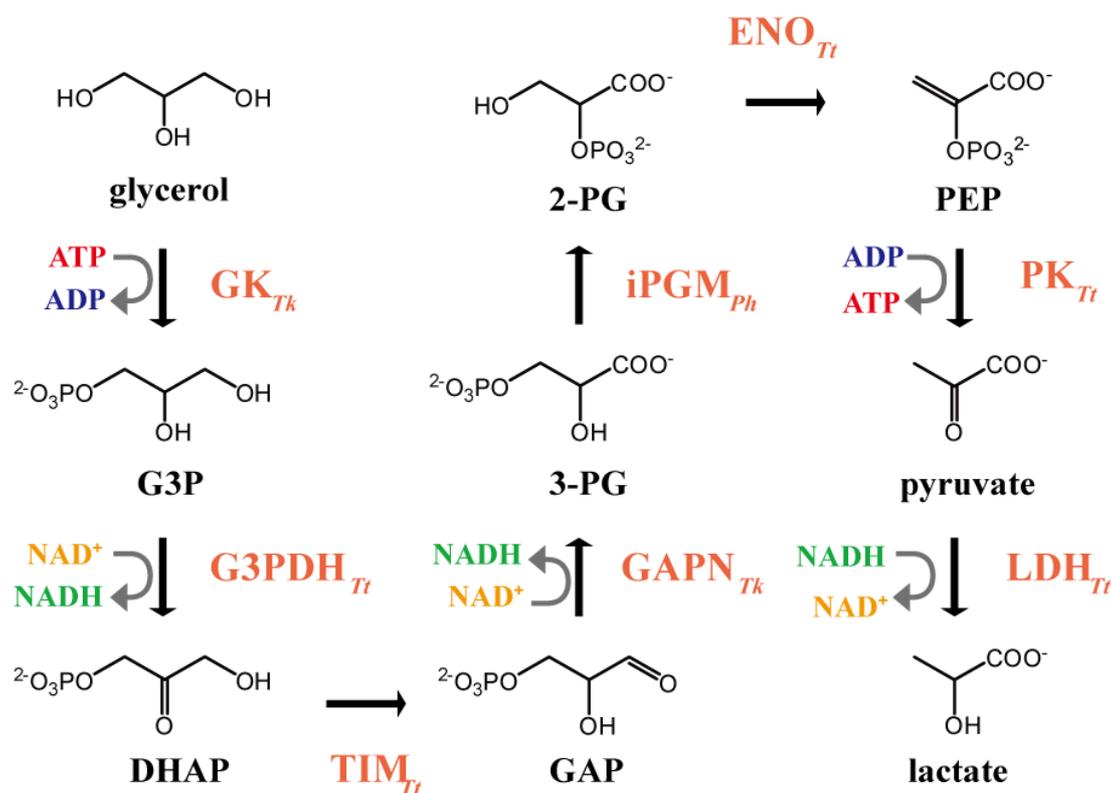


Figure 1 Schematic illustration of the *in vitro* synthetic pathway constructed in this study.

Abbreviations: G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate.

Enzyme stability

The enzyme stability was assessed by measuring the remaining activity of enzymes after the incubation at 60 °C (Figure 2). Most enzymes could retain more than 80% of their initial activity for 8 h, except that PK_{Tl} lost 35% of the activity after the incubation for the same time period. We also investigated the effect of methanol, which is the primary impurity contained in crude glycerol, on the enzyme stability (Yang et al. 2012). Although residual activities of TIM_{Tl}, iPGM_{Ph}, ENO_{Tl}, and LDH_{Tl} were moderately lower than those in the absence of methanol (16-32% decrease), the destabilization profile of other enzymes were not significantly affected by at least up to 100 mM of methanol.

