



Conversion of D-glucose to L-lactate via pyruvate by an optimized cell-free enzymatic biosystem containing minimized reactions

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ABSTRACT

Cell-free synthetic enzymatic biosystem is emerging to expand the traditional biotechnological mode by utilizing a number of purified/partially purified enzymes and coenzymes in a single reaction vessel for the production of desired products from low-cost substrates. Here, a cell-free synthetic biosystem containing minimized number of reactions was designed for the conversion of D-glucose to L-lactate via pyruvate. This NADH-balanced biosystem was comprised of only 5 thermophilic enzymes without ATP supplementation. After optimization of enzyme loading amounts, buffer concentration and cofactor concentration, D-glucose was converted to L-lactate with a product yield of ~90%. Our study has provided an emerging platform with potentials in producing pyruvate-derived chemicals, and may promote the development of cell-free synthetic enzymatic biosystems for biomanufacturing.

1. Introduction

Cell-free synthetic enzymatic biosystem is an emerging biomanufacturing platform containing a number of enzymes and coenzymes in a single reaction vessel for the production of chemicals [1–3]. This biosystem has some obvious advantages compared with traditional microbial fermentation, including high product yields, fast reaction rates, broad reaction conditions, uncomplicated scale up, easy reaction control and optimization [4–6]. To date, a variety of products, such as alcohols [7], organic acids [8], carbohydrates [9], isoprene [10], fine chemicals [11], bioplastic [12], hydrogen [13], and electricity [14] have been produced by many cell-free synthetic enzymatic biosystems. The design principles for such cell-free synthetic enzymatic biosystems on an industrial scale include using thermophilic enzymes without any cofactor or with a closed cofactor balance at least, minimizing the number of enzymes, and making the last step of the biosystem irreversible for the high product yield [3].

Pyruvate, the final product of glycolysis, is an important platform chemical in biochemistry. Many studies have used cell-free synthetic enzymatic biosystems to produce pyruvate from D-glucose, further to produce high value products, like n-butanol [15], isoprene [10], monoterpenes [16], 2,3-butanediol [17], and malate [8]. In previous

studies, pyruvate is always produced *in vitro* from D-glucose by about 10 glycolytic enzymes, generating two net NADH and two net ATP from one D-glucose. Numerous enzymes and non-balanced ATP of such cell-free biosystems result in system complexity, and thus limited the application of these biosystems on an industrial scale.

Inspired by the non-phosphorylative Entner-Doudoroff-Pathway (np-ED) derived from hyperthermophilic archaea [18], we designed a cell-free synthetic biosystem for the conversion of D-glucose to L-lactate via pyruvate. This biosystem is comprised of only 5 enzyme-catalyzed reactions, with balanced nicotinamide cofactors and without ATP involved (Fig. 1). These five enzymes are glucose dehydrogenase (EC 1.1.1.47, GDH) from *Sulfolobus solfataricus*, dihydroxy acid dehydratase (EC 4.2.1.9, DHAD) from *S. solfataricus*, 2-keto-3-deoxygluconate aldolase (EC 4.1.2.14, KDGA) from *Sulfolobus acidocaldarius*, glyceraldehyde dehydrogenase (EC 1.2.1.3, ALDH) from *Thermoplasma acidophilum*, and L-lactate dehydrogenase (EC 1.1.1.27, L-LDH) from *Thermotoga maritima*. These 5 enzymes were all from thermophilic strains, enabling simple enzyme preparation, and hence increasing their potential in industrial application. High-yield L-lactate (more than 90%) was produced through the optimization of reaction conditions including enzyme loading amounts, buffer concentration, and cofactor concentration. This thorough optimization sheds light on applying this cell-

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Table 1
The list of enzymes and their properties.

