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Effective production of succinic acid from coconut water (*Cocos nucifera)* by metabolically engineered *Escherichia coli* with overexpression of *Bacillus subtilis* pyruvate carboxylase

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ABSTRACT

Succinic acid is an important acid which is used in medicine and pharmaceutical companies. Metabolically engineered *Escherichia coli* strain was used for the effective production of succinic acid using *Cocos nucifera* water, which contained 5.00 ± 0.02 g/L glucose, 6.10 ± 0.01 g /L fructose and 6.70 ± 0.02 g /L sucrose. Fermentation of *C. nucifera* water with *E. coli* M6PM produced a final concentration of 11.78 ± 0.02 g/L succinic acid and yield of 1.23 ± 0.01 mol/mol, 0.66 ± 0.01 g/g total sugars after 72 h dual-phase fermentation in M9 medium while modeled sugar was 0.38 ± 0.02 mol/mol total sugars. It resulted in 72% of the maximum theoretical yield of succinic acid. Here we show that novel substrate of *C. nucifera* water resulted in effective production of succinic acid. These investigations unveil the importance of *C. nucifera* water as a substrate for the production of biochemicals.
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1. Introduction

Succinic acid is a platform chemical that has been identified as one of the top building block chemicals [1,2]. Succinic acid and its derivatives have many applications in medicine, chemical, pharmaceutical, agriculture, polymers and plastics industries, with a potential market of $15 billion [3]. The future economic and environmental profits of a bio-based succinate manufacturing have driven investigation and improvement of succinate-producing organisms. [3]. There are numerous biotechnological companies utilizing metabolic engineered microorganisms to manufacture succinic acid, for example Reverdia using *Saccharomyces cerevisiae* [4], Myriant using *Escherichia coli* [5], Bioamber using *Pichia kudriavzevii* [6] and Succinity using *Basfia succiniciproducens* [7]. Presently, succinic acid production by hydrogenation of the petroleum-derived maleic anhydride is expensive for utilization. Inexpensive microbial production of succinic acid from sugars has emerged over the past two decades.

E. coli is a facultative anaerobic gram-negative microorganism and effective in the utilization of carbon sources for heterotrophic growth [8]. It is acknowledged as an excellent microorganism due to its rapid growth, simple genetic manipulation and recognized metabolism [9–11]. *E. coli* can produce succinic acid during aerobic and anaerobic processes [12,13]. During the anaerobic processes, it utilizes the reductive branch of the tricarboxylic acid (TCA) pathway function in *E. coli* as the key pathway for the production of succinic acid. However, during the aerobic processes succinic acid is produced utilizing the whole oxidative TCA cycle. The anaerobic process for the production of succinic acid is more profitable [14], since it can guarantee a high yield of the main chemical because of the CO_2 fixation at the stage of oxaloacetate (OAA) formation, the main precursor metabolite of the reductive branch of the TCA

Abbreviations: HPLC, High performance liquid chromatography; O.D, optical density; rpm, revolution per minutes; IPTG, L isopropyl-β-D-thiogalactopyranoside; *ldh*A, lactate dehydrogenase A; *pta-ack*A, phosphotranacetylase acetate kinase A; *pfl*B, pyruvate formate lyase B; *pox*B, pyruvate oxidase B; *pgi*, phosphoglucose isomerase; *mre*C, murein cluster C; *pyc*, pyruvate carboxylase; *ppc*, phosphoenol pyruvate carboxylase; *zwf*, glucose 6-phosphate dehydrogenase; *pgl*, 6-phosphogluconolactonase; *gnd*, 6-phosphogluconate dehydrogenase; *tkt*, transketolase; *tal*, transaldolase.

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pathway [15]. Other microorganisms which can produce succinic acid includes *Anaerobiospirillum succiniciproducens* [16], *Mannheimia succiniciproducens* [12], *Corynebacterium glutamicum* [17], *Actinobacillus succinogen* [18], *B. succiniproducens* [19], *S. cerevisiae* [20] and *Yarrowia lipolytica* [21].