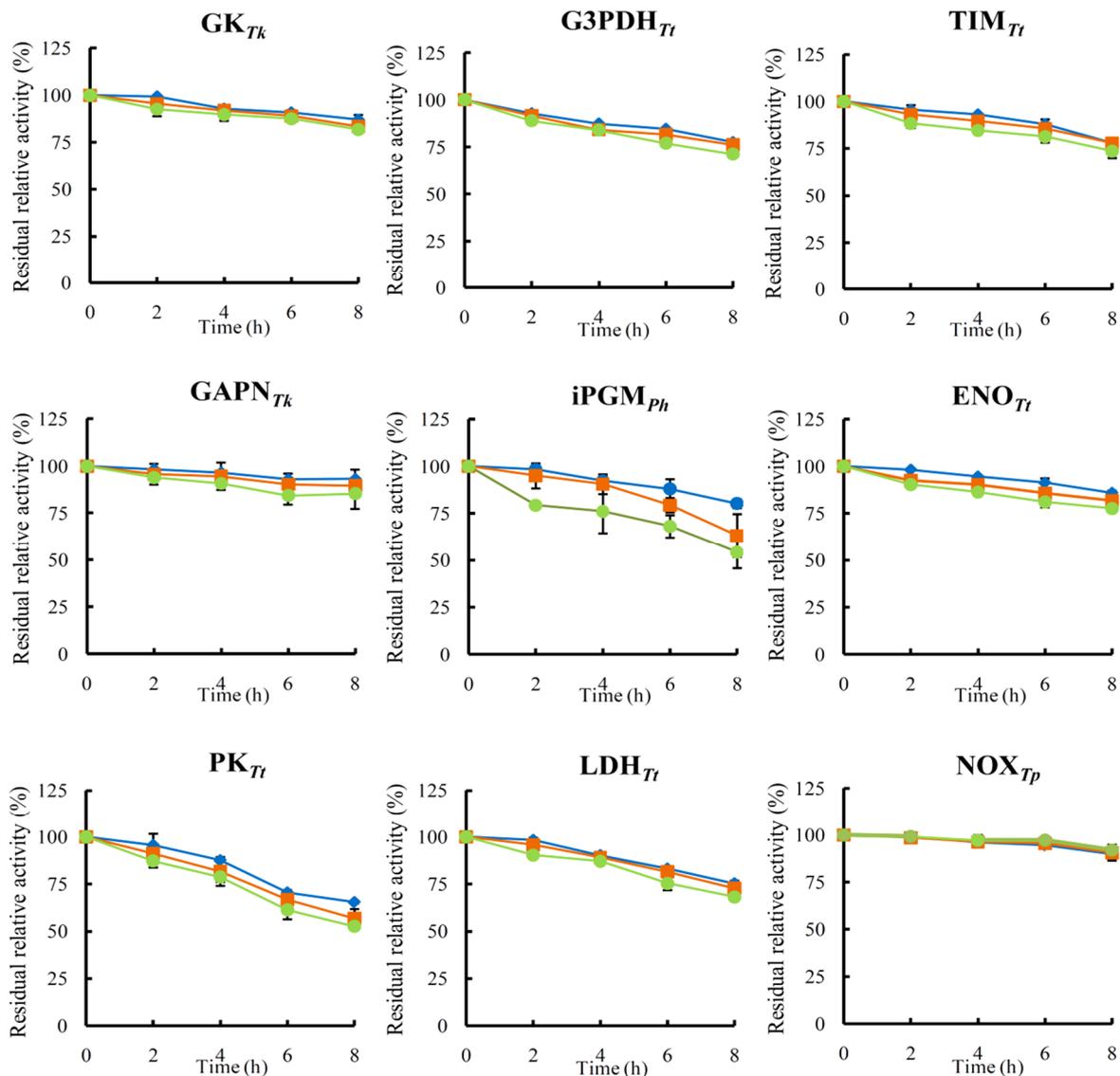


Figure 2 Enzyme stability. Enzyme solutions were incubated at 60 °C for indicated time periods and residual activities were determined under the standard assay conditions (blue diamond). Similarly, enzyme stabilities were also assessed at 60 °C in the presence of 50 (orange square) and 100 mM methanol (green circle).

