Production of succinic acid by engineered *E. coli* strains using soybean carbohydrates as feedstock under aerobic fermentation conditions

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Abstract

E. coli strains HL2765 and HL27659k harboring pRU600 and pKK313 were examined for succinate production under aerobic conditions using galactose, sucrose, raffinose, stachyose, and mixtures of these sugars extracted from soybean meal and soy solubles. HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) consumed 87 mM and 98 mM hexose of soybean meal extract and produced 83 mM and 95 mM succinate, respectively. While using soy solubles extract, HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) consumed 160 mM and 187 mM hexose and produced 158 mM and 183 mM succinate, respectively. Succinate yield of HL2765(pKK313)(pRU600) was low as compared to that of HL27659k(pKK313)(pRU600) while using acid hydrolysate of soybean meal or soy solubles extracts. Maximum succinate production of 312 mM with a molar yield of 0.82 mol/mol hexose was obtained using soy solubles hydrolysate by HL27659k(pKK313)(pRU600). This study demonstrated the use of soluble carbohydrates of the renewable feedstock, soybean as an inexpensive carbon source to produce succinate by fermentation.

Keywords

Succinate, *Escherichia coli*, Aerobic fermentation, Soybean carbohydrates, Soy solubles

1. Introduction

Succinic acid has been considered an important specialty chemical that can be produced by microorganisms. However, the placement of this molecule on the US Department of Energy (DOE) list of twelve platform chemicals from biomass (Werpy and Petersen, 2004) has generated widespread attention toward biobased succinate manufacturing (McKinlay et al., 2007). Various valuable molecules derived from succinic acid by known chemical processes are 1,4-butanediol, maleic anhydride, succinimide, 2-pyrrolidinone, and tetrahydrofuran; all of which have large markets and can be converted to a wide range of other valuable molecules for use in polymers (Xu and Guo, 2010), industrial solvents, and specialty chemicals, for example, biodegradable succinate esters (Budarin et al., 2007; Zeikus et al., 1999). Succinate is also used as a flavoring agent in food and as a counterion in many pharmaceutical formulations. Sodium dioctyl sulfosuccinate is used as a surfactant and wetting agent in textile and printing products and is a component of the Corexit dispersant used in the recent Gulf oil-spill cleanup operations (Thakker et al., 2012).

Major succinate producing organisms include *Escherichia coli*, *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae* and *Basfia succiniciproducens*. Much work has been reported using these organisms to produce succinate with high yield and productivity and efforts are being made to commercialize succinate process by various groups. However, bio-based succinate still faces the challenge of becoming cost competitive against petrochemical-based processes (McKinlay et al., 2007). In order to develop a bio-based industrial production of succinic acid, it will be important to use low cost sugar rich feedstocks

and develop a robust strain capable of catalyzing a wide range of sugar feedstocks to produce succinic acid in good yields so that the cheapest available raw material can be used.

Some of the above mentioned organisms were examined for their ability to use agricultural feedstocks for succinate production and increasingly the focus has been on the use of lignocellulosic-derived biomass as a feedstock (Borges and Pereira, 2011; Kim et al., 2004; Lee et al., 2003; Luo et al., 2010; Shanmugam and Ingram, 2008). *A. succinogenes* (Guettler et al., 1996; Li et al., 2011), *M. succiniciproducens* (Lee et al., 2008), *A. succiniciproducens* (Lee et al., 2008) can produce succinic acid from a broad range of carbon sources such as glucose, galactose, sucrose, maltose, lactose, xylose, and fructose. Most reported efforts to enhance production of succinate using *A. succiniciproducens*, *A. succinogenes*, and *M. succiniciproducens* have been performed under anaerobic conditions. These efforts have often been hampered by the limitations of NADH availability, poor cell growth, and slow production. In contrast, aerobic cell culture conditions have many advantages over anaerobic conditions that favor implementation on an industrial scale due to higher biomass generation, faster carbon throughput and product formation.

E. coli remains an attractive organism for testing new succinate technologies due to the extensive knowledge of its genome, proteome, availability of genetic tools, simple nutrient requirements, and facile cultivation. Lin et al. (2005a) strategically designed aerobic succinate producing recombinant *E. coli* strains in our laboratory. Based on pathway modeling and optimization, the maximum theoretical succinate yield that can be achieved using engineered *E. coli* under aerobic condition is 1.0 mol of succinate per mol of glucose consumed (Lin et al., 2005a and 2005b). Recently, Wang et al. (2011) demonstrated the production of succinate from

sucrose (1.6-1.9 mol succinate/mol sucrose) under aerobic conditions using the engineered *E. coli* strains HL2765k and HL27659k harboring pHL413 and pUR400 plasmids.

In this study, we further explored the use of strains HL2765 and HL27659k harboring pKK313 (Lin et al., 2005a and 2005b) and pRU600 (Schmid and Schmitt, 1976; Aslanidis et al., 1989) for co-metabolizing various sugar substrates present in soybeans for succinate production.