Cocos nucifera (coconut tree) belong to the palm tree family (Arecaceae) [22,23]. Coconut water (coconut liquid endosperm), is very useful and multipurpose natural product which makes it to be abundant [24]. Due to its abundance in the region where it is cultivated and grown the liquid may be wasted. It is a revitalizing beverage that is naturally disbursed worldwide because it is beneficial and valuable for health. Coconut water is conventionally utilized for plant tissue culture or micropropagation [25]. The comprehensive uses of coconut water can be acceptable by its distinctive chemical composition of sugars, vitamins, minerals, amino acids and phytohormones [26]. Hence we utilized *C. nucifera* water for the production of succinic acid due to its importance, abundance and may be wasted. *Cocos nucifera* water is a wastewater originating from the southeastern part of Asia. About 200,000 tons of *C. nucifera* water was produced in Thailand in 2001 [27]. It can be collected free of charge when there is available transportation for the waste. It can be used for the production of sports drinks and food gels. The major sugars that can be derived from it include glucose, fructose, and sucrose [28]. Purified *C. nucifera* water from Brazil showed elevated reductase activity at ambient temperature in aliphatic, aromatic aldehydes and ketones, which indicates that *C. nucifera* water investigation for the synthesis of organic compounds is still underexplored [29]. Investigation of *C. nucifera* water is deprived, mainly focusing on specific uses, biochemical compositions, and preservation techniques. Therefore, in this investigation genetically engineered strain was constructed and established for succinic acid production from *C. nucifera* water. Three major components of *C. nucifera* water (glucose, fructose, and sucrose) were investigated for the production of succinic acid in the dual-phase fermentation.

2. Materials and methods

2.1. Strains and plasmids

Strains, plasmids and primers used in this study are presented in Supplementary Table 1. Wild-type *E. coli* K-12 strain MG1655 and all the genetic modifications were performed on the strain or derivatives. The pyruvate carboxylase (*pyc)* gene was amplified from *Bacillus subtilis* and plasmid-pTrcHisA *pyc* was developed using restriction enzymes *Kpn* I and *Hind* III. Here, we use *cat*A*sac*B selection marker for the gene deletion, which is better than λ-red recombinase and group II introns retrotransposition which leave scars in the genome and limits allelic exchange application. Forward and reverse primers contained 50 bp homologous sequences upstream or downstream of the target gene followed by 25 bp sequences corresponding to the chloramphenicol acetyltransferase and levansucrase cassette (*cat*A*sac*B). The DNA fragment was amplified from peasy*cat*A*sac*B and subjected to electroporation of *E. coli* MG1655 or derivatives carrying pKD46 for homologous recombination. The resistant strain *cat*A*sac*B and target gene deleted was screened and verified by colony PCR and sequencing of the DNA. The *cat*A*sac*B was then removed by culturing in 10 ml LB medium in a 50 ml beaker overnight (around 12 h). The next day the 1 ml of the cultures were inoculated into 10 ml LB medium with 10% sucrose, 0.5% yeast and 1% tryptone. After grown to the late stationary phase, the cultures were streaked on an LB agar. The grown colonies were then transferred with and without chloramphenicol simultaneously. Finally, colonies, which lost the chloramphenicol resistance, were screened for the *cat*A*sac*B removal [30].

2.2. Media

All modified strains were grown in LB medium (per liter): 10 g tryptone, 5 g yeast and 10 g NaCl. Other supplements include 100 mg/mL ampicillin, 50 mg/L kanamycin and 34 mg/mL chloramphenicol. M9 minimal medium contained 800 mL H_2O, with 64 g Na_2HPO_4-$7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5.0 g NH_4Cl. The solution was stirred until all salt dissolved and adjusted to 1000 mL with distilled H_2O and sterilized in an autoclave. 700 mL distilled H_2O (sterilized), 200 ml of M9 salts, 2 mL of 1 M $MgSO_4$ (sterilized), 20 ml of 20% glucose (or other source of carbon) and 100 μL of 1 M $CaCl_2$ (sterilized) was added and the solution adjusted to 1000 mL with distilled H_2O.

2.3. Cloning and overexpression of the gene pyruvate carboxylase (pyc)

The plasmid pTrchisA was used as the template for cloning the *pyc* gene with the trc and its promoter. The *pyc* gene was amplified with primers *pyc*-F and *pyc*-R (Table 1). Both primers 4 μM, synthesized at Sangon Company Beijing China were mixed with 10 ng template, 4 μL dNTPs and 0.5 μl Taq DNA polymerase and sterile water to form a 50 μL PCR mixture all from Beijing Trans Gen Biotech Company China. The PCR conditions were 94 °C for 4 min., 94 °C for 20 s, 60 °C for 20 s, 72 °C for 1 min. with a final step of 72 °C for 8 min. for 35 cycles. The PCR was confirmed using the agarose gel electrophoresis and PCR products were extracted using the Axygen (Axygen Scientific Inc). The pTrchisA was extracted and digested using *Kpn* I and *Hind* III overnight and constructed using gibson assembly mix. The plasmid was then transformed into *E. coli* to generate the strains shown in Table 1. The strains were grown in 100 ml LB medium at 37 °C by adding 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