NO.	Plasmid	Enzyme	Abbreviation	Source	KEGG ORF	EC	Opt. T(°C)	Reaction	Specific activity ^a (U/mg)
1	pET28a-Ssgdh	Glucose dehydrogenase	GDH	<i>Sulfolobus Solfataricus</i>	SSO3003	1.1.1.47	70	D-glucose + NAD ⁺ → D-glucono-1,5-lactone + NADH + H ⁺	12.5
2	pET28a-Ssdhad	Dihydroxy acid dehydratase	DHAD	<i>S. solfataricus</i>	SS03107	4.2.1.9	70	(1) D-gluconate → 2-keto-3-deoxygluconate + H ₂ O (2) Glycerate → Pyruvate + H ₂ O	0.1 0.008
3	pET28a-Sackdga	2-keto-3-desoxygluconate aldolase	KDGA	<i>Sulfolobus acidocaldarius</i>	Saci0225	4.1.2.14	99 [7]	2-keto-3-deoxygluconate → Pyruvate + Glyceraldehyde	1.2
4	pET28a-Taaldh	Glyceraldehyde dehydrogenase	ALDH	<i>Thermoplasma acidophilum</i>	Ta0809	1.2.1.3	63	Glyceraldehyde + NAD ⁺ + H ₂ O → Glycerate + NADH + H ⁺	1.0
5	pET20b-Tmldh	L-lactate dehydrogenase	L-LDH	<i>Thermotoga maritima</i>	TM1867	1.1.1.27	85	Pyruvate + NADH + H ⁺ → L-Lactate + NAD ⁺	98

^a The specific activity of enzymes was determined at 50 °C.

of NADH at 340 nm ($\epsilon_{\text{NADH}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). The assay mixture contained 50 mM D-glucose, 2.0 mM NAD⁺, and 5.0 mM MgCl₂ in 100 mM HEPES-NaOH buffer (pH 7.0) [7].

The activity of DHAD for gluconate was assayed at 50 °C by measuring the reduction of NADH at 340 nm. The assay mixture contained 100 mM HEPES-NaOH buffer (pH 7.0), 5.0 mM MgCl₂, 0.3 mM NADH, 1.0 U/mL TmLDH, 20 mM gluconate, and excess amount of SackDGA [7,23].

The activity of KDGA was assayed at 50 °C by measuring the reduction of NADH at 340 nm. The assay mixture contained 100 mM HEPES-NaOH buffer (pH 7.0), 5.0 mM MgCl₂, 0.3 mM NADH, 2.0 U/mL SsdHAD, 1.0 U/mL TmLDH, and 20 mM gluconate [24].

The activity of ALDH was assayed at 50 °C by measuring the increase of NADH at 340 nm. The assay mixture contained 100 mM HEPES-NaOH buffer (pH 7.0), 5.0 mM MgCl₂, 4.0 mM NAD⁺, and 5.0 mM D,L-glyceraldehyde [7,19].

The activity of DHAD for glycerate was assayed at 50 °C by measuring the reduction of NADH at 340 nm. The assay mixture contained 100 mM HEPES-NaOH buffer (pH 7.0), 5.0 mM MgCl₂, 0.3 mM NADH, 1.0 U/mL TmLDH, and 1.0 mM glycerate.

The activity of lLDH was assayed at 50 °C by measuring the reduction of NADH at 340 nm. The assay mixture contained 100 mM HEPES-NaOH buffer (pH 7.0), 5.0 mM MgCl₂, 0.3 mM NADH, and 1.0 mM pyruvate [25].

One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of product per minute. Unless otherwise stated, each measurement was conducted in triplicate.

2.5. L-lactate synthesis and intermediate assays

One-pot biosynthesis of L-lactate was conducted in a 1.0-mL reaction system containing 100 mM HEPES-NaOH buffer (pH 7.0), 27.8 mM D-glucose, 5.0 mM NAD⁺, 5.0 mM MgCl₂, as well as GDH, DHAD, KDGA, ALDH, and LDH (equal unit, 1U/mL) at 50 °C. An aliquot (65 μL) of the reaction sample was withdrawn and mixed with 35 μL of 1.9 M HClO₄ to stop the reaction. The pH value of the reaction solution was then adjusted to neutral with 13 μL of 5.0 M KOH. Gluconate, pyruvate, glycerate, D-, L-glyceraldehyde and L-lactate were determined by high performance liquid chromatography (HPLC) (Shimadzu, Japan) equipped with a Bio-Rad HPX-87H column with 5.0 mM H₂SO₄ as a mobile phase and a refractive index detector. The concentration of D-glucose was determined by D-glucose assay kit (Appligen Technologies Inc, Beijing, China).