Soybeans, an important agriculture feedstock, are a major ingredient in non-ruminant animal diets. Soybeans contain approximately 37% crude protein and 20% oil. Defatted soybean meal has been widely used as a protein source in poultry, swine and other feed. Carbohydrate is the 2nd most predominant component in soybean seeds representing approximately 35% of soybean meal dry matter (Giannoccaro et al., 2006). Cellulose, hemicellulose, pectin, and trace amounts of starch represent insoluble carbohydrates of soybeans (Liu 1997). Soluble carbohydrates consist of stachyose, raffinose, sucrose, glucose, fructose, and trace amounts of arabinose, rhamnose, fucose, ribose, xylose, and mannose (Eldridge et al., 1979). Consumption of the indigestible oligosaccharides, raffinose and stachyose results in flatulence and abdominal discomfort, so these are often removed during production of soy protein products.

Since the use of an inexpensive source of carbohydrates is an effective method among other possible alternatives to reduce the cost of fermentation process, we therefore aimed to demonstrate the use of the soluble carbohydrates including the nondigestible oligosaccharide fraction of soybean meal to produce succinate by aerobic fermentation using engineered *E. coli* strains.

2. Materials and methods

2.1. Oligosaccharides and soybean feedstock

Raffinose and stachyose were obtained from B&K Technology Group Co. Ltd., China. Soybean meal was obtained from Hieden Feed and Supply, Inc. (Houston, USA). Soy solubles obtained from Solae (St. Louis, MO) soy processing facility and stored in 50 mL aliquots at -20°C.

2.2. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. In our laboratory, succinate producing recombinant *E. coli* strains HL2765 [$\Delta sdhAB$, $\Delta(ackA-pta)$, $\Delta poxB$, $\Delta iclR$] and HL27659k [$\Delta sdhAB$, $\Delta(ackA-pta)$, $\Delta poxB$, $\Delta iclR$, $\Delta ptsG::Km^R$] (Lin et al., 2005a) were constructed from GJT001 (Tolentino et al., 1992).

Plasmid pKK313, carrying the S8D mutation in the *Sorghum vulgare pepc* gene (Wang et al., 1992), was used to produce high levels of phosphoenol pyruvate carboxylase (PEPC) in the mutant *E. coli* strains (Lin et al., 2005a and 2005b). PEPC catalyzes the carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA) and mediates the fixation of CO₂. OAA is then sequentially reduced to malate and eventually converted to succinate. The pKK313 plasmid confers ampicillin resistance and harbors altered *pepc* having a site-directed mutation that advantageously relieves malate feedback inhibition from the enzyme (Wang et al., 1992). Plasmids pRU600 and pUR400 were provided by Dr. Kurt Schmid (Universitat Osnabruck, Germany). The plasmid pRU600 bears chloramphenicol resistance and harbors *rafA* (α -galactosidase), *rafB* (raffinose permease) and *rafD* (sucrose hydrolase) involved in the catabolism of raffinose (Schmid and Schmitt, 1976; Aslanidis et al., 1989). The plasmid pRU600

allows the engineered *E. coli* strain HL2765 and HL27659k to metabolize raffinose and sucrose either individually in pure form or as a mixture under aerobic condition. The pUR400 plasmid (70 kb) has a tetracycline resistant marker and expresses *scrK* (fructokinase), *scrY* (sucrose-specific porin), *scrA* (enzyme II^{scr} of the phosphotransferase system), *scrB* (β -D-fructofuranoside fructohydrolase) and *scrR* (regulator) genes, which allows the metabolism of sucrose by converting sucrose 6-phosphate to β -D-fructose and α -D-glucose 6-phosphate (Schmid et al., 1982 and 1988). The pUR400 plasmid was conjugated in *E. coli* strains as described by Wang et al. (2011). The plasmid pHL413 (Lin et al., 2004) overexpresses the *pyc* gene from *Lactococcus lactis*, which encodes the enzyme pyruvate carboxylase that converts pyruvate to oxaloacetate (OAA).

2.3. Media, shake flask culture and cultivation conditions

Luria–Bertani (LB) medium was used for culture cultivations. Appropriate concentrations of ampicillin (100–200 mg/L), kanamycin (30 mg/L) or chloramphenicol (25 mg/L) were added to the medium. Precultures were grown from glycerol stock in a 15 mL tubes containing 5 mL of LB medium with appropriate antibiotics. Overnight grown precultures were washed twice with LB and used as 1% (v/v) inoculum.

A two-step culture technique, adapted from Sanchez et al. (2005), was used to examine succinate production. The first growth phase step was followed by the second step, the succinate production phase. Cells were aerobically grown at 37°C with shaking at 250 rpm for 16-17 h in 2 L flasks containing 400 mL of LB medium with appropriate antibiotics. Cells were harvested by centrifugation and the supernatant was discarded. The cells were then resuspended in 10 mL of soybean meal extract, soy solubles extract or its hydrolysate to a final concentration of 20 OD₆₀₀.

While using raffinose, stachyose, sucrose or galactose as the sole carbon source to examine succinate production, the cells were resuspended in 10 mL LB containing an appropriate concentration of the sugar. After resuspension, the cultures were transferred aseptically to 250 mL previously autoclaved flasks containing 0.3 g of MgCO_3 . Sterile foam stoppers were used as cap for 2 L and 250 mL flasks. All cultures were cultivated at 37°C and 250 rpm. Samples were collected at 24-48 h for HPLC analysis.