2.4. Dual-phase fermentation on pure glucose in the shaking flasks

M9 medium was used for aerobic and anaerobic fermentation. The medium was supplemented with glucose, 100 mg/L ampicillin, and 50 mg/L kanamycin. 1 mL of seed inoculum from an overnight 10 mL culture was added into 250 mL flask containing 50 mL M9 medium for aerobic growth at 37 °C and 200 rpm agitation speed.

Table 1
Dual-phase fermentation of the different engineered strains after 48 h in M9 medium using 20 g/L glucose.

Strains	DCW [a] (g/L)	Glucose consumed [a] (g/L)	Fermentation products [a] (g/L)				Yield [a] (mol/mol)
			Succinate	Pyruvate	Acetate	Formate	
MG 1655	0.58 ± 0.01	18.00 ± 0.03	1.08 ± 0.07	0.13 ± 0.01	5.00 ± 0.06	0.32 ± 0.01	0.08 ± 0.01
MGG	0.47 ± 0.01	17.00 ± 0.02	0.44 ± 0.05	0.25 ± 0.03	4.00 ± 0.09	N.D	0.04 ± 0.02
M6P	0.62 ± 0.06	17.00 ± 0.02	2.65 ± 0.01	0.58 ± 0.02	N.D	N.D	0.23 ± 0.01
M6PM	0.54 ± 0.05	19.00 ± 0.01	1.82 ± 0.05	0.68 ± 0.01	N.D	N.D	0.15 ± 0.01

[a] Each values is the mean of three parallel replicates ± standard deviation.
[b] ND not detected.
Yield was calculated as mol succinate produced per mol glucose metabolized.

Preliminary optimization of transitional time anaerobic fermentation was carried out after 6 h of aerobic incubation (OD_{600} = 2.0). 10 g/L glucose, 1 g $MgCO_3$, and 0.2 mM IPTG were placed in an anaerobic incubator (CIMO Medical Instrument Manufacturing Ltd., Shanghai, China) without shaking at 37 °C for 48 h. Samples at 0 h and 48 h in anaerobic phase were collected and stored for analysis of biomass and glucose.

2.5. Dual-phase fermentation of a modeled sugar mixture and C. nucifera *water in 5 L- bioreactor by engineered* E. coli *M6PM*

Dual-phase fermentation of modeled sugar mixture of *C. nucifera* water was carried out in 5-L bioreactor (New Brunswick Scientific Co., Inc. USA) containing 2 L M9 medium. In aerobic phase, 5% seed cultures were inoculated. The flow rate of sterile air was controlled at 5 L/min with 350 rpm of agitation speed with the temperature kept at 37 °C. The anaerobic phase was started when OD_{600} reached 2.0, CO_2 gas was sparged with a flow rate of 2 L/min and agitation speed decreased to 150 rpm. Simultaneously, the concentrated mixture was gradually fed into the medium. The pH was kept at about 7.0 with 10 M NaOH and 10% H_2SO_4. The dual-phase fermentation of *C. nucifera* water was performed using the same method as described above. Sugars from *C. nucifera* water were supplemented at the transition time from aerobic phase to anaerobic phase. All bioreactor fermentation experiments were performed in triplicate.

2.6. Analytical methods

The OD_{600} was determined to monitor cell growth and correlated to the dry cell weight (DCW): DCW (g/L) = 0.36 × OD_{600}. Glucose, fructose, sucrose and fermentation products (succinate, acetate, formate, and pyruvate) were quantified by high-performance liquid chromatography (HPLC) equipped with UV absorbance detector and refractive index detector (Agilent Technologies, USA). The Aminex HPX-87H ion-exchange column was used for analysis (Bio-Rad, USA) and operated at 50 °C with a mobile phase of 5 mM H_2SO_4 solution at a flow rate of 0.6 mL/min.

