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ORIGINAL PAPER

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Effect of carbon sources differing in oxidation state and transport route on succinate production in metabolically engineered *Escherichia coli*

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Abstract In mixed-acid fermentation, succinate synthesis requires one mole of phosphoenolpyruvate (PEP), one mole of CO_2, and two moles of NADH for every mole of succinate to be formed. Different carbon sources with different properties were used to address these requirements. Sorbitol generates one more mole of NADH than glucose. Fermentation of sorbitol was shown in this study (and by others) to produce significantly more succinate than fermentation of glucose, due to increased NADH availability. Xylose fermentation conserves the intracellular PEP pool, since its transport does not require the phosphotransferase system normally used for glucose transport. The extra PEP can then be assimilated in the succinate pathway to improve production. In this study, fermentation of xylose did yield higher succinate production than glucose fermentation. Subsequent inactivation of the acetate and lactate pathways was performed to study metabolite redistribution and the effect on succinate production. With the acetate pathway inactivated, significant carbon flux shifted toward lactate rather than succinate. When both acetate and lactate pathways were inactivated, succinate yield ultimately increased with a concomitant increase in ethanol yield.

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Introduction

Succinate is a C_4-dicarboxylic acid with broad industrial value. It acts as a potential precursor for many commercial chemicals, such as 1,4-butanediol, with applications in the pharmaceutical, agricultural, fine chemical, and polymer industries [23]. Traditionally, succinate is produced through petrochemical processes that are expensive and can lead to pollution problems. Recently, efforts shifted toward microbial fermentation as a viable alternative for the production of succinate. The success of fermentation processes with renewable feedstocks can potentially supplant energy-intensive processes that use nonrenewable sources [12].

The use of recombinant DNA technology to alter pathways in *Escherichia coli* has been successful in increasing succinate production. By amplifying enzymatic steps involved in the succinate pathway, higher succinate production can be achieved. An example of this was demonstrated when native *E. coli* phosphoenolpyruvate carboxylase (PEPC) was overexpressed in *E. coli* [9], which increased succinate production. Conversion of fumarate to succinate was enhanced by overexpressing fumarate reductase in *E. coli* [5, 19]. Certain pathways are not indigenous in *E. coli*, but can potentially improve succinate production. By introducing pyruvate carboxylase from *Rhizobium etli* into *E. coli*, succinate production was enhanced [4]. Other metabolic engineering strategies include inactivating competing pathways of the desired product. Inactivating pyruvate formate lyase and lactate dehydrogenase (LDH) while overexpressing malic enzyme significantly increased succinate production [14]. An inactive glucose phosphotransferase system (PTSG) was also shown to yield higher succinate production and better cell growth in the same mutant *E. coli* [3].

Under anaerobic conditions, *E. coli* undergoes mixed-acid fermentation to produce acetate, ethanol, formate, and lactate, with succinate as a minor product. The succinate synthesis pathway branches from the glycolytic backbone at the phosphoenolpyruvate (PEP) node (Fig. 1). PEP is converted to oxaloacetate (OAA) by PEPC through a carboxylation reaction. For every mole of succinate to be formed, one mole of CO_2 and two moles of NADH are required (Fig. 1). PEPC is important in enhancing succinate production [9] and various PEPC enzymes from different species have been characterized [1, 8, 10, 11, 15, 16, 17]. In particular, PEPCs from plant species draw much attention due to their natural involvement in CO_2 fixation. For this reason, a mutant PEPC from a plant species (*Sorghum*) is overexpressed in all the strains used in the study. This mutant PEPC is particularly insensitive to malate feedback inhibition [20]. Therefore, the mutation is advantageous and favorable for improving succinate production.

Different carbon sources are used to examine the pathway requirements of succinate. Sorbitol, with an oxidation state of −1, generates 1 mol of NADH more than glucose per mole consumed. Therefore, the use of sorbitol in fermentation increases the NADH availability. This, in turn, should increase succinate production compared with glucose. PEP is the first precursor in the succinate pathway. This substrate can be limiting, since it is also assimilated in glycolysis and by the PTSG (Fig. 1). Xylose can be used as a carbon source to conserve the PEP pool for the succinate pathway. The transport of xylose does not require the phosphotransferase system, unlike glucose. It is transported by high-affinity permease driven by ATP [7]. With PTSG, 1 mol of PEP is converted to 1 mol of pyruvate while 1 mol of glucose is transported and phosphorylated. For this reason, xylose fermentation should yield higher succinate production than glucose fermentation. The acetate (*ackA-pta*) and lactate (*ldhA*) pathways are also inactivated in appropriate strains to study the effect on metabolite redistribution and the response to different carbon sources. Inactivating these pathways could eliminate competition for carbon flux with the succinate pathway, therefore improving succinate production.