2.4. Extraction of soluble carbohydrates from soybean meal and hydrolysis of oligosaccharides

Twenty grams of soybean meal was added to 100 mL water and stirred at room temperature for 15 min. The content was centrifuged at 10,000×g for 10 min to remove wet soybean meal and the supernatant, herein referred as soybean meal extract, containing soluble carbohydrates was recovered. To prepare soybean meal hydrolysate, 0.3% (v/v) concentrated H_2SO_4 was added to the soybean meal extract and mixed by stirring for 1-2 min followed by incubation at 100°C for 1 h. After cooling to room temperature, the content was centrifuged at 10,000×g for 15 min to remove debris. The supernatant, referred to as soybean meal hydrolysate, was recovered and neutralized to pH 7.0 with 8 M NaOH.

Twenty grams of a thick slurry of soy solubles was added to water with a final volume of 100 mL and stirred at room temperature for 15 min. The content was centrifuged at 10,000×g for 10 min and the supernatant, referred as soy solubles extract, containing soluble carbohydrates was recovered. To prepare soy solubles hydrolysate, concentrated H_2SO_4 (1% v/v) was added to soy solubles extract and mixed by stirring for 1-2 min followed by incubation at 100°C for 1 h. After cooling to room temperature, the content was centrifuged at 10,000×g for 15 min to remove debris. The supernatant, referred as soy solubles hydrolysate, was recovered and

neutralized to pH 7.0 with 8 M NaOH. Soybean meal extract, soybean meal hydrolysate, soy solubles extract and soy solubles hydrolysate solutions were filtered through a 0.2 µm filter assembly **prior to their** use in fermentation experiments.

2.5. Analytical techniques

Cell density was measured at 600 nm using a UV–Vis spectrophotometer (DU800, Beckman Coulter, USA). Samples were diluted appropriately to the linear range with 0.9% **(w/v)** NaCl. For analyzing the extracellular metabolites, 1 mL of fermented broth was centrifuged for 1 min at 10,000×g in a microcentrifuge and the supernatant was then filtered through a 0.2 µm syringe filter for HPLC analysis. The fermentation products and residual sugars were quantified using a HPLC system (Shimadzu, Japan), equipped with a cation-exchange column (Aminex HPX-87H; Bio-Rad, USA), a UV detector (Shimadzu SPD-10A), and a differential refractive index detector (Waters, USA). A mobile phase of 2.5 mM H₂SO₄ solution at a 0.4 mL/min flow rate was used and the column operated at 28°C. Succinate, formate, acetate, fumarate, stachyose, raffinose, sucrose, glucose, galactose and fructose were measured by the RI detector and pyruvate was measured by the UV detector at 210 nm. Galactose and fructose were found to have similar retention times and therefore, quantified as residual galactose and or fructose.

While using galactose, sucrose and raffinose individually in pure form, the succinate yield was expressed as mol of succinate produced per mol of sugar consumed. In case of soluble sugars mixture containing glucose, galactose, fructose, sucrose, raffinose and stachyose, the succinate yield was expressed as mol of succinate produced per mol of hexose consumed. Each of these sugars was individually quantified by HPLC and the amount converted to monomeric hexose units to calculate total hexose consumed during fermentation.

3. Results and discussion

3.1. Fermentation of galactose, sucrose, raffinose and stachyose

In this study, we first examined HL2765 and HL27659k strains harboring pKK313 and pRU600 plasmids to see how well both strains are able to produce succinate using various concentrations of stachyose, raffinose, sucrose and galactose individually in pure form before testing them on soy based substrates that contain a mixture of these sugars.

Galactose metabolism and succinate production by HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) were examined using different concentrations of initial galactose in the range of 10 to 30 g/L (Fig. 1A and B). Strain HL2765(pKK313)(pRU600) consumed 52, 111.21 and 160.30 mM galactose in 24 h and produced 51.74, 82.81 and 114.55 mM succinate with succinate yields of 0.99, 0.74 and 0.71 mol/mol galactose while using 10, 20 and 30 g/L galactose, respectively. Whereas, strain HL27659k(pKK313)(pRU600) consumed 55.60, 111.60 and 163.15 mM galactose in 24 h and produced 53.16, 92.30 and 120.63 mM succinate with succinate yields of 0.95, 0.82 and 0.74 mol/mol galactose while using 10, 20 and 30 g/L galactose, respectively. Trace amount of byproducts such as formate, acetate and fumarate were also observed for both strains, specifically, a high concentration of acetate (17 – 20 mM) was seen while using 30 g/L galactose (Fig. 1A and B). It was observed that with increasing galactose concentration, succinate production increased, however, succinate yield decreased in both strains. Both strains were capable to efficiently utilize almost all of the initial available galactose within 24 h.

