Biochemical Engineering Journal 171 (2021) 108004

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Enzymatic direct carboxylation under supercritical CO_2

Kristian Ray Angelo Are, Shusuke Ohshima, Yusuke Koike, Yoshihisa Asanuma, Shogo Kashikura, Mayumi Tamura, Tomoko Matsuda *

Department of Life Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa, 226-8501, Japan

ARTICLE INFO

Keywords:
Enzymatic carboxylation
CO_2
Isocitrate dehydrogenase
Glucose dehydrogenase
Cofactor regeneration

ABSTRACT

Thermodynamically-constrained carboxylation reaction requires high temperature and pressure in industrial processes. Research endeavors are aimed towards developing safer and greener alternatives. Enzymatic systems are considered as potential substitutes. In this study, we conducted direct carboxylation *via* a bi-enzymatic reaction with *Thermoplasma acidophilum* isocitrate dehydrogenase (*Ta*IDH) and *T. acidophilum* glucose dehydrogenase (*Ta*GDH) to produce isocitric acid from of 2-ketoglutaric acid under supercritical CO_2. Coupling with the *Ta*GDH reaction (for cofactor regeneration) enabled the equilibrium of the reactions to shift towards carboxylation, resulting in a 16-fold increase in yield. Furthermore, the yield was increased by optimizing the reaction parameters such as pH, substrate concentration, temperature, and time, and by testing various metal ions. A preparative scale reaction was conducted successfully.

1. Introduction

The global climate crisis has compelled various sectors to minimize carbon emissions from domestic and industrial sources. Several carbon capture and storage (CCS) and CO_2 valorization technologies are being developed [1]. As CO_2 is a cheap and non-toxic compound, it can be a convenient one-carbon building block to produce valuable organic products by carboxylation reactions. However, such reactions are inconveniently thermodynamically-constrained. Carboxylation reactions done in industries today, such as urea production, Kolbe-Schmitt reaction, and cyclic organic carbonates synthesis, require high temperatures and pressures, which translates to high energy demand, negative environmental impact, and hazardous working environments [2–5]. Research is directed towards the development of green and benign alternatives [6,7]. Enzymatic systems are considered as promising solutions [8] because of high specificity, fast reaction kinetics, mild reaction conditions, and biodegradability, which allow enzymatic reactions to proceed with higher yields, better purity, and lesser waste generation. According to a comparative environmental assessment, enzymatic processes have lower greenhouse gas emissions, acidification, eutrophication, photochemical ozone formation, and energy consumption compared to their conventional counterparts [9].

Overcoming the thermodynamic constraints is also the main challenge in enzymatic carboxylation research. Unlike organic reactions, enzymes can only work in a limited range of conditions. A few studies were pursued to improve the performance of enzymatic carboxylation, resulting in increasing the yield [10,11] and improving the enzyme resistance against denaturants [12]. Salting-out of product using quaternary salts shifts the equilibrium towards carboxylation [13]. Supercritical CO_2 solvent also affects the yield of the reaction. Its use dramatically increased the yield of carboxylation of pyrrole producing pyrrole-2-carboxylate by *B. megaterium* PYR 2910, with $KHCO_3$ as carbon source [14]. In the production of isobutanol and isopentanol from 2-ketoisovalerate by an engineered strain of *B. megaterium* SR7, it is energetically feasible and comparable to other *in situ* extraction techniques, when done under supercritical CO_2 and if sufficient product titers can be achieved [15]. Despite these previous researches, enzymatic carboxylation is yet to attain preparative-scale reaction for most of the cases. Furthermore, these reported reactions need the addition of carbonates (*e.g.* $KHCO_3$ [10,13,14] or $NaHCO_3$ [11]); and gaseous CO_2 has not been directly used as a substrate, except in a few recent cases [12,15]. Therefore, further development for this important reaction is necessary.

In this study, enzymatic carboxylation of 2-ketoglutaric acid to produce isocitric acid using CO_2 as a substrate was conducted. *Thermoplasma acidophilum* isocitrate dehydrogenase (*Ta*IDH) and *T. acidophilum* glucose dehydrogenase (*Ta*GDH) were used because enzymes derived from thermophilic archaeon were reported to be high

* Corresponding author.
E-mail address: tmatsuda@bio.titech.ac.jp (T. Matsuda).

