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O-Succinyl-L-homoserine-based C4-chemical production: succinic acid, homoserine lactone, γ-butyrolactone, γ-butyrolactone derivatives, and 1,4-butanediol

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Abstract There has been a significant global interest to produce bulk chemicals from renewable resources using engineered microorganisms. Large research programs have been launched by academia and industry towards this goal. Particularly, C4 chemicals such as succinic acid (SA) and 1,4-butanediol have been leading the path towards the commercialization of biobased technology with the effort of replacing chemical production. Here we present *O*-Succinyl-L-homoserine (SH) as a new, potentially important platform biochemical and demonstrate its central role as an intermediate in the production of SA, homoserine lactone (HSL), γ-butyrolactone (GBL) and its derivatives, and 1,4-butanediol (BDO). This technology encompasses (1) the genetic manipulation of *Escherichia coli* to produce SH with high productivity, (2) hydrolysis into SA and homoserine (HS) or homoserine lactone hydrochloride, and (3) chemical conversion of either HS or homoserine lactone HCL (HSL·HCl) into drop-in chemicals in polymer industry. This production strategy with environmental benefits is discussed in the perspective of targeting of fermented product and a process direction compared to petroleum-based chemical conversion, which may reduce the overall manufacturing cost.

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Introduction

Increasing oil prices, depleting petroleum reserves, and the uncertain political environments compel the use of alternative resources as the raw material for the continued production of bulk and commodity chemicals [19]. Agriculture-derived sugars offer an attractive alternative to petroleum because they are renewable, environmental friendly and therefore, sustainable. Currently, the technology for biochemical production has been shown with some successful examples. 1,3-Propandiol for the production of plastic, polytrimethylene terephthalate (PTT) was produced by recombinant *Escherichia coli* using glucose as a renewable resource [11]. This bioprocess was reported to be superior to the incumbent petroleum-based one with 40 % less energy utilization and 20 % reduction of greenhouse gas emissions [9]. Other chemicals such as 1,4-butanediol, isobutanol, and succinic acid (SA) are also close to commercialization by biobased process [3, 19, 20]. The recent advances in metabolic engineering and associated technology for making efficient microbial cell factories are enabling the transformation of chemical industry. Despite the success, there are still challenges for bioprocesses to replace petro-chemical processes. The primary hurdle is the high manufacturing cost associated with producing biochemical. Several factors contribute to the cost such as the price of carbon source, product yield and productivity, and level of purification. Through the optimization of metabolic pathways, it has been possible to achieve near-maximal

product yields for bioproducts particularly, under anaerobic conditions to prevent CO_2 loss [1]. However, attaining high productivity seems to be a greater challenge at commercial scale [8]. Construction of a cell factory that is capable of high specific carbon uptake rate with metabolizing high amount of carbon sources in short time, i.e. for high productivity with high titer, is relatively more difficult than engineering the cell that has proper carbon flux distribution, i.e. for high yield. The lack of an economic process for the purification of the biochemical from fermentation broth is another critical factor contributing to the high cost of biochemicals; generally the required purity level of chemicals as monomers or additives for producing polymer is higher than 99.9 %. Since bioprocesses use different raw materials, impurity such as ions, organic acids, water content significantly differs from those of petro-based processes. This difference has been the barrier for biochemical production over the last few years.

Among fermentation products, C4 chemicals have advanced closest to commercialization, for example, succinic acid, isobutanol, 1,4-butanediol, and so on. The structural and chemical versatility resulting in diverse applications, large market potential, and the promise of replacing petro-derived alternatives are the main factors driving the success [17]. The recent decline in the cost of shale gas renews interest in the production of C2 and C3, but has not aided the production of C4 chemicals [13]. Commercial production of SA is planned early next year by BioAmber, and the technology of 1,4-butanediol production developed by Genomatica has been employed by BASF, which is the biggest producer of petro-1,4-butanediol.