Optimization of reaction conditions

The lactate production rate through the synthetic pathway was determined at different pH and temperatures by incubating 0.1 U ml⁻¹ each of GK_{Tk}, G3PDH_{Tl}, TIM_{Tl}, GAPN_{Tk}, iPGM_{Ph}, ENO_{Tl}, PK_{Tl}, LDH_{Tl}, and NOX_{Tp} with 10 mM glycerol and appropriate concentrations of

cofactors and metal ions (Figure 3). Glucose-1-phosphate (G1P) was put in the reaction mixture as an activator for GAPN_{7k} (Matsubara et al. 2011). When the reaction was carried out at 60 °C in different buffers, the highest lactate production rate of 0.036 $\mu\text{mol ml}^{-1} \text{min}^{-1}$ was observed in HEPES-NaOH (pH 7) (Figure 3A). The reaction was then performed in this buffer at 50, 60, and 70 °C (Figure 3B) but at 70 °C, the degradation of cofactors and intermediates easier than 60 °C then optimum condition was pH 7 and 60 °C (Ye et al. 2012).

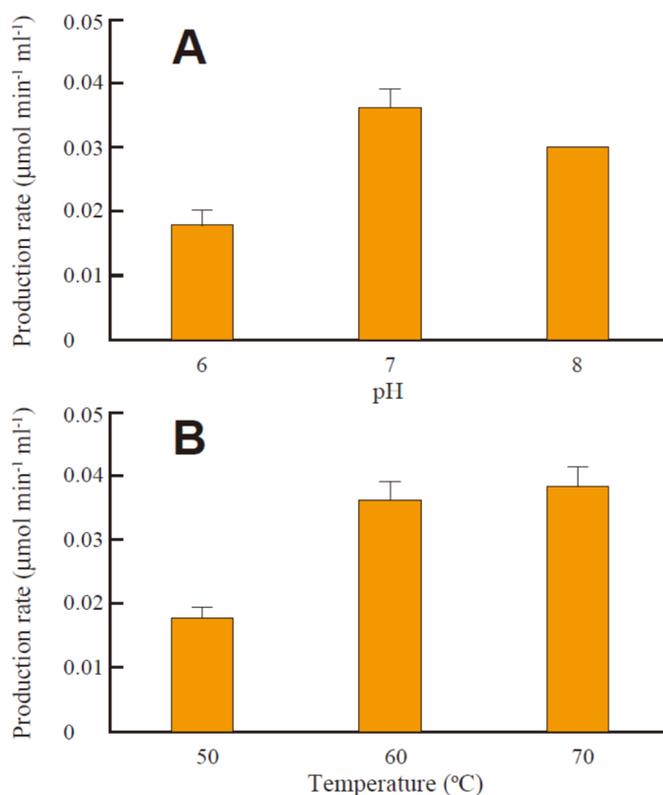


Figure 3 Effect of pH (A) and temperature (B) on the lactate production through the *in vitro* synthetic pathway.

Discussion

The *in vitro* artificial pathway was designed to balance the intrapathway consumption and regeneration of energy and redox cofactors. All enzymes involved in the *in vitro* pathway exhibited an acceptable level of stability at high temperature (60 °C) and their stability was not markedly affected by the co-existing of up to 100 mM methanol. Many thermophilic enzymes exhibit higher stability not only at high temperatures but also in the presence of denaturants such as detergents and organic solvents than their mesophilic counterparts. Although almost identical production rates were observed at 60 and 70 °C, the reaction temperature of 60 °C was employed for further studies to mitigate the thermal inactivation of the enzymes and the decomposition of thermo-labile intermediates and cofactors.

Conclusion

In this study, we constructed an artificial *in vitro* metabolic pathway for the conversion of glycerol to lactate. The *in vitro* pathway consisted of 9 thermophilic and hyperthermophilic

enzymes and designed to balance the intrapathway consumption and regeneration of cofactors. The one-pot conversion of glycerol to lactate through the *in vitro* pathway could be achieved in an almost stoichiometric manner. The implementation of the *in vitro* bioconversion with a real crude glycerol would be needed in next studies.

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