2.6. Effect of enzyme and coenzyme concentrations on L-lactate production

Enzyme loading amounts were optimized in 100 mM HEPES-NaOH buffer (pH 7.0) containing 27.8 mM D-glucose, 5.0 mM NAD⁺ and

5.0 mM MgCl₂ at 50 °C (the initial reaction condition). The concentration of L-lactate was determined at the reaction time of 12 h. The concentration of GDH was varied from 0 to 2.0 U/mL while the other four enzymes were loaded at 1.0 U/mL for each. After the optimal loading amount of GDH was settled, DHAD was changed from 0 to 3.0 U/mL while the other three enzymes, KDGA, ALDH, and LDH, were loaded at 1.0 U/mL for each. After the optimal loading amounts of GDH and DHAD were both settled, the concentration of KDGA was adjusted from 0 to 2.0 U/mL, while ALDH and LDH were loaded at 1.0 U/mL. Then, after the optimal loading amounts of GDH, DHAD, and KDGA were settled, ALDH was varied from 0 to 2.0 U/mL, while LDH was loaded at 1.0 U/mL. And finally, after the concentrations of GDH, DHAD, KDGA and ALDH were settled, the concentration of LDH was varied from 0 to 2.0 U/mL to determine the optimal loading amount. The coenzyme of NAD⁺ was varied from 0 to 10 mM for optimization after the concentrations of all enzymes were determined.

2.7. Effect of buffer concentration on L-lactate production

The effect of different concentrations of HEPES-NaOH buffer was determined in a reaction system containing 27.8 mM D-glucose, 5.0 mM MgCl₂ at 50 °C with the optimal enzyme ratios and coenzyme concentration for the production of L-lactate from glucose. The concentration of L-lactate was measured at the reaction time of 12 h. The concentrations of HEPES-NaOH buffer was ranged from 0 to 400 mM.

3. Result

3.1. Pathway design

A cell-free enzymatic synthetic biosystem containing only 5 enzymes has been constructed to convert D-glucose to pyruvate, and further to L-lactate (Fig. 1A). Within this biosystem, D-Glucose is first converted to gluconate by GDH, generating 1 NADH from 1 NAD⁺. Gluconate is converted to 2-keto-3-deoxygluconate by DHAD, and then to pyruvate and glyceraldehyde by KDGA. Subsequently, glyceraldehyde is converted to glycerate by ALDH, generating 1 NADH from 1 NAD⁺. And glycerate is converted to pyruvate by DHAD. Finally, pyruvate is converted to L-lactate by lLDH which consumes NADH and produces NAD⁺. As a result, 2 molecules of L-lactate can be synthesized from 1 molecule of D-glucose, while NAD⁺/NADH in the system was balanced. The standard Gibbs free energy changes (ΔG°) of overall reaction is -188.6 kJ/mol at pH 7.0 and ionic strength = 0.1 M (<http://equilibrator.weizmann.ac.il/>) (Fig. 1B). Although the reaction of converting 2-keto-3-deoxygluconate to pyruvate and glyceraldehyde by KDGA is thermodynamically unfavorable, the downstream reaction steps are all exergonic, especially the one catalyzed by LDH. LDH catalyzes the last step of the cascade reaction with a standard Gibbs free

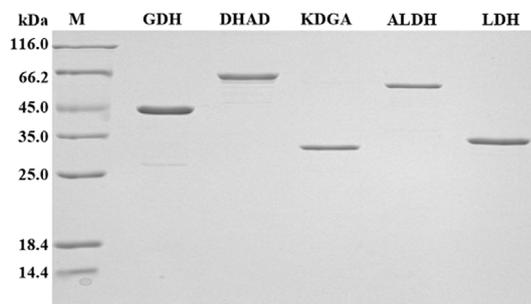


Fig. 2. SDS-PAGE gel analysis of recombinant enzymes in one-pot biosynthesis of L-lactate from D-glucose. M, protein marker. The molecular weights of GDH, DHAD, KDGA, ALDH, and LDH were approximately 42, 60, 33, 57, and 35 kDa according to the protein marker, respectively.

energy change of -27.5 kJ/mol, indicating this reaction is almost irreversible. The irreversible reaction of LDH, together with the exergonic reactions of ALDH and DHAD, help to push the overall reaction toward completeness. The analysis of thermodynamic data indicates the yield of L-lactate based on D-glucose can be almost 100%.