https://doi.org/10.1016/j.bej.2021.108004
Received 22 December 2020; Received in revised form 3 March 2021; Accepted 22 March 2021
Available online 26 March 2021
1369-703X/

temperature resistant and have higher activity at acidic pH compared to other fungal enzymes [16–20]. Among many thermophiles, *Ta*IDH is used because it was reported to have fewer amino acids on the N-terminal side compared to other IDHs, the reason for its incredible heat resistance [21]. Enzymes derived from thermophiles have been used for the first time under supercritical CO_2 conditions to the best of our knowledge. *Ta*IDH and *Ta*GDH genes were overexpressed in *E. coli* host cells, and thermal and pressure stabilities of the enzymes were assessed. Enzymatic reactions were conducted in a biphasic system (aqueous and supercritical CO_2), wherein supercritical CO_2 was also the carbon source. *Ta*IDH catalyzed the carboxylation reaction, while *Ta*GDH catalyzed the cofactor (nicotinamide adenine dinucleotide phosphate (NADPH)) regeneration (Fig. 1). It was found that coupling of NADPH regeneration reaction made the carboxylation reaction thermodynamically-favorable. pH, substrate concentration, reaction temperature, metal ion, and reaction time were optimized to improve yield. A preparative-scale reaction was performed successfully.

2. Materials and methods

2.1. Equipment and reagents

The polymerase chain reaction (PCR) was carried out using GeneAmp PCR System 9700 from Applied Biosystems (USA). Cell disruption was done using Ultrasonic Generator (Insonator 201 M) from Kubota (Tokyo, Japan). The UV-spectroscopic analysis was done using UV-1900-UV–vis spectrophotometer from Shimadzu (Kyoto, Japan). All high-pressure experiments were conducted using 10 mL of SUS316 pressure-resistant vessel from Taiatsu Techno (Tokyo, Japan). CO_2 was sent by Supercritical Peltier CO_2 Pump PU-2080−CO2 from JASCO (Tokyo, Japan). ^{1}H NMR spectra were recorded at 400 MHz on a Brucker Biospin Avance III 400A spectrometer at room temperature.

Reagents were purchased from Nacalai Tesque, Inc. (Japan) or Wako Pure Chemical Industries, Ltd. (Japan) and used without purification unless indicated otherwise. Genomic DNA of *Thermoplasma acidophilum* ATCC 25905D-5 for the cloning of *Ta*IDH and NBRC 15155 G for the cloning of *Ta*GDH was purchased from National Institute of Technology and Evaluation. Porcine heart IDH (phIDH) and *S. cerevisiae* glucose-6-phosphate dehydrogenase (G6PDH, Type XV, lyophilized powder) was purchased from Sigma-Aldrich®.

2.2. Cloning and overexpression of TaIDH and TaGDH

*Ta*IDH gene (*Ta*0117, KEGG) was amplified by PCR using commercially available purified genomic DNA of *T. acidophilum* ATCC, 25905D-5 using the following primers: FW (5′ - AAAAAGCTTATGGCATATTCAAGTGAAGGAG - 3′) and RV (5′ - AAAGAGCTCTTAGTGAACAGGTTTTTTCATCCT - 3′). *Ta*GDH (*Ta*0897, KEGG) was amplified from genomic DNA of *T. acidophilum* NBRC 15155 G using the following primers: FW (5′ - ACATAATGACTGAACAGAAAGCCAT - 3′) and RV (5′ - GCCATACACTGCCACTTTATCACCG - 3′). Genes encoding *Ta*IDH and *Ta*GDH were inserted to pET21b(+), and transformed to *E. coli* BL21 (DE3) and Rosetta™(DE3)pLysS, respectively.

Antibiotics used for cultivation was 125 μg/mL carbenicillin for BL21(DE3)-pET21b(+)-*Ta*IDH, and both of 125 μg/mL carbenicillin and 20 μg/mL chloramphenicol for Rosetta™(DE3)pLysS-pET21b(+)-*Ta*GDH. BL21(DE3)-pET21b(+)-*Ta*IDH and Rosetta™(DE3)pLysS-pET21b(+)-*Ta*GDH were pre-cultured in 5 mL Luria-Bertani (LB) medium at 37 °C and 250 rpm for 16−18 h. 2.5 mL of pre-cultured *E. coli* were transferred to 250 mL LB medium and cultured until OD_{600} reaches 0.5−0.7. Then, 2.5 mL of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, and it was cultured for 20 h at 20 °C and 250 rpm, centrifuged at 8000 G for 10 min, washed with 50 mL of 0.85 % (*m/v*) NaCl solution, centrifuged at 8000 G for 10 min, and homogenized with 10 mL of 100 mM KH_2PO_4-K_2HPO_4 buffer (pH 7) with 1 mM 1,4-dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF).Then, the solution underwent an ultrasonic lysis treatment (100 W, 30 min, 0 °C). The lysate was centrifuged at 12000 G for 30 min at 4 °C. The supernatant was heat-treated at 60 °C for 10 min and centrifuged at 12000 G for 30 min. Protein concentration was measured by the Bradford method. The expression levels and purity were analyzed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S1, Table S1). The resulting enzyme solutions (*Ta*IDH: 8 mL (19.1 mg protein/mL), *Ta*GDH: 8 mL (18.1 mg protein/mL)) were used directly for the small scale (section 2.5.1) and preparative scale (section 2.6) reactions. 20–40 times diluted solution was used for activity assay (sections 2.3 and 2.4). 10 times diluted solution was used as a product analysis reagent (section 2.5.2).