The yield of succinic acid was calculated with the following formula respectively:

$$\text{Succinic acid yield from mono sugar } (g/g) = \frac{\text{Succinic acid produced } (g/L)}{\text{Glucose consumed}(g/L)} \quad (1)$$

$$\text{Succinic acid yield from mono sugar } (mol/mol) = \frac{\text{Succinic acid produced } g/L/(118 g/mol)}{\text{Glucose consumed } g/L/(180 g/mol)} \quad (2)$$

$$\text{Succinic acid yield of sugar mixture } (g/g) = \frac{\text{Succinic acid produced}(g/L)}{(\text{Glucose} + \text{Fructose} + \text{Sucrose})\text{consumed}(g/L)} \quad (3)$$

$$\text{Percentage yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \times 100\% \quad (4)$$

3. Results and discussion

3.1. Dual-phase fermentation of the different engineered strains using glucose

During the dual-phase fermentation of the different engineered strain using glucose only about 5% of the maximum theoretical yield of succinic acid was produced in the wild-type *E. coli* while the metabolic flux was directed into the production of byproducts. It could be seen vividly that during the deletion of only the 6-phosphogluconate dehydrogenase (*gnd)* there was no significant difference from the wild-type (Table 1). This shows that the deletion of only the *gnd* is not effective for the production of succinic acid because there is still formation of byproducts which is not important for the production of succinic acid. The 6-phosphogluconate dehydrogenase (*gnd*) is involved in the production of ribulose 5-phosphate and functions in the pentose phosphate pathway as the main generator of cellular NADPH. It is located in the boundary of oxidative and non-oxidative pentose phosphate pathway. Our result for the MGG in which only *gnd* was deleted is in consonant with previous research with titer value of 0.35 g/L succinic acid of using glucose [31].

Hence the byproducts pathways were deleted by the inactivation of the *ldh*A, *pta-ackA*, *pox*B, *pfl*B, *pgi,* and down-regulation of *ppc* with overexpression of *pyc* (Fig. 1). There was a significant increase in synthesis of succinic acid by 13.4% maximum theoretical yield compared to the wild-type (Table 1). This

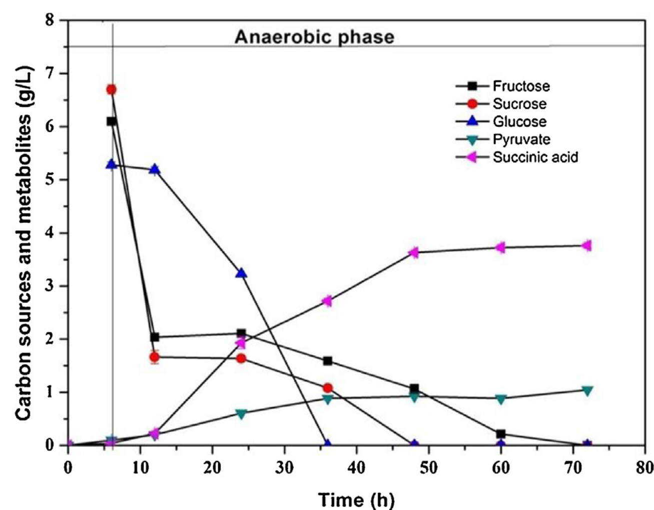

Fig. 1. Production of succinic acid using modeled sugar in 5 L bioreactor.

Table 2
Dual-phase fermentation of the different engineered strains after 48 h in M9 medium using 20 g/L fructose.

Strains	DCW [a] (g/L)	Fructose consumed [a] (g/L)	Fermentation products [a] (g/L)				Yield [a] (mol/mol)
			Succinate	Pyruvate	Acetate	Formate	
MG 1655	0.58 ± 0.01	18.00 ± 0.03	1.08 ± 0.07	0.13 ± 0.01	5.00 ± 0.06	0.32 ± 0.01	0.08 ± 0.01
MGG	0.48 ± 0.01	19.00 ± 0.02	1.57 ± 0.05	0.29 ± 0.03	4.00 ± 0.09	N.D	0.13 ± 0.01
M6P	0.42 ± 0.02	10.00 ± 0.02	1.75 ± 0.01	0.60 ± 0.02	N.D	N.D	0.27 ± 0.01
M6PM	0.47 ± 0.03	12.20 ± 0.01	2.00 ± 0.05	0.78 ± 0.01	N.D	N.D	0.30 ± 0.01

[a] Each values is the mean of three parallel replicates ± standard deviation.
[b] ND not detected.
Yield was calculated as mol succinate produced per mol fructose metabolized.

Table 3
Dual-phase fermentation of the different engineered strains after 48 h in M9 medium using 20 g/L sucrose.