Materials and methods

Strains and plasmid

Our laboratory wild-type strain GJT001, a spontaneous *S*-(2-aminoethyl)-L-cysteine-resistant mutant of MC4100, Δ*lac* strain (*arg-lac*) *U169 rspL150 relA1* [18], was used for all genetic manipulations. YBS121 is GJT001Δ(*ackA-pta*) [21] with the acetate pathway inactivated. YBS132 is GJT001Δ(*ackA-pta*, *ldhA*) [22] with both the acetate and lactate pathways inactivated.

Plasmid pKK313, carrying the S8D mutation in *Sorghum PEPC* [20], was used to overexpress PEPC in GJT001, YBS121, and YBS132. Plasmid pKK313 confers ampicillin resistance. The altered *Sorghum PEPC* has a site-directed mutation that advantageously relieves malate feedback inhibition from the enzyme [20]. This plasmid, pKK313, was transformed into GJT001, YSB121, and YSB132. GJT(pKK313) was used as the basic host for all experiments. To study pathway inactivation, GJT(pKK313) was used as the control for YBS121(pKK313) and YBS132(pKK313).

Medium

The medium used was Luria–Bertani broth (LB) adjusted to pH 7.4. It was supplemented with 40 g/L of carbon source (glucose, sorbitol, xylose), depending on

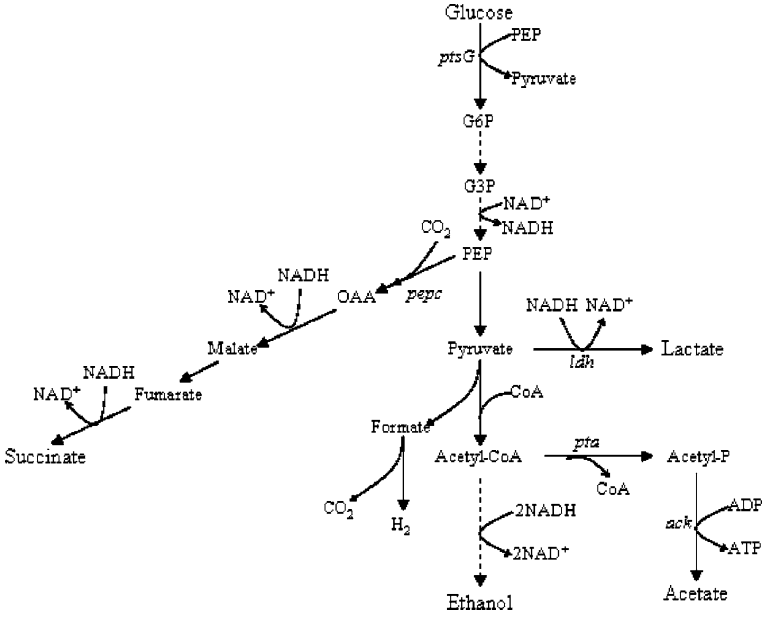

Fig. 1 Mixed-acid fermentation pathways in *E. coli*

the experiment. An isopropyl thiogalactose concentration of 0.1 mM was added to the medium to induce the expression of the *Sorghum* PEPC under the control of the *trc* promoter. An ampicillin concentration of 50 mg/L was used for selection of the plasmid.