2.3. Activity assay

2.3.1. TaIDH decarboxylation activity assay

950 μL of 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH buffer (pH 6.5), 10 μL of 10 mM DL-isocitrate, 10 μL of 10 mM $MgCl_2$, 10 μL of *Ta*IDH solution were mixed and incubated at 37 °C for 15 min. 20 μL of a 10 mM $NADP^+$ was added, and the change in absorbance at 340 nm was measured. One unit of enzyme activity was defined as the micromoles of NADPH released by the decarboxylation and oxidation of DL-isocitrate per minute under the above-mentioned conditions.

2.3.2. TaGDH activity assay

960 μL of 100 mM HEPES-NaOH buffer (pH 6.5), 10 μL of 1 M D-glucose, 10 μL of *Ta*GDH solution were mixed and incubated at 37 °C for 15 min. 20 μL of 10 mM $NADP^+$ was added, and the change in absorbance at 340 nm was measured. One unit of enzyme activity was defined as the micromoles of NADPH released by the oxidation of D-glucose per minute under the above-mentioned conditions.

2.4. Stability of TaIDH and TaGDH

2.4.1. Thermostability

*Ta*IDH and *Ta*GDH aqueous solutions were incubated at 50 °C and 70 °C for 4 h. Then, the residual activities were measured using the

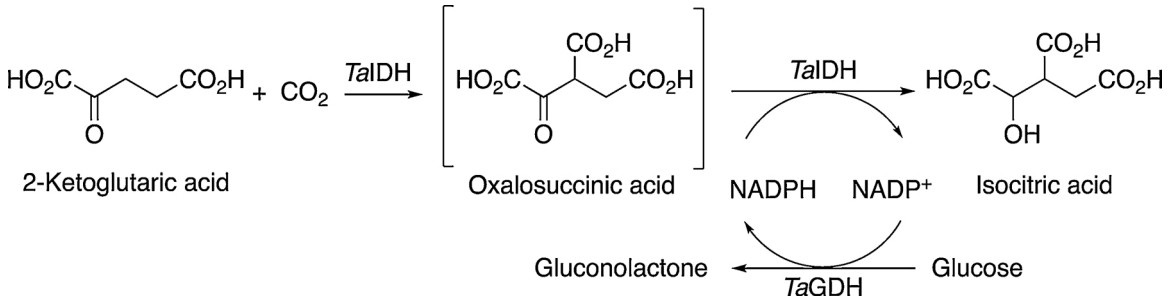

Fig. 1. Carboxylation of 2-ketoglutaric acid by the coupled-enzymatic reactions.

method in 2.3.

2.4.2. CO_2 pressure stability

Enzyme aqueous solutions were placed in 10 mL of SUS316 pressure-resistant vessel, pressurized with CO_2 to 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 MPa for *Ta*IDH and to 0.5, 1, 2, 5, 10, 15 MPa for *Ta*GDH, and kept at 35 °C for 1 h. CO_2 was liquefied by placing the vessel in ice and depressurized. Then, the residual activity was measured using the method in 2.3.

2.5. Analytical-scale carboxylation reaction

2.5.1. Carboxylation reaction procedure

0.9 g of D-glucose (final concentration of 1 M) dissolved in 3 mL of 500 mM HEPES buffer (pH 7), 100 μL of 1 M $MnCl_2$ solution (final concentration of 20 mM), 250 μL of 10 mM NADPH (final concentration of 0.5 mM), 2-ketoglutaric acid (20 mM), 1.25 mL of *Ta*GDH solution, 0.625 mL of *Ta*IDH solution, and 500 mM HEPES buffer (pH 7) to make the total volume of 5 mL were mixed in pressure-resistant vessel (10 mL). Then, CO_2 was pumped until the pressure is 10 MPa. The solution was stirred by a magnetic stirrer for 12 h at 30 °C. Reaction was quenched by depressurization. Above reactions were repeated at various pH (6, 6.5, 7, 7.5, 8, or 8.5), at various substrate concentration (10, 20, 30, or 40 mM), at various temperature (20, 30, 35, 40, 50, or 60 °C), using different metal salts with the same concentration ($MnCl_2$, $MgCl_2$, $CdCl_2$, $NiCl_2$, $CoCl_2$, $CaCl_2$, $FeCl_3$, Fe(II)SO_4, $CuSO_4$, KCl, or NaCl) or for various reaction time (0.5, 1, 3, 5, 12 or 16 h). The 2-ketoglutaric acid concentration was 10 mM for pH and metal ion investigation and 20 mM for the temperature and time course investigation.