Strains	DCW [a] (g/L)	Sucrose consumed[a] (g/L)	Fermentation products[a] (g/L)				Yield[a] (mol/mol)
			Succinate	Pyruvate	Acetate	Formate	
MG 1655	0.58 ± 0.01	18.00 ± 0.03	1.08 ± 0.07	0.13 ± 0.01	5.00 ± 0.06	0.32 ± 0.01	0.08 ± 0.01
MGG	0.54 ± 0.01	17.00 ± 0.04	2.92 ± 0.05	0.28 ± 0.03	4.00 ± 0.09	N.D	0.27 ± 0.01
M6P	0.54 ± 0.02	10.00 ± 0.02	2.65 ± 0.01	0.52 ± 0.02	N.D	N.D	0.40 ± 0.01
M6PM	0.64 ± 0.01	18.20 ± 0.01	1.80 ± 0.05	0.77 ± 0.01	N.D	N.D	0.15 ± 0.01

[a] Each values is the mean of three parallel replicates ± standard deviation.
[b] ND not detected.
Yield was calculated as mol succinate produced per mol sucrose metabolized.

Table 4
Dual-phase fermentations of *E. coli* M6PM after 48 h in M9 medium with different carbon sources.

Medium	DCW [a] (g/L)	Glucose consumed [a] (g/L)	Fructose consumed [a] (g/L)	Sucrose consumed [a] (g/L)	Fermentation products [a] (g/L)				Yield [a] (g/g)
					Succinate	Pyruvate	Acetate	Formate	
Medium A	0.60 ± 0.01	5.20 ± 0.02	6.00 ± 0.02	6.00 ± 0.03	5.08 ± 0.02	0.13 ± 0.01	4.00 ± 0.06	0.32 ± 0.01	0.30 ± 0.01
Medium B	1.69 ± 0.01	5.00 ± 0.01	6.10 ± 0.01	6.70 ± 0.04	7.64 ± 0.03	0.25 ± 0.03	4.00 ± 0.09	0.22 ± 0.01	0.43 ± 0.02

Yield was calculated as succinate produced (g/L)/ glucose consumed g/L + fructose consumed (g/L) + sucrose consumed (g/L).
Medium A represents the sugar mixture (Glucose, fructose and sucrose).
Medium B represents *C. nucifera water*.

demonstrates that genetically engineering of the pathway may enhance the yield of succinic acid production.

3.2. Dual-phase fermentation of the different engineered strains using fructose

Fructose which is one of the major sugars present in the *C. nucifera* water was utilized as a substrate for the production of succinic acid. Fructose can be referred to as a C_6 polyhydroxyketone. Table 2 shows that after 48 h dual-phase fermentation on 20 g/L fructose, the concentrations of succinic acid in the wild-type *E coli* MG 1655, MGG, M6P and M6PM were 1.08 ± 0.08 g/L, 1.57 ± 0.05 g/L, 1.75 ± 0.01 g/L, and 2.00 ± 0.05 g/L respectively. Utilization of carbon source for the production of succinic acid in the MGG alone is not sufficient to disallow the accumulations of undesired products. Hence mutation, down-regulation and overexpression were done to enhance the synthesis of succinic acid.

3.3. Influence of sucrose substrate on dual-phase fermentation of the different engineered strains

Gene mutation, down-regulation of phosphoenolpyruvate carboxylase and overexpression of *Bacillus subtilis* pyruvate carboxylase in *E. coli* MGG, M6P and M6PM enhanced the production of succinic acid by 170%, 145% and 67%, respectively and thus shows the efficiency of the gene mutation and overexpression of the *Bacillus subtilis* pyruvate carboxylase gene utilizing surcrose (Fig. 3). Here, the percentage increase of succinic acid production during the deletion of 6-phosphogluconate dehydrogenase (*gnd)* during the utilization of sucrose may be due to the fact that the pentose phosphate pathway has been known to be major routes of sugar catabolism, generating reducing equivalent and production of cellular metabolites for cellular activities [32]. Little is known about the metabolism of sucrose in the central metabolic pathway if *gnd* is deleted in the pentose phosphate pathway. Hence, blockage of the PPP may redirect the metabolic flux and activate the alternative mechanism. 6-phosphogluconate dehydrogenase (*gnd)* is located between the oxidative and non-oxidative pentose phosphate pathway which may have induced activation of the pyruvate carboxylase (Fig. 3). Therefore, deletion of only *gnd* could lead to the accumulation of 6-phosphogluconate during dual-phase fermentation stemming from the inability of the oxidative pathway to function effectively, and may also reduce the functions of ED pathway (Table 3).