Metabolism of disaccharide sucrose in the range of 10 to 30 g/L and succinate production was examined using HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) (Fig. 2A

and B). Both strains were capable to efficiently metabolize sucrose to succinate in presence of plasmid pRU600 harboring *rafD* (sucrose hydrolase). With increasing sucrose concentration up to 30 g/L, succinate production by these two strains also increased. The strain HL2765(pKK313)(pRU600) consumed 9.25, 48.44, 79.26 mM sucrose in 24 h and produced 17.70, 88.52 and 129 mM succinate with succinate yields of 1.91, 1.82 and 1.62 mol/mol sucrose while using 10, 20 and 30 g/L sucrose, respectively. In comparison, strain HL27659k(pKK313)(pRU600) consumed 16.20, 51 and 80.50 mM sucrose in 24 h and produced 29, 85.80 and 134.85 mM succinate with succinate yields of 1.78, 1.68 and 1.67 mol/mol sucrose while using 10, 20 and 30 g/L sucrose, respectively. Trace amounts (0.7 – 1 mM) of acetate were also observed (Fig. 2A and B). Previously, Wang et al. (2011) reported the production of succinate from sucrose using HL2765k(pHL413)(pUR400) and HL27659k(pHL413)(pUR400). HL2765k(pHL413)(pUR400) was reported to produce 22 mM succinate in 46 h with succinate yield of 1.60 mol/mol sucrose, whereas, HL27659k(pHL413)(pUR400) produced 24 mM succinate with succinate yield of 1.84 mol/mol sucrose. Results of these two studies suggest that strains HL2765 and HL27659k harboring plasmids pHL413 and pUR400 or pKK313 and pRU600 are capable to efficiently metabolize sucrose to succinate.

Raffinose fermentation by HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) to succinate was examined using 5 and 10 g/L raffinose (Fig. 3A and B). The strain HL2765(pKK313)(pRU600) produced 29 mM and 47.4 mM succinate from 9.87 mM and 19 mM raffinose with succinate yields of 2.93 and 2.49 mol/mol raffinose, respectively (Fig. 3A). Whereas, HL27659k(pKK313)(pRU600) produced 29 mM and 53.88 mM succinate from 9.71 mM and 19.72 mM raffinose with succinate yields of 2.98 and 2.73 mol/mol raffinose, respectively (Fig. 3B). Both strains were capable to efficiently utilize almost

all of the initial available raffinose within 24 h. The efficient catabolism of raffinose by HL2765 and HL27659k was due to the presence of the pRU600 plasmid harboring *rafA* (α -galactosidase), *rafB* (raffinose permease) and *rafD* (sucrose hydrolase) genes (Aslanidis et al., 1989). Cornelis et al. (1978) reported that raffinose can be transported by the lactose permease, and isolated *lacI* constitutive mutants of *E. coli* K-12 capable of raffinose uptake and metabolism. Moniruzzaman et al. (1997) isolated raffinose positive mutants of KO11, a derivative of *E. coli* B, by culture enrichment in presence of raffinose and the adapted strain may contain an analogous mutation.

Strains HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) were examined for stachyose catabolism using 5 and 10 g/L stachyose. However, both strains showed poor utilization (1-3 mM in 24 h) of stachyose (data not shown). This could be due to the lack of specific glycoporin for stachyose transport in the host strains used in this study. In *E. coli* the uptake of tetrasaccharides, such as stachyose, and longer oligosaccharides, like maltodextrins, requires glycoporins (Ulmke et al., 1997). Results of the above experiments and previous studies (Lin et al., 2005a; Wang et al., 2011) showed the potential of engineered *E. coli* strains HL2765 and HL27659k harboring either pHL413 and pUR400 or pKK313 and pRU600 to efficiently metabolize glucose, galactose, fructose, sucrose and raffinose under aerobic conditions to produce succinate.

3.2. Production of succinate using soybean meal extract and its hydrolysate

HPLC analysis of soybean meal extract showed the presence of 19 mM stachyose, 2 mM raffinose, 48 mM sucrose, 5 mM galactose and or fructose, and 10 mM glucose which correspond to a total hexose concentration of about 193 mM. In comparison, soybean meal hydrolysate contains 1 mM stachyose, 13 mM raffinose, 25 mM sucrose, 55 mM galactose and

or fructose, and 42 mM glucose, which corresponds to a total hexose concentration of about 190 mM.

Succinate production using soybean meal extract by strains HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) at 24 h is shown in Fig. 4A. HL2765(pKK313)(pRU600) consumed 1.62 mM stachyose, 2 mM raffinose, 33.72 mM sucrose, 2.62 mM galactose and or fructose and 4.28 mM glucose which corresponds to about 87 mM hexose out of 193 mM total hexose available in soybean meal extract and produced 83 mM succinate in 24 h with a succinate yield of 0.95 mol/mol hexose. Whereas, HL27659k(pKK313)(pRU600) consumed 1.5 mM stachyose, 2 mM raffinose, 37.22 mM sucrose, 2.67 mM galactose and or fructose and 8.90 mM glucose which corresponds to about 98 mM hexose out of 193 mM total hexose available in soybean meal extract and produced 95 mM succinate with succinate yield of 0.97 mol/mol hexose. Minor amounts (about 1 mM) of byproducts acetate and pyruvate were also observed for both strains (Fig. 4A).