2.5.2. Analytical method to determine yield of carboxylation

The carboxylation yield was determined by enzymatic analysis, a method used in determining the isocitric acid content of fruit juices [22, 23]. A calibration curve was prepared as follows: 20 μL of 1 M D-glucose, 20 μL of *Ta*IDH solution, 20 μL of 1.00 M $MgCl_2$, and 920 μL of various concentrations of isocitrate (0, 0.05, 0.075, 0.1, 0.15, 0.2 mM) in 500 mM pH 8.5 HEPES-NaOH buffer were incubated at 37 °C for 10 min. 20 μL of 5 mM $NADP^+$ was added, and the change in absorbance at 340 nm was measured to construct calibration curves (Fig. S2).

The yield of carboxylation reaction in 2.5.1 was determined as follows: the carboxylation reaction mixture (2.5.1) was heat-treated at 85 °C for 10 min and centrifuged at 12000 G, 4 °C for 20 min. 10 μL of the supernatant, 20 μL of *Ta*IDH solution, 20 μL of 1 M Mg^{2+}, and 930 μL of 500 mM pH 8.5 HEPES-NaOH buffer were incubated at 37 °C for 10 min. 20 μL of 5 mM $NADP^+$ was added. Then, the change in absorbance at 340 nm was measured. The concentration of the isocitric acid in the supernatant from the carboxylation reaction in 2.5.1 was determined using the calibration curve (Fig. S2, Table S2). The yield shown in Figs. 2–4 was calculated according to the following equations.

$$\text{Yield}(\%) = \frac{\text{Concentration of isocitric acid after reaction}}{\text{Initial concentration of 2-ketoglutaric acid}} \times 100$$

2.6. Preparative-scale carboxylation reaction

20 mM 2-ketoglutaric acid (58.4 mg, 0.4 mmol), 1 M glucose (3.6 g, 20 mmol), 20 mM $MnCl_2$ (50.4 mg, 0.4 mmol), 0.5 mM NADPH (7.44 mg, 0.01 mmol), 2.5 mL of *Ta*IDH solution, 5 mL of *Ta*GDH solution, and 500 mM pH 7.5 HEPES-NaOH buffer were mixed to make 20 mL of reaction mixture which was divided into 6 batches in pressure-resistant vessels (10 mL). Then, CO_2 was pumped until pressure was 10 MPa. The solution was stirred by a magnetic stirrer for 15 h at 35 °C. Reaction was quenched by depressurization.

The product, isocitrate, was esterified with ethanol to isolate from the aqueous solution (Fig. S3) as follows. The reaction solution was stored in −80 °C for a day and was freeze-dried (−49 °C, 9.8 mmHg) for 15 h. The resulting solid was mixed with 70 mL of dried ethanol, and 15 drops of 95 % sulfuric acid, and stirred and heated under reflux at 85 °C for 12 h. The reaction was quenched by immersing the flask in an ice bath and by adding 4 M NaOH solution dropwise until the pH becomes 6.5−7. Then, the product, triethyl isocitrate, was extracted, purified by silica gel chromatography (eluent hexane : ethyl acetate = 1 : 1), and identified by ^{1}H NMR analysis (Fig. S4) which was identical with that of the standard sample prepared by esterification of standard DL-isocitric acid trisodium salt. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 1.25−1.33(m, 9 H), 2.64(dd, 1H, J = 17.0, 6.2 Hz), 2.91(dd, 1H, J = 17.0, 8.2 Hz), 3.15(d, 1H, J = 5.6 Hz), 3.48−3.53(m, 1 H), 4.13−4.36(m, 7 H). Yield 53 % (58.8 mg, 0.213 mmol) for enzymatic carboxylation and esterification.

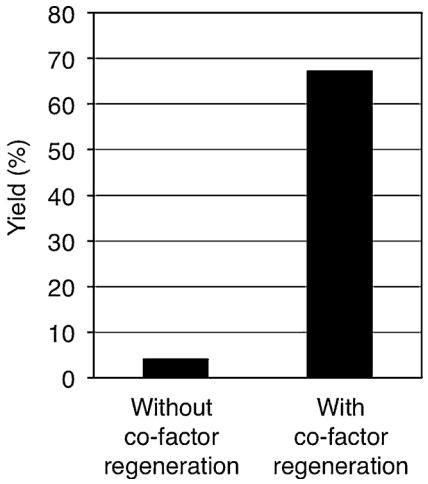

Fig. 2. Effect of co-factor regeneration on carboxylation of 2-ketoglutaric acid by *Ta*IDH. Initial condition: 20 mM 2-ketoglutaric acid, 0.625 mL *Ta*IDH, and 20 mM Mn^{2+}. For reaction without co-factor regeneration, 13.5 mM NADPH was added. For reaction with co-factor regeneration, 0.5 mM NADPH, 1 M glucose, and 1.25 mL *Ta*GDH were added. 500 mM HEPES-NaOH (pH 7) buffer was added to make the total volume to 5 mL. Reaction was conducted at 30 °C and 10 MPa for 16 h.