3.2. Enzyme preparation and enzyme activities

All the enzymes were expressed in the soluble form in *E. coli* BL21(DE3). All the enzymes were purified by heat precipitation, followed by nickel-resin purification (Fig. S1). All the enzymes were purified to homogeneity as shown in Fig. 2. The molecular weights of GDH, DHAD, KDGA, ALDH, and LDH were approximately 42, 60, 33, 57, and 35 kDa, respectively, which were in agreement with their theoretical molecular weight values. The specific activities of GDH, KDGA, ALDH, and LDH were 12.5, 1.2, 1.0, and 98 U/mg at 50 °C, respectively. The specific activity values of DHAD (purified by heat-treatment) [23] on gluconate and glycerate were 0.1 and 0.008 U/mg at 50 °C, respectively (Table 1).

3.3. L-lactate synthesis under initial reaction conditions

HPLC was used to quantify the concentrations of L-lactate. The height of L-lactate peak is proportional to the concentration of L-lactate (Fig. S2), and the retention time of L-lactate was 12.9 min which showed no significant overlap with any of the substrate or intermediates (Fig. S3 and Table S1). The same HPLC method was also able to separate the other two intermediates, glycerate and glyceraldehyde. The amount of D-glucose was determined by the glucose assay kit, as the retention time of D-glucose analyzed by HPLC was 9.4 min which had a significant overlap with that of the reaction intermediate, gluconate (Fig. S3 and Table S1).

Because ALDH was not stable enough at temperatures above 50 °C, and Maillard reaction between D-glucose and proteins, which can deactivate enzymes, happen at high temperatures easily [26], and the optimal pH of DHAD, which is the rate-limited enzyme, was 7.0 in HEPES-NaOH buffers, the one-pot biosynthesis of L-lactate by the cell-free synthetic biosystem containing GDH, DHAD, KDGA, ALDH, and LDH (1.0 U/mL for each) was conducted at 50 °C to convert D-glucose to L-lactate in 100 mM HEPES-NaOH buffer (pH 7.0) containing 27.8 mM D-glucose, 5.0 mM NAD^+ , and 5.0 mM MgCl_2 . As shown in Fig. 3 and Fig. S4, the concentration of L-lactate was 31.2 mM with a product yield of 56% after reacting at 50 °C for 20 h. The further increase of L-lactate can be ignored, and the residual D-glucose was approximately 3.72 mM at 20 h. The concentration of glycerate was about 8.29 mM after 20 h, accounting for the most part of the intermediates.

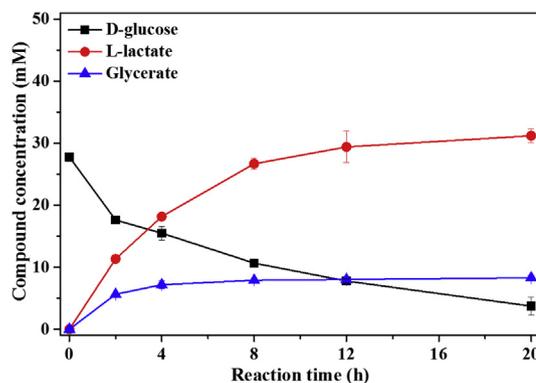


Fig. 3. L-lactate production from D-glucose via pyruvate by *in vitro* enzymatic synthetic biosystem under initial reaction conditions. The reaction was performed at 50 °C in 100 mM HEPES-NaOH buffer (pH 7.0) containing 27.8 mM D-glucose, 5.0 mM MgCl_2 , 1.0 U/mL GDH, 1.0 U/mL DHAD, 1.0 U/mL KDGA, 1.0 U/mL ALDH, 1.0 U/mL LDH, and 5.0 mM NAD^+ . Values shown are means of triplicate determinations.