We also examined the capability of strains HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) to produce succinate using soybean meal hydrolysate containing 190 mM hexose sugars (Fig. 4B). HL2765(pKK313)(pRU600) consumed 12 mM raffinose, 14 mM sucrose, 50 mM galactose and or fructose and 25 mM glucose which corresponds to about 139 mM hexose to produce 87 mM succinate in 24 h. The molar succinate yield achieved for this strain was 0.62 mol/mol hexose. The strain produced about 3 mM formate and 5 mM acetate as byproducts (Fig. 4B). As compared to HL2765(pKK313)(pRU600), the strain HL27659k(pKK313)(pRU600) consumed 13 mM raffinose, 14.5 mM sucrose, 52 mM galactose and or fructose and 23 mM glucose which corresponds to about 143 mM hexose to produce 120 mM succinate, 2 mM formate and 0.4 mM acetate in 24 h (Fig. 4B). The molar succinate yield

for HL27659k(pKK313)(pRU600) was 0.83 mol/mol hexose, which was 1.3 fold higher than HL2765(pKK313)(pRU600). Both strains consumed more hexose sugars present in soybean meal hydrolysate as compared to soybean meal extract. However, molar succinate yield obtained using soybean meal hydrolysate was lower as compared to soybean meal extract. It is not clear why the molar succinate yield dropped while using soybean meal hydrolysate.

3.3. Production of succinate using soy solubles extract and its hydrolysate

We obtained soy solubles slurry, a byproduct rich in soluble sugars, from a soy processing industry. Soluble sugars were recovered from soy solubles as explained in materials and methods and used to demonstrate succinate production using HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600). HPLC analysis of soy solubles extract showed the presence of 45 mM stachyose, 13 mM raffinose, 90 mM sucrose, 12 mM galactose and or fructose, and 30 mM glucose which corresponds to a total hexose concentration of 441 mM. Whereas, soy solubles hydrolysate contains 37 mM raffinose, 31 mM sucrose, 147 mM galactose and or fructose, and 111 mM glucose, which corresponds to a total hexose concentration of about 431 mM.

Succinate production by strains HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) using soy solubles extract is shown in Fig. 5. HL2765(pKK313)(pRU600) consumed 13 mM raffinose, 53.82 mM sucrose, 5.11 mM galactose and or fructose and 8.22 mM glucose which corresponds to 160 mM hexose out of 441 mM total hexose available in soy solubles extract and produced 158 mM succinate, 18 mM formate and 9 mM acetate in 24 h. The molar succinate yield was 0.98 mol/mol hexose. Whereas, HL27659k(pKK313)(pRU600) consumed 2 mM stachyose, 13 mM raffinose, 60.43 mM sucrose,

4.72 mM galactose and or fructose and 14.42 mM glucose which corresponds to 187 mM hexose out of 441 mM total hexose available in soy solubles extract and produced 183 mM succinate, 19 mM formate and 0.56 mM acetate in 24 h. The molar succinate yield was 0.97 mol/mol hexose (Fig. 5). Thus, both strains were capable of producing succinate with close to maximum theoretical succinate yield using extracted soluble sugars of soy solubles.

Soy solubles extract was treated with concentrated H_2SO_4 to hydrolyze stachyose to raffinose, sucrose and hexose sugars for metabolisms by engineered *E. coli* strains HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600). Succinate production and hexose consumption by these two strains using soy solubles hydrolysate is shown in Fig. 6A and B. HL2765(pKK313)(pRU600) consumed 22.61 mM raffinose, 5.68 mM sucrose, 139.62 mM galactose and or fructose and 75.19 mM glucose which corresponds to 294 mM hexose out of total 431 mM hexose present in soy solubles hydrolysate and produced 204 mM succinate with succinate yield of 0.69 mol/mol hexose in 24 h. Additionally, the strain produced 20 mM formate, 46 mM acetate and 39 mM pyruvate. Whereas in 48 h, the strain consumed 30 mM raffinose, 21.55 mM sucrose, 140 mM galactose and or fructose and 80 mM glucose which corresponds to 353 mM hexose sugars and produced 246 mM succinate with a succinate yield of 0.7 mol/mol hexose. Byproducts were 17 mM formate, 110 mM acetate and 24 mM pyruvate (Fig. 6A). As compared to HL2765(pKK313)(pRU600), the strain HL27659k(pKK313)(pRU600) consumed 32.52 mM raffinose, 24.98 mM sucrose, 79.68 mM galactose and or fructose and 78.80 mM glucose which corresponds to 306 mM hexose out of total 431 mM hexose present in soy solubles hydrolysate and produced 250 mM succinate, 26 mM formate and 30 mM acetate in 24 h. Whereas in 48 h, the strain consumed 37 mM raffinose, 26.69 mM sucrose, 135.84 mM galactose and or fructose and 80 mM glucose which corresponds

to 380 mM hexose and produced 312 mM succinate, 27 mM formate and 50 mM acetate (Fig. 6B). The molar succinate yields for HL27659k(pKK313)(pRU600) at 24 h and 48 h was 0.81 and 0.82 mol/mol hexose, respectively. These results showed that both strains were capable of efficiently consuming hexose up to 48 h of fermentation and produced high succinate without decreasing molar succinate yields. It was interesting to observe that HL2765(pKK313)(pRU600) consumed less raffinose and sucrose but more galactose and or fructose in 24 h as compared to HL27659k(pKK313)(pRU600).