3. Results and discussions

3.1. Cloning and overexpression of TaIDH and TaGDH

*Ta*IDH and *Ta*GDH were overexpressed in the BL21(DE3) and Rosetta™(DE3)pLysS. *Ta*IDH obtained from both *E. coli* showed similar activities. On the other hand, Rosetta™(DE3)pLysS produced a higher concentration of *Ta*GDH than BL21(DE3). Therefore, *Ta*IDH was overexpressed in BL21(DE3), and *Ta*GDH was overexpressed in Rosetta™(DE3)pLysS, for further study. The SDS-PAGE result in Fig. S1(a) showed a large band at about 42 kDa derived from the overexpressed *Ta*IDH which is estimated to be 42 kDa from its gene size, and that in Fig. S1(b) showed a large band at about 44 kDa derived from the overexpressed *Ta*GDH that was estimated to be 44 kDa from its gene size. These results indicate the success of overexpression of *Ta*IDH and *Ta*GDH. The *Ta*IDH and *Ta*GDH were purified by 1.4 and 1.3 fold by heat treatment, resulting in the activity of 1.50 and 3.83 μmol/mg/min, respectively. Many *E. coli*-derived proteins can be removed by heat treatment since enzymes derived from *E. coli* have been reported to be inactivated at 60 °C or lower [24,25].

3.2. Thermal and pressure stability of enzymes

First, the thermostability of both *Ta*IDH and *Ta*GDH were examined. > 90 % of activities for both *Ta*IDH and *Ta*GDH were kept at 50 °C after 4 h. Moreover, at 70 °C, 42 % of activity for *Ta*IDH and 34 % of