Analytical techniques

Samples were centrifuged at 6000 *g* and 4°C for 10 min. Cell density could not be measured accurately due to interference from the $MgCO_3$ used in the experimental setups. The supernatant was then filtered through a 0.45-μm syringe filter and stored in HPLC vials at −20°C. The carbon source and extracellular metabolites (succinate, lactate, formate, acetate, ethanol) were quantified using a HPLC system (Thermo-Separation Products) equipped with a cation-exchange column (HPX-87H; BioRad Labs, Hercules, Calif.) and a differential refractive index detector (Waters). A mobile phase using 2.5 mM H_2SO_4 solution at 0.6 mL/min was applied to the column. The 2.5 mM H_2SO_4 solution was prepared with Milli-Q water (Milli-Q water system; Millipore, Bedford, Mass.) and filtered through a 0.45-μm membrane filter. The column was operated at 55°C.

Experimental setup A

For experimental setup A, 45-mL capped tubes containing 18 mL of medium were used. The tubes were sealed airtight, allowing no exchange of gas with the outside atmosphere. The air in the headspace was replaced with 100% CO_2 at 101 kPa by purging CO_2 into the tube at a flow rate of 1 L/min for 8 s. The condition in this experimental setup was anaerobic. A seed inoculum of 45 μL from an overnight 5-mL culture was used for each tube. A $MgCO_3$ concentration of 27.78 g/L was also supplied in the medium to buffer the pH during growth and to act as an indirect means of supplying CO_2. All experiments were performed in triplicates. Cultures were grown in an orbital shaker at 250 rpm and 37°C. Results were used only when all of the carbon source had been consumed.

Experimental setup B

For experimental setup B, 250-mL capped non-baffled flasks containing 10 mL of medium were used. The flasks were sealed airtight allowing no exchange of gas with the outside atmosphere. The headspace was not replaced with CO_2. This setup was used when studying mutant *E. coli* strains, since it allowed the strains to grow faster in the presence of oxygen. This helps generate biomass before mixed-acid fermentation takes place under an anaerobic atmosphere when oxygen is depleted. The condition in this experimental setup was microaerobic. A seed inoculum of 40 μL from an overnight 5-mL culture was used for each flask. $MgCO_3$ at a concentration of 27.78 g/L was also supplied to the medium. All experiments were performed in triplicates. Cultures were grown at 37°C and 250 rpm. Results were examined only when all of the carbon source had been consumed. The CO_2 and biomass contributions were not determined.

Results

Effect of carbon sources with different oxidation states and transport routes on succinate production

Satisfying the cofactor requirement of metabolic pathways is essential in facilitating product formation. One of the requirements for succinate formation is the cofactor NADH: 2 mol of NADH are needed for the production of 1 mol of succinate from PEP (Fig. 1). The maximum theoretical succinate yield under anaerobic conditions is 2 mol of succinate formed for 1 mol of glucose consumed if excess CO_2 is available. This theoretical yield for succinate is not achievable, though, under natural conditions since NADH is limiting when metabolizing glucose. For 1 mol of glucose metabolized via glycolysis, only 2 mol of NADH are formed. This, therefore, only allows 1 mol of succinate to be produced. To study the effect of increased NADH availability on succinate production, sorbitol was used as a carbon source for comparison to glucose. Studies showed the importance of sorbitol's reducing power in enhancing succinate production [6]. This is related to NADH formation since the catabolism of sorbitol produces more NADH than glucose [13]. Sorbitol has an oxidation state of −1, whereas glucose has an oxidation state of 0 [13]. For 1 mol of sorbitol consumed, 3 mol of NADH are formed, compared with only 2 mol of NADH formed for 1 mol of glucose consumed.

The PTSG is utilized to transport glucose into the cytoplasm of the cell. The transport process is mediated at the expense of 1 mol of PEP converted to 1 mol of pyruvate per mole of glucose transported (Fig. 1). During this process, glucose is phosphorylated with the phosphate group from PEP. Since PEP is the first precursor in the succinate synthesis pathway, the depletion of PEP by PTSG should hamper succinate productivity. To study the importance of the PEP requirement in succinate production, a carbon source that does not use the PTS system for transport was used and compared with glucose. Xylose is a five-carbon sugar with the same oxidation state, 0, as glucose. Its uptake into the cell uses a non-PTS system. In this regard, using xylose as a carbon source is expected to conserve more PEP for succinate synthesis, therefore yielding higher succinate production than glucose. Sorbitol like glucose is transported into the cell by the PTS system. Sorbitol and xylose were compared with glucose to examine the effect of different oxidation states and transport routes on succinate production.