Despite the fact that the acetate producing pathways were knocked out, we noticed that both strains accumulated acetate, specifically increased acetate accumulation in 48 h. This could be due to a number of minor acetate producing pathways such as acetylornithine deacetylase and citrate lyase (Sanchez et al., 2005). It was interesting to observe accumulation of pyruvate in culture broth of the strain HL2765(pKK313)(pRU600) (Fig.6A). However, the strain HL27659k(pKK313)(pRU600) did not show accumulation of pyruvate. This could be due to the PtsG system which is active in the strain HL2765(pKK313)(pRU600). Pyruvate accumulation in the strain HL2765(pKK313)(pRU600) can be reduced by increasing OAA through coexpression of PEPC and *L. lactis* PYC (Lin et al., 2005c) that could result in higher succinate production.

Wang et al. (2011) reported efficient succinate production from sucrose under aerobic conditions using *E. coli* strains HL2765k and HL27659k harboring pUR400 and pHL413. As pUR400 expresses sucrose catabolizing genes, we thought using this plasmid along with pRU600 might help in efficient uptake and metabolism of sucrose by engineered *E. coli* strains. Therefore, we studied the effect of using pUR400 in combination of pRU600 and pKK313. Succinate production was examined using soy solubles hydrolysate using HL27659k(pKK313)(pRU600) and HL27659k(pKK313)(pRU600)(pUR400) strains. However,

the molar succinate yield dropped to 0.6 mol/mol hexose for the strain HL27659k(pKK313)(pRU600)(pUR400) (data not shown) as compared to 0.81 mol/mol hexose obtained by HL27659k(pKK313)(pRU600). HL27659k(pKK313)(pRU600)(pUR400) did not show significant improvement in sucrose uptake. Similarly, we examined the succinate production from soy solubles hydrolysate using HL27659k(pKK313)(pRU600) and HL27659(pHL413)(pRU600) to evaluate the effect of PYC overexpression as compared to PEPC overexpression. The strain HL27659k(pHL413)(pRU600) consumed only about 50 mM hexose and produced 40 mM succinate in 24 h (data not shown).

Lignocellulosic biomass rich in cellulose, hemicellulose and lignin, has attracted much attention as a source of reducing sugars to produce biofuels by fermentation. However, in order to use this material in bioprocesses, various chemical, mechanical, thermo-chemical and biochemical pretreatments have been investigated, including acid hydrolysis, alkali hydrolysis, steam explosion, or ammonia fiber explosion (Yoo et al., 2011). Thermo-mechanical extrusion pretreatment for the conversion of cellulosic sugars present in soybean hulls to fermentable sugars and their use to produce ethanol by *Saccharomyces cerevisiae* have been reported (Yoo et al., 2011 and 2012). Fermentation inhibitors, such as furfural, 5-(hydroxymethyl)-2-furaldehyde (HMF), and acetic acid, were found in the extrusion pretreated soybean hulls and hydrolysate. However, their concentrations were below the known thresholds for inhibition (Yoo et al., 2012). As compared to the harsh lignocellulosic biomass pretreatments, in the present study we used diluted sulphuric acid to hydrolyze soluble oligosaccharides, specifically stachyose, into fermentable sugars. However, it remains to be examined if there are any fermentation inhibitors present in soybean meal extract hydrolysate or soy solubles extract hydrolysate and at what

concentrations. In such circumstances, further pretreatment methods could be examined to minimize inhibitory effects and thereby to improve sugar metabolism and succinate production.

This study successfully demonstrated the use of soybean derived soluble sugar feedstock for the aerobic production of succinate. Further improvement in succinate yield and titer could be achieved by performing batch or fed-batch fermentation under controlled conditions using a bioreactor. To further reduce the process cost, attempts are being made to replace complex and expensive Luria broth medium with soybean meal extract or soy solubles based medium for culture growth and succinate production. In addition, there are other oilseeds that contain valuable carbohydrates. In this context, we have examined cottonseed meal extract carbohydrates in a similar process and obtained succinate with these strains. Additionally, *pepc* and raffinose operon genes could be integrated into the HL27659k strain for more stable gene expression and thereby eliminating the need for addition of antibiotics during fermentation process.

4. Conclusions

In the development of a succinic acid production process, the cost of raw material is an important factor. In this study, an efficient succinic acid production process using metabolically engineered *E. coli* strains HL2765 and HL27659k harboring pRU600 and pKK313 plasmids was demonstrated using soluble sugars extracted from soybean meal and soy solubles. Both strains showed capability to simultaneously metabolize multiple sugars and osmotolerance to high initial sugar concentration during the aerobic succinate fermentation process with close to theoretical molar succinic acid yield. HL27659k(pRU600)(pKK313) could be a potential strain for economic aerobic succinate production from renewable substrates such as soybeans.