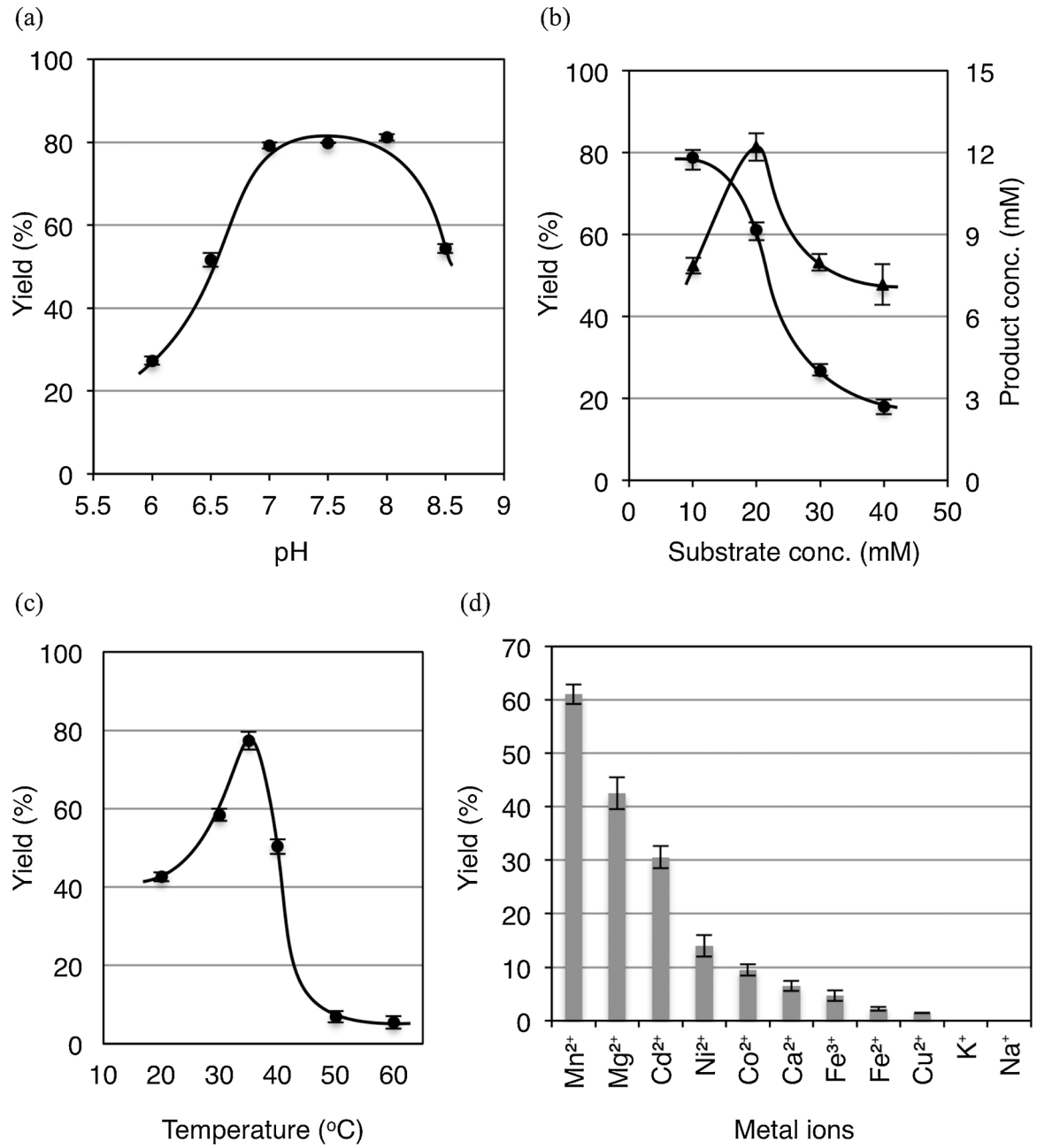

Fig. 3. Effect of (a) initial pH, (b) substrate concentration (circle: yield, triangle: product concentration), (c) reaction temperature, (d) metal ion on the carboxylation reactions. Initial condition: 10 mM (a, d) or 20 mM (c) 2-ketoglutaric acid, 1 M glucose, 0.625 mL *Ta*IDH, 1.25 mL *Ta*GDH, 0.5 mM NADPH, and 20 mM Mn^{2+}, 500 mM HEPES-NaOH buffer (pH 7) added to make the total volume to 5 mL, 10 MPa, 30 °C unless otherwise indicated.

activity for *Ta*GDH were kept after 4 h. The thermo-stabilities of both enzymes were excellent since they are derived from a thermophilic organism. Next, the CO_2 pressure stabilities of *Ta*IDH and *Ta*GDH were investigated at various pressure at 35 °C for 1 h. As examples of the commercially-available mesophilic enzymes, porcine heart IDH (phIDH) and *S. cerevisiae* glucose-6-phosphate dehydrogenase (*Sc*G6PDH) were also used to examine the CO_2 pressure stabilities. *Ta*IDH and *Ta*GDH maintained most of the activities (Fig. S5). On the other hand, phIDH and *Sc*G6PDH lost their activity to 3.6 % and 1%, respectively, even when pressurized at 1 MPa. *Ta*IDH and *Ta*GDH were shown to be suitable for high-pressure reactions. The excellent stabilities of enzymes from *T. acidophilum* under the high CO_2 pressure conditions are likely related to the structural rigidity. Thermostable proteins form a rigid conformation caused by salt bridges and hydrogen bonds, which results in compactness [26,27]. *Ta*IDH also contains structures that contribute to its thermostability such as an aromatic cluster which strengths subunit interactions [21]. This rigidity of *Ta*IDH can help in lessening denaturation under high CO_2 pressure conditions. A correlation of thermostability to pressure stability of enzymes applied to organic synthesis is suggested for the first time to the best of our knowledge, although the studies emphasized on the nutritional preservation of fruit enzymes have investigated thermostability and pressure stabilities of enzymes [28–31].

3.3. Optimization of carboxylation

First, carboxylation in an analytical scale was conducted using *Ta*IDH and NADPH in aqueous-supercritical CO_2 biphasic solvent. Previous studies on enzymatic carboxylation used various carbonates such as $KHCO_3$ [10,13,14] or $NaHCO_3$ [11] as a carbon source, but in this

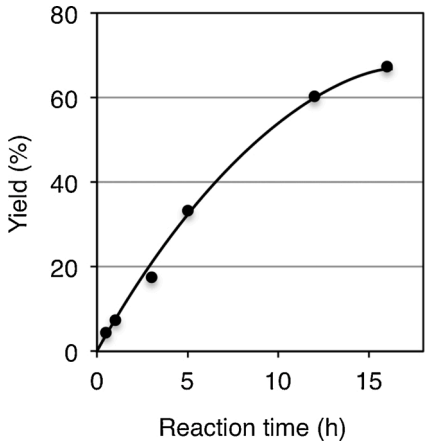

Fig. 4. Time-course of carboxylation reaction. Initial condition: 20 mM 2-ketoglutarate, 1 M of glucose, 0.625 mL *Ta*IDH, 1.25 mL *Ta*GDH, 0.5 mM NADPH, and 20 mM Mn^{2+}. 500 mM HEPES-NaOH buffer (pH 7) was added to make the total volume to 5 mL. Reaction was conducted at 30 °C and 10 MPa.