3.4. Dual-phase fermentations of the different engineered strains

`*C. nucifera* water is composed mainly of fructose, glucose and sucrose, which makes it an important substrate for the production of succinic acid. Firstly, dual-phase fermentation of MP6M strain was investigated after 48 h in M9 medium with *C. nucifera* water in shaking flasks. The *C. nucifera* water and sugar mixture was added into the fermentation medium with about 17.8 g/L initial total sugars. It could be seen that MP6M could consume 5.20 ± 0.02 g/L glucose, 6.00 ± 0.02 g/L fructose and 6.00 ± 0.03 g/L sucrose in the modeled sugar which produced a final yield of 0.30 ± 0.01 mol/mol of succinic acid. *E.coli* M6PM consumed all the sugars in the *C. nucifera* water completely within 48 h of fermentation and produced a final yield of succinic acid of 0.43 ± 0.02 g/g (Table 4) which was slightly better than the modeled sugar. It was favored than that of M6P and MGG which were 0.37 ± 0.03 g/g (Table 5)

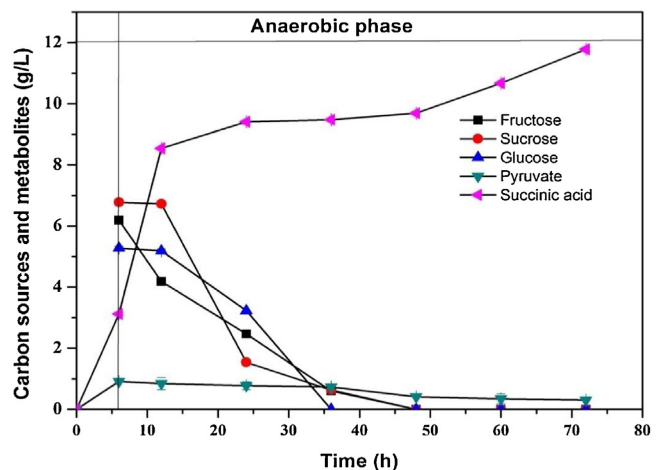

Fig. 2. Production of succinic acid using *Cocos nucifera* water in 5 L bioreactor.

Table 5
Dual-phase fermentations of *E.coli* M6P after 48 h in M9 medium with different carbon sources.

Medium	DCW [a] (g/L)	Glucose consumed [a] (g/L)	Fructose consumed [a] (g/L)	Sucrose consumed [a] (g/L)	Fermentation products [a] (g/L)				Yield [a] (g/g)
					Succinate	Pyruvate	Acetate	Formate	
Medium A	0.68 ± 0.01	5.20 ± 0.02	6.00 ± 0.02	6.00 ± 0.03	5.00 ± 0.02	0.53 ± 0.01	4.00 ± 0.06	0.32 ± 0.01	0.29 ± 0.01
Medium B	1.67 ± 0.01	5.00 ± 0.01	6.10 ± 0.01	6.70 ± 0.04	6.54 ± 0.03	0.65 ± 0.03	4.00 ± 0.09	0.22 ± 0.01	0.37 ± 0.03

Yield was calculated as succinate produced (g/L)/ glucose consumed g/L + fructose consumed (g/L) + sucrose consumed (g/L).
Medium A represents the sugar mixture (Glucose, fructose and sucrose).
Medium B represents *C. nucifera water.*