3.4. Optimization of reaction conditions for a high L-lactate yield

We performed the optimization of enzyme loading amounts, cofactor concentration, and buffer concentration to achieve a high yield of L-lactate produced from glucose. Enzyme loading amounts were optimized under the initial reaction condition. We first adjusted the loading amount of GDH and found that the yield of L-lactate rapidly increased from 0 to 52% when GDH concentration increased from 0 to 1.0 U/mL. When more than 1.0 U/mL of GDH was used, the L-lactate yield leveled off (Fig. 4A). Therefore, the optimal GDH loading was 1.0 U/mL (As shown in red circle) which was used for further optimization of the loading amounts of other enzymes. When more than 2.0 U/mL of DHAD was used, the L-lactate yield leveled off (Fig. 4B), suggesting that the optimal DHAD loading was 2.0 U/mL. Using the same method, the optimal enzyme loading values of KDGA, ALDH, and LDH were all found to be 1.0 U/mL (Fig. 4C–E). After the enzyme loading values were determined, the loading concentration of cofactor was optimized to be 5 mM for L-lactate production (Fig. 4F). The effect of different concentrations of HEPES-NaOH buffer on L-lactate production from D-glucose was also determined. The L-lactate yield was increased as the concentration of HEPES-NaOH buffer (pH 7.0) increased (Fig. 5). However, high concentration of HEPES-NaOH buffer results in high product cost. Considering the trade-off of product cost and yield, 200 mM was chosen as the optimal HEPES-NaOH buffer concentration. As a result, the optimal reaction condition for converting 27.8 mM D-glucose to L-lactate was 200 mM HEPES-NaOH buffer (pH 7.0) containing 5.0 mM NAD^+ , with the loadings of GDH, DHAD, KDGA, ALDH, and L-LDH being 1.0, 2.0, 1.0, 1.0, and 1.0 U/mL, respectively.

3.5. L-lactate production under optimized conditions

Under the optimal reaction condition, 27.8 mM D-glucose was used to produce L-lactate by cell-free synthetic enzymatic biosystem containing 5 enzymes at 50 °C. As shown in Fig. 6, at hour 8, the concentration of D-glucose decreased to about 0, while the concentration of L-lactate reached 48.2 mM, and then leveled off. The yield of L-lactate from D-glucose was about 90%, which was much higher than the yield (56%) under non-optimized conditions. 4.7 mM glycerate was found when the reaction reach equilibrium, which was much lower than the glycerate concentration under the initial reaction condition as shown in Fig. 3.