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References

- Aslanidis, C., Schmid, K., Schmitt, R., 1989. Nucleotide sequences and operon structure of plasmid-borne genes mediating uptake and utilization of raffinose in *Escherichia coli*. J. Bacteriol. 171(12), 6753-6763.
- Borges, E.R., Pereira, N., Jr., 2011. Succinic acid production from sugarcane bagasse hemicellulose hydrolysate by *Actinobacillus succinogenes*. J. Ind. Microbiol. Biotechnol. 38(8), 1001-1011.
- Budarin, V., Luque, R., Macquarrie, D.J., Clark, J.H., 2007. Towards a bio-based industry: benign catalytic esterifications of succinic acid in the presence of water. Chemistry. 13(24), 6914-6919.
- Cornelis, G., Luke, R. K. J., Richmond, M. H., 1978. Fermentation of raffinose by lactose-fermenting strains of *Yersinia enterocolitica* and by sucrose-fermenting strains of *Escherichia coli*. J. Clin. Microbiol. 7, 180-183.
- Eldridge, A.C., Black, L.T., Wolf, J.W., 1979. Carbohydrate composition of soybean flours, protein concentrates, and isolates. J. Agric. Food Chem. 27, 799-802.
- Giannoccaro, E., Wang, Y.J., Chen, P., 2006. Effects of solvent, temperature, time, solvent-to-sample ratio, sample size, and defatting on the extraction of soluble sugars in soybean. J.

Food Sci. 71, C59-C64.

Guettler, M.V., Jain, M.K., Rumler, D. 1996. Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. US Patent 5,573,931.

Kim, D.Y., Yim, S.C., Lee, P.C., Lee, W.G., Lee, S.Y., Chang, H.N., 2004. Batch and continuous fermentation of succinic acid from wood hydrolysate by *Mannheimia succiniciproducens* MBEL55E. Enzyme Microb. Technol. 35, 648-653.

Lee, P.C., Lee, S.Y., Chang, H.N., 2008. Succinic acid production by *Anaerobiospirillum succiniciproducens* ATCC 29305 growing on galactose, galactose/glucose, and galactose/lactose. J. Microbiol. Biotechnol. 18(11), 1792-1796.

Lee, P.C., Lee, S.Y., Hong, S.H., Chang, H.N., Park, S.C., 2003. Biological conversion of wood hydrolysate to succinic acid by *Anaerobiospirillum succiniciproducens*. Biotechnol. Lett. 25(2), 111-114.

Lee, S.Y., Kim, J.M., Song, H., Lee, J.W., Kim, T.Y., Jang, Y.S., 2008. From genome sequence to integrated bioprocess for succinic acid production by *Mannheimia succiniciproducens*. Appl. Microbiol. Biotechnol. 79(1), 11-22.

Li, J., Zheng, X.Y., Fang, X.J., Liu, S.W., Chen, K.Q., Jiang, M., Wei, P., Ouyang, P.K., 2011. A complete industrial system for economical succinic acid production by *Actinobacillus succinogenes*. Bioresour. Technol. 102(10), 6147-6152.

Lin, H., Bennett, G.N., San, K.Y., 2005a. Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield. Metab. Eng. 7(2), 116-127.

Lin, H., Bennett, G.N., San, K.Y., 2005b. Fed-batch culture of a metabolically engineered

- Escherichia coli* strain designed for high-level succinate production and yield under aerobic conditions. *Biotechnol. Bioeng.* 90(6), 775-779.
- Lin, H., San, K.Y., Bennett, G.N., 2005c. Effect of *Sorghum vulgare* phosphoenolpyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 67(4), 515-523.
- Lin, H., Vadali, R.V., Bennett, G.N., San, K.Y., 2004. Increasing the acetyl-CoA pool in the presence of overexpressed phosphoenolpyruvate carboxylase or pyruvate carboxylase enhances succinate production in *Escherichia coli*. *Biotechnol. Prog.* 20(5), 1599-1604.
- Liu, K., 1997. Soybeans: chemistry, technology, and utilization. Chapman and Hall, New York.
- Luo, L., van der Voet, E., Hupples, G., 2010. Biorefining of lignocellulosic feedstock-Technical, economic and environmental considerations. *Bioresour. Technol.* 101(13), 5023-5032.
- McKinlay, J.B., Vieille, C., Zeikus, J.G., 2007. Prospects for a bio-based succinate industry. *Appl. Microbiol. Biotechnol.* 76(4), 727-740.
- Moniruzzaman, M., Lai, X., York, S.W., Ingram, L.O., 1997. Extracellular melibiose and fructose are intermediates in raffinose catabolism during fermentation to ethanol by engineered enteric bacteria. *J. Bacteriol.* 179(6), 1880-1886.
- Sanchez, A.M., Bennett, G.N., San, K.Y., 2005. Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity. *Metab. Eng.* 7(3), 229-239.
- Schmid, K., Ebner, R., Altenbuchner, J., Schmitt, R., Lengeler, J.W., 1988. Plasmid-mediated sucrose metabolism in *Escherichia coli* K12: mapping of the *scr* genes of pUR400. *Mol. Microbiol.* 2(1), 1-8.

Schmid, K., Schmitt, R., 1976. Raffinose metabolism in *Escherichia coli* K12. Purification and properties of a new α -galactosidase specified by a transmissible plasmid. Eur. J. Biochem. 67, 95-104.

Schmid, K., Schupfner, M., Schmitt, R., 1982. Plasmid-mediated uptake and metabolism of sucrose by *Escherichia coli* K-12. J. Bacteriol. 151(1), 68-76.