study, supercritical CO_2, which also served as a solvent, was the carbon source. The yield of reaction by *Ta*IDH and NADPH was only 4% yield (Fig. 2). To improve the poor yield, a co-factor regeneration system was investigated; *Ta*GDH and D-glucose were added. Amounts of the enzymes added for the reaction using 10 mL of reactor were fixed to be 87 U for *Ta*GDH (1.25 mL, 18.1 mg/mL, 3.83 U/mg) and 18 U for *Ta*IDH (0.625 mL, 19.1 mg/mL, 1.50 U/mg, U is defined as enzyme's decarboxylation activity.). In this system, the *Ta*GDH catalyzed reaction produces NADPH, which is necessary to overcome energetically unfavorable *Ta*IDH catalyzed carboxylation. Hence, higher a ratio of *Ta*GDH to *Ta*IDH was added. Besides, both enzymes were added in a large excess to achieve a high yield. Then, the carboxylation with coenzyme regeneration system resulted in a 67 % yield with a 16-fold increase (Fig. 2). Carboxylation reactions were achieved without the need of carbonates such as $NaHCO_3$. As shown in Fig. 1, the *Ta*GDH reaction functions as the exergonic component of the coupled reaction, aside from co-factor regeneration. At equimolar concentrations of $NADP^+$ and NADPH, and D-glucose and D-glucono-1,5-lactone, GDH reaction is exergonic from pH 6.6 towards more alkaline pH; while at equimolar concentrations of substrates and products, IDH reaction is exergonic from pH 5 towards acidic pH [32].

The effects of pH, substrate concentration, reaction temperature, and metal ions on the carboxylation reaction were analyzed. First, the effect of initial pH was examined. As shown in Fig. 3(a), the yield was optimal at the initial pH of 7–8 and decreased at pH 6.5 and lower pH than that or pH 8.5. However, it is reported that an acidic reaction solution (lower than pH 7) would render carboxylation reaction more thermodynamically favored [33] than decarboxylation. We suppose that the actual pH during the reaction was lower than the initial pH. As CO_2 gas is absorbed in the reaction solution, the formation of carbonic acid (H_2CO_3) occurs which would decrease the pH of the solution. Also, *Ta*GDH catalyzed NADPH regeneration reaction, increasing its net change in Gibbs energy at acidic pH [32], could be the reason for this. Therefore, the optimum initial pH is between 7–8, and higher pH than the optimum would cause a carboxylation reaction unfavorable, and lower pH would cause *Ta*GDH catalyzed reaction unfavorable. As IDHs have been reported to accept CO_2 molecule, but not bicarbonate ion (HCO_3^-), as a substrate [34], this enzyme may also accept CO_2 molecule. The pH affects the equilibrium position among CO_2(aq), H_2CO_3, and HCO_3^-, which in turn influences the CO_2(aq) concentration, the substrate of this reaction.

Next, the effect of substrate concentration on the carboxylation was examined. The range of substrate examined was from 10 mM to 40 mM since the preparative scale carboxylation reaction with the substrate concentration lower than 10 mM would be difficult, and the reaction above 40 mM was anticipated to result in low yields. The result is shown in Fig. 3(b). Yield decreases as substrate concentration increases. Examining the product concentration revealed that the solution with 20 mM substrate produced the highest concentration of product with 12 mM. While that with 10, 30, or 40 mM substrate produced about 8 mM of product. Therefore, 20 mM of substrate was used for preparative-scale reaction. The results of the highest yield at 10 mM and the highest product concentration at 20 mM can lead to a speculation that the rate of the reaction is higher with 20 mM than 10 mM. It might be due to the Michaelis constant, Km, of the enzyme with the substrate, while Km for the 2-ketoglutaric acid has been reported to be 0.85 mM for IDH from *Saccharomyces cerevisiae* and 1.1 mM for IDH from *Chlorobium limicola* at ambient pressure [34]. Another factor to be considered to explain the experimental results is an equilibrium of the reaction between carboxylation and decarboxylation. The equilibrium might reach at 10 mM but might not reach at 20 mM. However, it may not be reasonable since an excess amount of NADPH was supplied by the reaction of excess amounts of glucose and *Ta*GDH. The plausible reason for the lower yields and product concentrations at 30 and 40 mM may be due to the substrate inhibition.

The effect of reaction temperature on the carboxylation was examined. The optimal temperature was found to be 35 °C (Fig. 3(c)). The increase in the yield from 25 °C to 35 °C is probably due to the improvement in the enzyme activities; the *Ta*IDH activity was reported to be highest at 75 °C at ambient pressure [21], and the optimum temperature for *Ta*GDH activity is also estimated to be much higher than 25 °C since it is also from thermoacidophilic archaeon, *T. acidophilum* [21]. However, a sudden decrease in yield from 40 °C to 50 °C was observed despite that *Ta*IDH and *Ta*GDH are supposed to increase their activity along with temperature at ambient conditions. The enzyme activities may decrease at higher temperatures than 35 °C at high pressure (10 MPa), as reported previously for the case of other enzymes [35,36]. The lower yields from 40 °C onwards could be also due to lower gas (CO_2) absorption to the aqueous layer which results in lower yield. The carbon dioxide concentration in the aqueous layer is affected by both pH and temperature, which in turn may relate to the yield.