Sorbitol was a more effective carbon source in succinate production than glucose with experimental setup A when strain GJT(pKK313) was examined. Succinate production and yield with sorbitol were 96% and 81% higher than glucose, respectively (Tables 1, 2). This effect was also observed in studies by others [6]. The results show the importance of NADH as a rate-limiting factor in the succinate synthesis pathway. Succinate productivity increases when more NADH is available. Ethanol production also increased substantially with sorbitol as the carbon source, compared with glucose. Production and yield of ethanol with sorbitol increased by 75% and 62% over glucose, respectively (Tables 1, 2). Since formation of 1 mol of ethanol also requires 2 mol of NADH, the increased NADH pool generated by the use of sorbitol is expected to enhance ethanol production. Interestingly, lactate production and yield with sorbitol decreased substantially, compared with glucose (Tables 1, 2). Formation of lactate also requires NADH. The succinate and ethanol pathways could possibly be preferred routes for establishing redox balance under reduced environments when sorbitol is being consumed, since both pathways oxidize 2 mol of NADH for 1 mol of product formed. This could explain why lactate production decreased with sorbitol, since more carbon flux was channeled toward succinate and ethanol. Production and yield for acetate also decreased substantially with sorbitol, compared with glucose (Tables 1, 2). The partition of carbon flux at the acetyl-CoA node was imbalanced under sorbitol, with a molar ethanol:acetate ratio of approximately 4:1 (Table 1). This molar ratio under glucose is approximately 1:1, showing a more balanced partition between acetate and ethanol at the acetyl-CoA node (Table 1). These results show the significant effect of sorbitol in rendering reduced environments through the formation of extra NADH during its catabolism.

The use of xylose as a carbon source by strain GJT(pKK313) in experimental setup A resulted in higher succinate production than the use of glucose. Succinate production and yield with xylose increased by 51% and 17%, respectively (Tables 1, 2). Lactate production and yield with xylose decreased substantially, compared with glucose (Tables 1, 2). This decrease could be in part attributed to more carbon flux being directed toward the succinate pathway at the PEP node. Therefore, less carbon flux was being channeled toward pyruvate and the lactate pathway. Importantly, the ethanol:acetate ratio under xylose was approximately 1:1, similar to glucose (Table 1). This is the expected result, since the oxidation state of xylose and glucose is 0, which does not alter the redox state of the intracellular environment.

Effect of acetate and lactate pathway inactivation on metabolite production under glucose, sorbitol, and xylose fermentation

Inactivating competing pathways of succinate can channel more carbon flux toward the succinate synthesis pathway, therefore resulting in higher succinate production. An example is an engineered *E. coli* strain in which lactate dehydrogenase and pyruvate formate lyase have both been inactivated [2]. This inactivation in the central metabolic pathway, as a result, causes a net accumulation of pyruvate. The strategy is direct, but the effect is sometimes haphazard, since the cellular response to perturbation is often stochastic. The effect of inactivating the acetate pathway on metabolite production was characterized under glucose, sorbitol, and xylose fermentation. Subsequently, the lactate pathway was inactivated in combination with the acetate pathway to characterize further the effect on metabolite production.

Strain GJT(pKK313) was used in the study as the control strain for the pathway mutant strains. Strain YBS121(pKK313) is GJT(pKK313) with the acetate pathway inactivated (Δ*ackA-pta*). YSB132(pKK313) is GJT(pKK313) with both the acetate and lactate pathways inactivated (Δ*ackA-pta*, *ldhA*). Experimental setup

Table 1 Comparison of carbon source on metabolite production using strain GJT(pKK313). Experimental setup A was used, with 40 g/L of carbon source supplemented. Experiments were performed in triplicate

Carbon source	Succinate yield (mM)	Lactate yield (mM)	Acetate yield (mM)	Ethanol yield (mM)
Glucose	32.74 ± 0.14	45.66 ± 3.2	128.14 ± 2.38	150.35 ± 1.40
Sorbitol	64.11 ± 0.66	6.270 ± 0.3	66.850 ± 0.99	263.08 ± 4.17
Xylose	49.54 ± 1.10	6.680 ± 0.6	163.30 ± 3.16	172.83 ± 3.28