Shanmugam, K.T., Ingram, L.O., 2008. Engineering biocatalysts for production of commodity chemicals. J. Mol. Microbiol. Biotechnol. 15(1), 8-15.

Thakker, C., Martinez, I., San, K.Y., Bennett, G.N., 2012. Succinate production in *Escherichia coli*. Biotechnol. J. 7(2), 213-224.

Tolentino, G.J., Meng, S.Y., Bennett, G.N., San, K.Y., 1992. A pH-regulated promoter for the expression of recombinant proteins in *Escherichia coli*. Biotechnol. Lett. 14, 157-162.

Ulmke, C., Lengeler, J.W., Schmid, K., 1997. Identification of a new porin, RafY, encoded by raffinose plasmid pRSD2 of *Escherichia coli*. J. Bacteriol. 179, 5783-5788.

Wang, J., Zhu, J., Bennett, G.N., San, K.Y., 2011. Succinate production from sucrose by metabolic engineered *Escherichia coli* strains under aerobic conditions. Biotechnol. Prog. 27(5), 1242-1247.

Wang, Y.H., Duff, S.M., Lepiniec, L., Cretin, C., Sarath, G., Condon, S.A., Vidal, J., Gadal, P., Chollet, R., 1992. Site-directed mutagenesis of the phosphorylatable serine (Ser8) in C4 phosphoenolpyruvate carboxylase from *Sorghum*. The effect of negative charge at position 8. J. Biol. Chem. 267(24), 16759-16762.

Werpy, T., Petersen, G., 2004. Top Value Added Chemicals from Biomass. USDOE, Washington DC.

- Xu, J., Guo, B.H., 2010. Poly(butylene succinate) and its copolymers: research, development and industrialization. *Biotechnol. J.* 5(11), 1149-1163.
- Yoo, J., Alavi, S., Vadlani, P., Amanor-Boadu, V., 2011. Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars. *Bioresour. Technol.* 102(16), 7583-7590.
- Yoo, J., Alavi, S., Vadlani, P., Behnke, K.C., 2012. Soybean hulls pretreated using thermo-mechanical extrusion-hydrolysis efficiency, fermentation inhibitors, and ethanol yield. *Appl. Biochem. Biotechnol.* 166(3), 576-589.
- Zeikus, J.G., Jain, M.K., Elankovan, P., 1999. Biotechnology of succinic acid production and markets for derived industrial products. *Appl. Environ. Microbiol.* 51, 545–552.

Figure Captions

Figure 1. Galactose metabolism by [A] HL2765(pKK313)(pRU600) and [B] HL27659k(pKK313)(pRU600) at 24 h

Figure 2. Sucrose metabolism by [A] HL2765(pKK313)(pRU600) and [B] HL27659k(pKK313)(pRU600) at 24 h

Figure 3. Raffinose metabolism by [A] HL2765(pKK313)(pRU600) and [B] HL27659k(pKK313)(pRU600) at 24 h

Figure 4. Succinate production using [A] soybean meal extract and [B] soybean meal hydrolysate by HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) at 24 h

Figure 5. Succinate production using soy solubles extract by HL2765(pKK313)(pRU600) and HL27659k(pKK313)(pRU600) at 24 h

Figure 6. Succinate production using soy solubles hydrolysate by [A] HL2765(pKK313)(pRU600) and [B] HL27659k(pKK313)(pRU600) at 24 h and 48 h

Table 1. Strains and plasmids used in this study

Strains / Plasmids	Genotype / Phenotype	Source
<i>Strains</i>		
HL2765	GJT001[Δ <i>sdhAB</i> , Δ (<i>ackA-pta</i>), Δ <i>poxB</i> , Δ <i>iclR</i>]	Lin et al., 2005a
HL27659k	GJT001[Δ <i>sdhAB</i> , Δ (<i>ackA-pta</i>), Δ <i>poxB</i> , Δ <i>iclR</i> , Δ <i>ptsG</i> ::Km ^R]	Lin et al., 2005a
<i>Plasmids</i>		
pKK313	S8D mutant <i>Sorghum vulgare</i> <i>pepc</i> in pKK233-2, Ap ^R / Mutant <i>pepc</i> overexpression relieves malate feedback inhibition and catalyzes the carboxylation of PEP to OAA	Wang et al., 1992
pHL413	<i>Lactococcus lactis</i> <i>pyc</i> in pTrc99A, Ap ^R / <i>pyc</i> overexpression increases the flux from pyruvate to OAA	Lin et al., 2004
pUR400	<i>scrK</i> (fructokinase), <i>scrY</i> (sucrose-specific porin), <i>scrA</i> (enzyme II ^{scr} of the phosphotransferase system), <i>scrB</i> (β -D- fructofuranoside fructohydrolase) and <i>scrR</i> (regulator) genes, Tc ^R / allows the metabolism of sucrose by converting sucrose 6-phosphate to β -D-fructose and α -D- glucose 6-phosphate	Schmid et al., 1988
pRU600	<i>rafA</i> (α -galactosidase), <i>rafB</i> (raffinose permease) and <i>rafD</i> (sucrose hydrolase) in pACYC184, Cm ^R / allows the metabolism of raffinose and sucrose	Schmid and Schmitt, 1976; Aslanidis et al., 1989

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5

Figure 6.