4. Discussion

In this study, we constructed a cofactor-balanced and ATP-free cell-

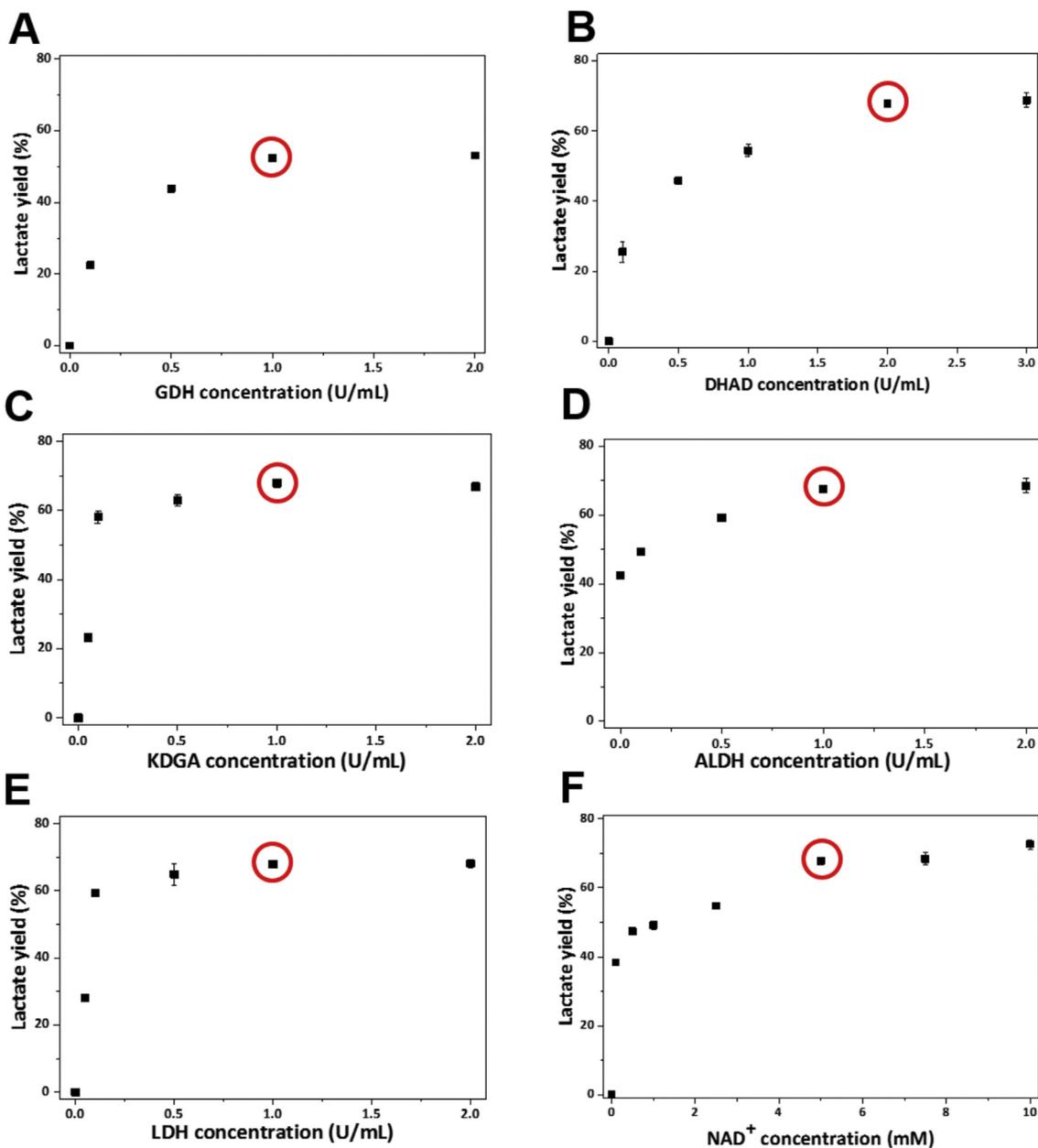


Fig. 4. Optimization of the reaction conditions for *L*-lactate production from *D*-glucose. The reaction was performed at 50 °C in 100 mM HEPES-NaOH buffer (pH 7.0) containing 27.8 mM *D*-glucose and 5.0 mM MgCl₂. (A) Effect of GDH concentration from 0 to 2.0 U/mL on *L*-lactate production in the presence of 1.0 U/mL DHAD, 1.0 U/mL KDGA, 1.0 U/mL ALDH, and 1.0 U/mL LDH. (B) Effect of DHAD concentration from 0 to 3.0 U/mL on *L*-lactate production in the presence of 1.0 U/mL GDH, 1.0 U/mL KDGA, 1.0 U/mL ALDH, and 1.0 U/mL LDH. (C) Effect of KDGA concentration from 0 to 2.0 U/mL on *L*-lactate production in the presence of 1.0 U/mL GDH, 2.0 U/mL DHAD, 1.0 U/mL ALDH, and 1.0 U/mL LDH. (D) Effect of ALDH concentration from 0 to 2.0 U/mL on *L*-lactate production in the presence of 1.0 U/mL GDH, 2.0 U/mL DHAD, 1.0 U/mL KDGA, and 1.0 U/mL LDH. (E) Effect of LDH concentration from 0 to 2.0 U/mL on *L*-lactate production in the presence of 1.0 U/mL GDH, 2.0 U/mL DHAD, 1.0 U/mL KDGA, and 1.0 U/mL ALDH. (F) Effect of coenzyme concentration on *L*-lactate production from 0 to 10 mM in the presence of 1.0 U/mL GDH, 2.0 U/mL DHAD, 1.0 U/mL KDGA, 1.0 U/mL ALDH, and 1.0 U/mL LDH. The points in the open red circle indicate the optimal condition for each parameter. Values shown are means of triplicate determinations.