Table 2 Comparison of carbon source on metabolite molar yield using strain GJT(pKK313). Experimental setup A was used, with 40 g/L of carbon source supplemented. Results are from the experiment in Table 1. Molar yield is moles of product formed per mole of carbon source consumed

Carbon source	Succinate molar yield	Lactate molar yield	Acetate molar yield	Ethanol molar yield
Glucose	0.172 ± 0.001	0.240 ± 0.017	0.675 ± 0.013	0.792 ± 0.007
Sorbitol	0.312 ± 0.003	0.031 ± 0.002	0.325 ± 0.001	1.280 ± 0.009
Xylose	0.201 ± 0.004	0.027 ± 0.002	0.663 ± 0.013	0.701 ± 0.013

Table 3 Effect of pathway inactivation on metabolite production with various carbon sources. Experimental setup B was used, with 40 g/L of carbon source supplemented. Experiments were performed in triplicate

Strain	Succinate yield (mM)	Lactate yield (mM)	Acetate yield (mM)	Ethanol yield (mM)
Glucose				
GJT(pKK313)	80.02 ± 2.18	11.80 ± 5.25	220.6 ± 7.88	13.99 ± 4.40
YBS121(pKK313)	34.51 ± 2.74	190.5 ± 9.54	40.62 ± 3.83	3.140 ± 0.43
YSB132(pKK313)	49.68 ± 2.69	6.720 ± 0.70	13.82 ± 1.39	46.42 ± 1.67
Sorbitol				
GJT(pKK313)	102.3 ± 0.90	0	143.0 ± 4.44	87.57 ± 6.32
YBS121(pKK313)	105.1 ± 2.92	93.47 ± 5.00	26.51 ± 3.99	57.91 ± 9.00
YSB132(pKK313)	102.4 ± 9.75	8.990 ± 1.00	8.790 ± 0.73	101.9 ± 21.2
Xylose				
GJT(pKK313)	83.21 ± 1.79	28.95 ± 5.28	239.8 ± 8.38	10.62 ± 1.65
YBS121(pKK313)	83.30 ± 4.98	182.6 ± 11.3	42.85 ± 5.93	3.920 ± 0.51
YSB132(pKK313)	72.77 ± 4.95	6.650 ± 0.18	15.97 ± 1.13	47.42 ± 2.42

B (see Materials and methods) was employed for studying these strains, in order to promote faster cell growth. Succinate, lactate, acetate, and ethanol production and yield were examined.

The results of YBS121(pKK313) showed that inactivating the acetate pathway had a significant effect on metabolite redistribution. Under all three carbon sources (glucose, sorbitol, xylose), lactate production and yield increased substantially. For glucose, lactate production and yield both increased 15-fold (Tables 3, 4). For sorbitol, there was no lactate production in GJT(pKK313). Once the acetate pathway was inactivated, substantial lactate production was observed (Table 3). For xylose, lactate production and yield increased over 5-fold (Tables 3, 4). Acetate production decreased substantially in strain YSB121(pKK313) with all three carbon sources. Ethanol production also decreased as a result of the acetate pathway inactivation. The cell response to acetate pathway inactivation, apparently, is to channel significant excess carbon flux toward the lactate pathway. With glucose and sorbitol, succinate yield decreased in strain YBS121(pKK313), compared with the control strain GJT(pKK313) (Table 4). With xylose, the succinate yield of YSB121(pKK313) was relatively unchanged, compared with GJT(pKK313) (Table 4). The most significant metabolite redistribution observed with acetate pathway inactivation was a change in lactate production.

Since lactate production increased substantially upon acetate pathway inactivation, the lactate pathway was also inactivated along with the acetate pathway. Mutant strain YBS132(pKK313) had both the acetate and lactate pathways inactivated. The metabolite production of this strain was compared with YBS121(pKK313) and GJT(pKK313). In YBS132(pKK313), lactate production and yield decreased substantially, compared with YSB121(pKK313) (Tables 3, 4). Acetate production and yield of YBS132(pKK313) further decreased, compared with YSB121(pKK313).