All known IDHs require a divalent metal cation such as Mg^{2+}or Mn^{2+} for catalysis [21,37]. As a divalent metal cation, Ca^{2+} was also used for the structural study [37], and Mn^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, and Cu^{2+} were reported to activate IDH from germinating *Vigna mungo* (*Vm*IDH) [38]. Therefore, to optimize the carboxylation reaction, *i.e.* to increase the yield, the metal ion is one parameter wherein we might be able to increase yield by using other metal ions aside from Mg^{2+} and Mn^{2+}. Therefore, the effect of various metal ions such as Mn^{2+}, Mg^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Ca^{2+}, Fe^{3+}, Fe^{2+}, Cu^{2+}, K^+, and Na^+ on the carboxylation was examined. The concentrations of the metal ions were fixed to be 20 mM to make them to be adequate since it was reported that the concentration of the metal ion (Mn^{2+}) required for the expression of 50 % activity of *Vm*IDH was reported to be 3.3 μM [38], and 52 mM Ca^{2+} and Mg^{2+} were used as a crystal soaking solution for the structural study of *E. coli* IDH [37]. As a result of the examination of the effect of various metal ions of 20 mM on carboxylation yield, it was found that Mn^{2+} ion has the highest yield as compared to other metal ions assessed in this study (Fig. 3(d)). It is followed by Mg^{2+}, and the yield decreased using other divalent ions. It is in accordance with the fact that Mn^{2+} and Mg^{2+} are both natural metal ions of IDH [37]. It is also in agreement with the decarboxylation activity of *Vm*IDH (Mn^{2+} 370 %, Mg^{2+} 190 %, Co^{2+} 166 %, Zn^{2+} 150 %, Cu^{2+} 133 %) [38] and *Mf*IDH (Mn^{2+} 100 %, Mg^{2+} 58 %, None 17 %) [39]. On the other hand, no product was produced when monovalent ions (K^+ and Na^+) were used for carboxylation in this study, which is consistent with the proposed mechanism of the *E. coli* IDH; the bidentate chelation of Mg^{2+} by the C1 carboxylate and C2 hydroxyl of the isocitrate is necessary to stabilize the negative charges formed on the hydroxyl oxygen during the transition states [37]. Finally, the time course of the reaction was examined. The yield plateaued after 16 h as shown in (Fig. 4).

3.4. Preparative-scale reaction

A preparative scale reaction was conducted under the optimum reaction conditions (20 mM 2-ketoglutaric acid, 20 mM $MnCl_2$, pH 7.5, 35 °C, 15 h). Since the hydrophilic product, isocitrate, is difficult to be isolated from the aqueous reaction mixture, esterification of the product with ethanol shown in Fig. S3 was carried out to transform it into a non-polar compound. The product, triethyl isocitrate, was successfully isolated in 58.8 mg yield (0.213 mmol, 53 %) for enzymatic carboxylation and esterification. It was identified by ^{1}H NMR spectroscopy (Fig. S4). This result confirmed the success to use gaseous carbon dioxide directly in enzymatic carboxylation.

4. Conclusion

Direct bi-enzymatic carboxylation under supercritical CO_2 was accomplished by producing isocitric acid from 2-ketoglutaric acid wherein supercritical CO_2 also functioned as a substrate. *Ta*IDH (carboxylation) was coupled with *Ta*GDH (cofactor regeneration), and the addition of cofactor regeneration increased the yield by 16-fold. High-pressure stabilities of both enzymes were observed, which could be due to their rigidity, a common property of thermostable enzymes [26,27,40]. We suggest the relation of thermostability to the high (CO_2) pressure stability of enzymes. Hence, other thermostable enzymes may also be suitable for reactions under pressurized CO_2. The reaction system works optimally with initial pH 7–8. The optimal reaction temperature is 35 °C, and a further increase in temperature would result in lower yields. Mn^{2+} ion shows the highest yield compared to ten other metal ions. With the optimized parameters, a preparative reaction scale was conducted successfully.

Author contribution statement

Kristian Ray Angelo Are: Conceptualization, Investigation, Data curation, Writing-Original draft preparation, Funding acquisition.
Shusuke Ohshima: Investigation.
Yusuke Koike: Investigation.
Yoshihisa Asanuma: Investigation.
Shogo Kashikura: Investigation.
Mayumi Tamura: Investigation, Funding acquisition.
Tomoko Matsuda: Supervision, Writing-Reviewing and Editing, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

This work was supported by the JSPS KAKENHI Grant Number JP19K05560 and Kobayashi Foundation. The authors are thankful to the Biomaterials Analysis Division, Open Facility Center, Tokyo Institute of Technology, for DNA sequencing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2021.108004.

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