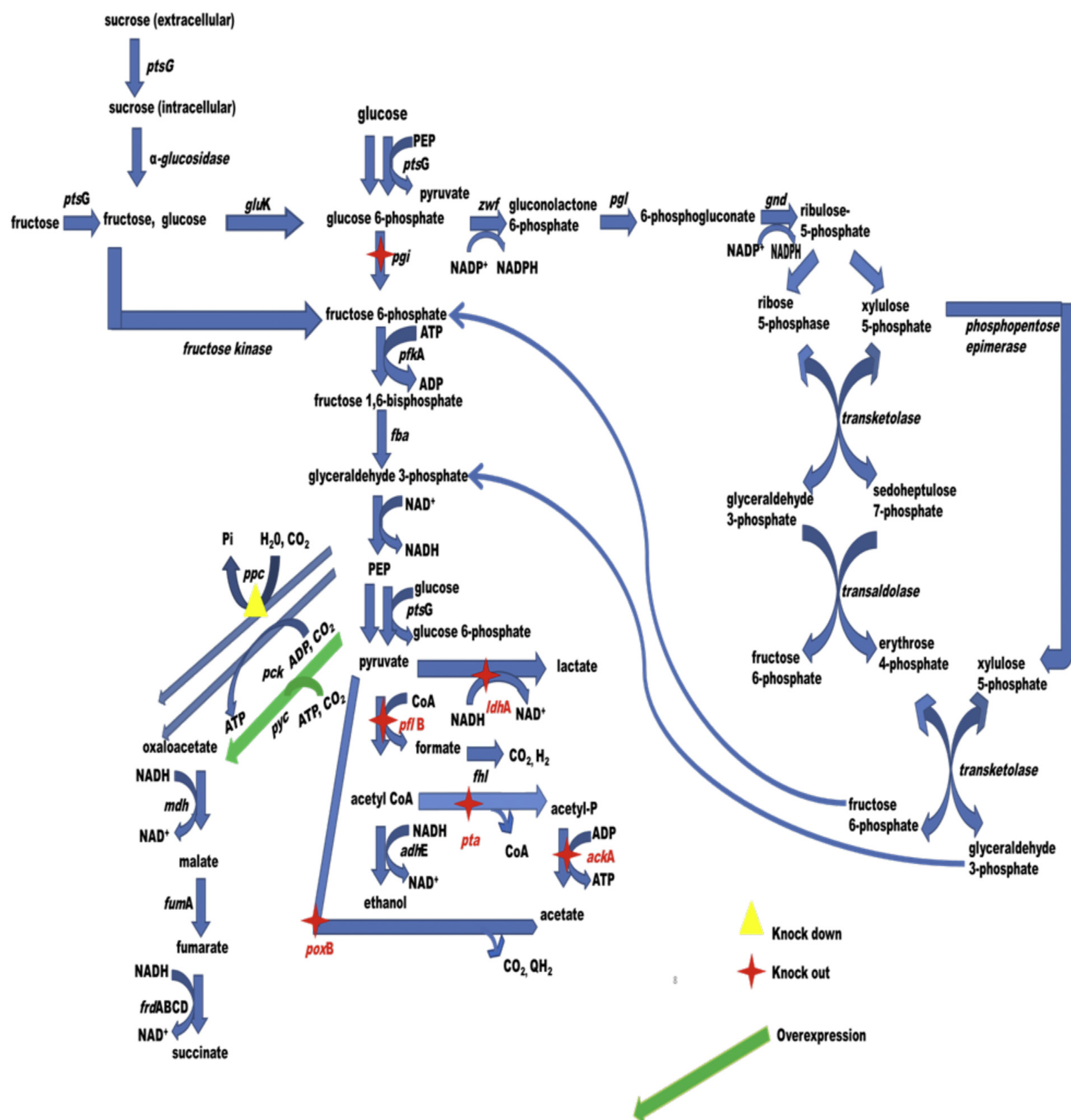

Fig. 3. Succinic acid production from recombinant *E.coli* using sucrose, fructose, and glucose.
NAD^+, oxidized nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$ oxidized nicotinamide adenine dinucleotide phosphate. Red star for knock out arrow green for overexpression and yellow triangle for knock down. lactate dehydrogenase A (*ldhA*), phosphotranacetylase acetate kinase A (*pta-ackA*), pyruvate formate lyase B (*pfl*B), pyruvate oxidase B (*pox*B), phosphoglucose isomerase (*pgi*), Glucose phosphotransferase system (*pts*G), pyruvate carboxylase(*pyc*), phosphoenol pyruvate carboxykinase (*pck*), phosphoenol pyruvate carboxlyase (*ppc*), galactose permease (*gal*P), phosphofructose kinase A (*pfk*A), fructose bisphosphate aldolase(fba), malate dehydrogenase (mdh), fumarase (fum), formate hydrogen lyase (*fhl*) glucose-6-phosphate dehydrogenase (*zwf*),6-phosphogluconate dehydrogenase (*gnd*), 6-phosphogluconolactonase (*pgl*).

Table 6
Dual-phase fermentations of *E. coli* MGG after 48 h in M9 medium with different carbon sources.