free synthetic enzymatic biosystem containing only 5 enzymes for the synthesis of *L*-lactate from *D*-glucose via pyruvate. The number of enzymes of this biosystem for the conversion of *D*-glucose to pyruvate is the fewest compared with the other biosystems which have the same input (glucose) and output (pyruvate) [8]. In our system, pyruvate is converted to *L*-lactate by one enzyme, enabling the balance of the intrapathway consumption and regeneration of cofactors. Under the initial reaction condition, the yield of *L*-lactate from 27.8 mM (5 g L⁻¹) *D*-glucose was only 56% which was accompanied with a relatively slow consumption of *D*-glucose and a high accumulation of the intermediate, glycerate. At 50 °C, Maillard reaction between *D*-glucose and proteins

may result in less *D*-glucose available as well as deactivated enzymes which may both account for the low yield of *L*-lactate. After optimization of the reaction conditions including enzyme loading amounts, cofactor concentration and buffer concentration, the consumption of *D*-glucose was more rapid, resulting in less accumulation of glycerate and an improvement of the yield of *L*-lactate from 56% to 90%.

Comparing with the initial reaction condition, two main parameters of the optimal condition were altered: the loading amount of DHAD was raised from 1.0 U/mL to 2.0 U/mL, and the concentration of HEPES-NaOH buffer was raised from 100 mM to 200 mM. These two factors might account for the low yield of *L*-lactate under the initial condition.

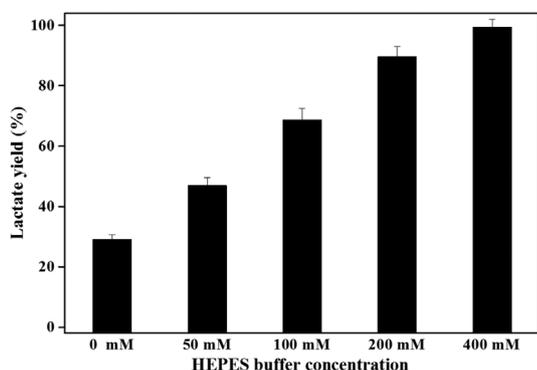


Fig. 5. Effect of concentration of HEPES-NaOH buffer (pH 7.0) on the L-lactate production from D-glucose. The reaction was performed at 50 °C in different concentration of HEPES-NaOH buffer (pH 7.0) containing 27.8 mM D-glucose, 5.0 mM NAD⁺, 5.0 mM MgCl₂, 1.0 U/mL GDH, 2.0 U/mL DHAD, 1.0 U/mL KDG, 1.0 U/mL ALDH, and 1.0 U/mL LDH. Values shown are means of triplicate determinations.

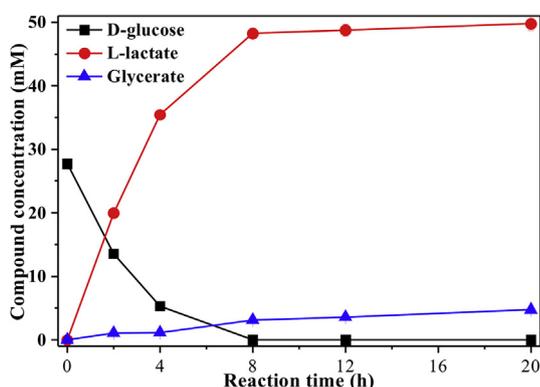


Fig. 6. L-lactate production from glucose under optimized conditions. The reaction was performed at 50 °C in 200 mM HEPES-NaOH buffer (pH 7.0) containing 27.8 mM D-glucose, 5.0 mM NAD⁺, 5.0 mM MgCl₂, 1.0 U/mL GDH, 2.0 U/mL DHAD, 1.0 U/mL KDG, 1.0 U/mL ALDH, and 1.0 U/mL LDH. Values shown are means of triplicate determinations.