The most significant shift in metabolite distribution upon lactate and acetate pathway inactivation was toward ethanol. The ethanol yield of YSB132(pKK313) increased substantially, compared with YSB121 (pKK313) and GJT(pKK313) under all three carbon sources (Table 4). The succinate yield was also high in YBS132(pKK313). With glucose, the succinate yield of YSB132(pKK313) was higher than YSB121(pkKK313), but not higher than GJT(pKK313) (Table 4). With sorbitol and xylose, the succinate yield of YSB132(pKK313) was higher than either YSB121 (pKK313) or GJT(pKK313) (Table 4). Ethanol production had the largest increase after the lactate and

Table 4 Effect of pathway inactivation on metabolite molar yield with various carbon sources. Experimental setup B was used, with 40 g/L of carbon source supplemented. Results are from the experiment in Table 3. Molar yield is moles of product formed per mole of carbon source consumed

Strain	Succinate molar yield	Lactate molar yield	Acetate molar yield	Ethanol molar yield
Glucose				
GJT(pKK313)	0.424 ± 0.012	0.063 ± 0.028	1.169 ± 0.042	0.074 ± 0.023
YBS121(pKK313)	0.183 ± 0.015	1.009 ± 0.051	0.215 ± 0.020	0.017 ± 0.002
YSB132(pKK313)	0.307 ± 0.016	0.042 ± 0.004	0.085 ± 0.009	0.287 ± 0.011
Sorbitol				
GJT(pKK313)	0.565 ± 0.004	0	0.790 ± 0.023	0.483 ± 0.030
YBS121(pKK313)	0.515 ± 0.014	0.458 ± 0.025	0.130 ± 0.020	0.284 ± 0.044
YSB132(pKK313)	0.661 ± 0.007	0.058 ± 0.006	0.057 ± 0.004	0.657 ± 0.120
Xylose				
GJT(pKK313)	0.342 ± 0.008	0.119 ± 0.022	0.986 ± 0.035	0.044 ± 0.007
YBS121(pKK313)	0.342 ± 0.002	0.751 ± 0.047	0.176 ± 0.024	0.016 ± 0.002
YSB132(pKK313)	0.433 ± 0.018	0.040 ± 0.002	0.095 ± 0.003	0.282 ± 0.008

acetate pathways were inactivated; indicating that the ethanol pathway was the cell's preferred pathway for relieving excess carbon flux and maintaining redox balance.

Among the three carbon sources, sorbitol still retained the highest succinate and ethanol production in YSB121(pKK313) and YSB132(pKK313), compared with glucose and xylose (Table 3). Lactate production in YBS121(pKK313) with sorbitol fermentation was the lowest, compared with glucose and xylose fermentation (Table 3). With xylose, succinate production in YSB121(pKK313) and YSB132(pKK313) was consistently higher than glucose. These results showed that the effect of sorbitol's oxidation state and xylose's transport property on succinate production remained the same, despite inactivation of the lactate and acetate pathways.

Discussion

The use of sorbitol as a carbon source increased succinate production substantially in *E. coli*, compared with glucose. This result showed that the cofactor NADH is a rate-limiting factor in the succinate synthesis pathway. Ethanol also increased, since its formation also requires 2 mol of NADH, like succinate. The effect of the reducing power of sorbitol can be observed by the molar ethanol:acetate ratio increasing substantially above 1. With glucose, this ratio is approximately 1:1. This altered ethanol:acetate ratio is a result of imbalanced carbon flux partitioning at the acetyl-CoA node, with more carbon flux directed toward the ethanol pathway. This is caused by the recycling of excess NADH generated from sorbitol catabolism.

PEP is an essential limiting precursor for driving succinate synthesis. This was observed by the use of xylose as a carbon source, which increased succinate production, compared with glucose. The transport of xylose does not require the PTS system, unlike glucose. As a result, more intracellular PEP is conserved, which can then be assimilated through the succinate pathway to increase production.

Attempts to redirect carbon flux toward a desired pathway by means of pathway inactivation cannot always be premeditated. Inactivating the acetate pathway only resulted in a substantial increase in lactate production rather than succinate production. Succinate production did finally increase when both the acetate and lactate pathways were inactivated. The effect of inactivating these two pathways, though, also caused ethanol production to increase substantially. The dynamic response of the cell to reach equilibrium due to perturbation in its metabolic network is sometimes difficult to foresee. Sequential pathway deletion in this study exemplifies the intricate nature of the metabolic network.

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