Medium	DCW [a] (g/L)	Glucose consumed [a] (g/L)	Fructose consumed [a] (g/L)	Sucrose consumed [a] (g/L)	Fermentation products [a] (g/L)				Yield [a] (g/g)
					Succinate	Pyruvate	Acetate	Formate	
Medium A	0.60 ± 0.01	5.20 ± 0.02	6.00 ± 0.02	6.00 ± 0.03	4.08 ± 0.02	0.13 ± 0.02	5.00 ± 0.02	2.52 ± 0.01	0.24 ± 0.01
Medium B	1.60 ± 0.01	5.00 ± 0.01	6.10 ± 0.01	6.70 ± 0.04	5.04 ± 0.03	0.20 ± 0.01	6.00 ± 0.05	2.60 ± 0.01	0.28 ± 0.02

Yield was calculated as succinate produced (g/L)/ glucose consumed g/L + fructose consumed (g/L) + sucrose consumed (g/L).
Medium A represents the sugar mixture (Glucose, fructose and sucrose).
Medium B represents *C. nucifera water.*

and 0.28 ± 0.02 g/g (Table 6) respectively. Hence *E. coli* M6PM was selected for dual-phase fermentation in the 5 L bioreactor.

3.5. Utilization of glucose, fructose, and sucrose for succinic acid production using E. coli *M6PM during dual-phase fermentation*

In Fig. 1, the modeled sugar was prepared with 5.20 ± 0.02 g /L glucose, 6.00 ± 0.02 g/L fructose, and 6.00 ± 0.03 g/L sucrose. The dry cell weight (DCW) was 1.15 ± 0.01 g/L, which yielded 3.76 ± 0.02 g/L succinic acid. The modeled sugar produced 0.38 ± 0.02 mol/mol total sugar after 72 h dual-phase fermentation. *E.coli* MP6M consumed 5.20 ± 0.02 g/L glucose, 6.00 ± 0.02 g/L fructose and 6.00 ± 0.03 g/L sucrose effectively within 36 h, 48 h and 72 h respectively. The average sugar consumption rates were 0.14 ± 0.01 $g L^{-1} h^{-1}$, 0.13 ± 0.01 $g L^{-1} h^{-1}$ and 0.08 ± 0.01 $g L^{-1} h^{-1}$, respectively, which revealed that both glucose and fructose was consumed faster than sucrose. The catabolic repression of glucose on non-preferred carbon sources may be responsible for the initial consumption of glucose [33–35].

3.6. Effective production of succinic acid from C. nucifera *using* E. coli *M6PM*

In order to verify the fermentation attributes of *C. nucifera* water as the substrate for the production of succinic acid dual-phase fermentation was carried out in a 5 L bioreactor (Fig. 2). Hence, 17.8 g/L total sugars were used in the *C. nucifera* investigation. *E. coli* M6PM exhibited different consumption of sugars from the modeled sugar. The three sugars were consumed simultaneously during the fermentation. Glucose was consumed more rapidly than fructose and sucrose. Glucose was consumed finally after 36 h while sucrose and fructose were utilized finally after 48 h. This may be due to the fact that murein cluster C gene (*mre*C) which is responsible for the rod shape [36,37], has been deleted, which allow the accumulation of succinic acid. The increased cell growth (1.7 ± 0.02 g/L DCW) may be as a result of the presence of good nutrient in the *C. nucifera* water which may enhance the production of succinic acid [28].

Fermentation of *C. nucifera* water with *E. coli* M6PM produced a final concentration of 11.78 ± 0.02 g/L succinic acid and yield of 1.23 ± 0.01 mol/mol, 0.66 ± 0.01 g/g total sugars after 72 h dual-phase fermentation in M9 medium. This makes up 72% of the maximum theoretical yield and is comparable to some results of succinic acid production in the literature [38–42]. Hence, *C. nucifera* water could be used for the production of succinic acid in large scale and other biochemicals. Therefore, metabolically engineering *E. coli* M6PM (MG1655Δ*ldh*AΔ*ptaack*AΔ*pox*BΔ*pfl*BΔ*pgi*ΔmreC*ppc pyc)*, may result in a better molar yield of succinic acid using *C. nucifera* water by enhancing the strain design.

4. Conclusions

E. coli M6PM was developed for succinic acid production using *C. nucifera* water which utilized the substrate wastewater effectively for the production of succinic acid. It produced 11.78 ± 0.02 g/L succinic acid and a yield of 1.23 ± 0.01 mol/mol, 0.66 ± 0.01 g/g total sugars after 72 h dual-phase fermentation in M9 medium, which resulted in 72% of the maximum theoretical yield. Hence, this work put forward that *C. nucifera* water could be used for the production of succinic acid and other biochemicals in industrial scale.

Declaration of Competing Interest

The authors declare no conflict of interest

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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.btre.2019.e00378.

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