In our cell-free biosystem, DHAD catalyzes both the conversion of gluconate to 2-keto-3-deoxygluconate and the conversion of glycerate to pyruvate. The specific activity of DHAD of these two reactions are only 0.1 and 0.008 U/mg at 50 °C, respectively, which are 1 or 2 order-of-magnitude lower than the activities of the other enzymes in our system. As the loading amount of DHAD was based on the enzyme activity on converting gluconate to 2-keto-3-deoxygluconate, the actual unit usage of DHAD on converting glycerate to pyruvate was only about 1/10 of converting gluconate to 2-keto-3-deoxygluconate. Thus, the utilization of glycerate by DHAD became a rate-limiting step, resulting in the accumulation of glycerate as shown in Fig. 3. Increasing the loading amount of DHAD decreased the accumulation of glycerate and enhanced lactate product yield, as shown in Figs. 4B and 6. However, such a low activity of DHAD resulted in a high enzyme loading amount (more than 20 mg/mL of DHAD) for a satisfactory L-lactate yield from D-glucose, which prohibits the future industrialization of this cell-free synthetic pathway. To tackle this problem, the activity of DHAD on converting glycerate to pyruvate should be increased by multiple protein engineering techniques including rational design and site-directed saturated mutagenesis [27,28]. The requirement for a high concentration of buffer to achieve a high product yield (Fig. 5) may due to the lowering of pH by the L-lactate produced. CaCO₃ and Ca(OH)₂ can be added to control the pH when this system is applied in an industrial scale in the future [29]. We also noticed that the consumption of D-glucose under the initial reaction condition was much lower than that

under the optimized condition. The slow metabolism of D-glucose provided a higher chance of Maillard reaction, which is a non-enzymatic condensation reaction of carbonyl groups on reducing sugar (for example, D-glucose) with amino groups on protein, peptides, and amino acids at high temperature [30,31]. Maillard reaction also causes the deactivation of enzymes [26], and may be another reason to explain the low product yield of L-lactate under the initial condition.

Pyruvate is an important platform chemical. The enzymatic pathway of converting glucose to pyruvate described in this study has the minimized number of enzymes, generating two net NADH per glucose. Many valuable chemicals can be produced from glucose via pyruvate using this cell-free biosystem by adding some specific enzymes. For example, L-alanine can be produced from pyruvate by L-alanine dehydrogenase (EC 1.4.1.1) with supplement of inorganic ammonia [32]. Terpenoids can be produced from pyruvate through the mevalonate pathway [16,33]. Polyhydroxybutyrate (PHB), ethanol, and isobutanol can also be produced from pyruvate by defined enzymes [7,12,34]. Therefore, the cell-free synthetic biosystem described in this study has the potential to serve as a next generation bio-production system. The optimization process of this cell-free synthetic biosystem converting D-glucose to L-lactate sheds light on further application of this biosystem in producing other valuable chemicals.

L-lactate can be produced by either fermentative production routes or *in vitro* synthetic routes. Currently, nearly all of the L-lactate around the world is produced by the fermentative method, which has been extensively investigated and well optimized [35]. For example, Pal and Dey [36] designed a three-stage membrane-integrated hybrid reactor system for L-lactate production by *Lactobacillus delbrueckii* (NCIM-2025) and obtained L-lactate at high yield (0.96 g g⁻¹), rapid productivity (12.4 g L⁻¹ h⁻¹), high concentration (250 g L⁻¹) and satisfactory purity (95%). Recently, with the development of *in vitro* synthetic biology, L-lactate has been produced by many *in vitro* synthetic enzymatic pathways from raw materials like D-glucose and glycerol [37,38]. For example, Honda and his coworkers demonstrated an alternative *in vitro* ATP-balanced enzymatic pathway that converted glycerol to L-lactate in an almost stoichiometric manner, and produced ~14.7 mM L-lactate in 7 h with a productivity of 0.04 mM/min [37]. Compared to their *in vitro* enzymatic biosystems, our system used fewer number of enzymes and obtained a higher L-lactate concentration (~48.2 mM in 8 h) as well as better productivity (0.10 mM/min). At present, the L-lactate concentration and productivity of *in vitro* biosystem cannot be comparable with the fermentative routes. Yet with the development of enzyme engineering and enzyme immobilization, we believe that the production of L-lactate by *in vitro* synthetic enzymatic biosystem would have a bright prospect [3].

In conclusion, we demonstrated the one-pot biosynthesis of L-lactate from D-glucose via pyruvate through a cell-free synthetic enzymatic biosystem containing minimized reactions using thermophilic enzymes. Approximately 49.8 mM L-lactate was produced from 27.8 mM D-glucose within 20 h after optimization of the reaction conditions including enzyme loading amounts, cofactor concentration, and buffer concentration. The optimized reaction conditions in this study provide some useful information to produce various chemicals with D-glucose as a starting material and pyruvate as an intermediate by this minimized cell-free synthetic biosystem.

Declarations of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.synbio.2018.05.003>.

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