In this study, we propose another process that can tap into the large market of C4 chemicals. First, *O*-Succinyl-L-homoserine (SH) was produced by fermentation using recombinant *E. coli* with high productivity. Next, SH was hydrolyzed by hydrogen chloride (HCl) generating SA and homoserine (HS) or homoserine lactone·HCl (HSL·HCl). Finally, the HSL·HCl was converted into γ-butyrolactone (GBL) and its derivatives and BDO with high yield. SH is a metabolite associated with methionine biosynthesis. Its accumulation was detected during the construction of methionine producing *E. coli* [15]. While the toxicity of the metabolites in methionine biosynthesis pathway such as HS and homocysteine has been reported, its production was not relatively harmful for cell growth [14]. We report the SH titers of over 100 g/l in 25 h. The production of SA from SH by acid hydrolysis is possible, which means that the production of SH may be one of the strategies for SA production. Furthermore, HS or HSL·HCl, also being a C4 chemical, could serve as a platform for conversion into other valuable chemicals. Here, we present a novel technology that can produce SH and a portfolio of C4 chemicals.

Materials and methods

Strains and plasmids construction

Threonine-producing *E. coli* FTR2533 was used as a host strain [12]. It was evolved to produce higher amount of threonine by classical random mutagenesis and amino acid analogue-based screening process. It had resistance to threonine analogue (α-amino-β-hydroxy valeric acid), lysine analogue (S-2-aminoethyl-L-cysteine), isoleucine analogues (α-aminobutyric acid), methionine analogues (ethionine) and the like. The gene deletion of *metB* and *thrB* was conducted using FRT one-step deletion method [2]. The mutant *metA* and *thrA* genes were amplified. Those were named as *metA11* and *thrAm*, which had resistance to feedback inhibition from methionine and threonine, respectively [6, 15]. The *metA11* gene was cloned into *pCL_Pcysk* vector that had been constructed by inserting of *E. coli* W3110 *cysK* promoter into *pCL1920* [7]. The constructed plasmid was named as *pCL_Pcysk-metA11*. The *thrAm* gene was inserted into the *pCL_Pcysk-metA11* resulting in *pCL_Pcysk-metA11-thrAm.* The second plasmid, *pBAC-scrKABR*, was constructed by cloning *scr* regulon. The *scr* regulon was obtained from a sucrose assimilative microorganism, *Streptococcus* species. The *scr* regulon of Gram-positive *Streptococcus* species was composed of four genes, *scrK*, *scrA*, *scrB*, and *scrR*. *Streptococcus* species (ATCC700610D-5) were purchased from American Type Culture Collection as a template [4]. The *scr* regulon PCR products were cloned into pCC1BAC/HindIII (EPICENTRE Cat. No. CCBAC1H) using infusion cloning kit (clontech Cat No.638910). The ligated circular DNAs were transformed with DH5a and spread on a MacConkey agar plate containing 0.5 % sucrose. Among the colonies, deep purple colonies were selected. FTR-SH strain was constructed by transforming these two plasmids into FTR2255 having inactivated *thrB* and *metB* (Table 1).

Fermentation

A seed culture was prepared at 33 °C overnight in 600 ml medium, using seed media (50.0 g/l sucrose, 2.5 g/l $MgSO_4 \cdot 7H_2O$, 7.0 g/l KH_2PO_4, 1.0 g/l NH_4Cl, 0.5 g/l NaCl, 10.0 g/l yeast extract, 50 mg/l spectinomycin dihydrochloride pentahydrate, 30 mg/l chloramphenicol) in 1-l bioreactors (Biott Corporation, Japan). Antibiotics were used only in seed culture. The pH was held at 6.5 using ammonia gas. The agitation rate was set at 900 rpm. Airflow was kept constant at 0.4 l/min. Fermentations were performed with 0.4 l initial culture volume in 1-l bioreactors (Biott Corporation, Japan) using main media (40.0 g/l sucrose, 4.2 g/l $MgSO_4 \cdot 7H_2O$, 3.0 g/l KH_2PO_4, 3.2 g/l yeast extract, 6.3 g/l ammonium sulfate, 0.5 g/l

Table 1 Strains and plasmids

Designation	Genotype or description	References
Strains		
FTR2533	A high L-threonine-producing strain, *E.coli* W3110 mutant generated by random mutagenesis	[12]
FTR-SH	FTR2533 Δ*metB,* Δ*thrB pCL-Pcysk-metA11*[a] *_thrAm*[b]*, pBAC-scrKABR*[c]	This study
Plasmids		
pCL-Pcysk-metA11_thrAm	*metA11, thrAm* amplification	This study
pBAC-scrKABR	Sucrose operon amplification	This study

[a] *metA11* [15]; [b] *thrAm* [6], [c] *scrKABR* [4]

L-methionine, 0.5 g/l L-threonine, 0.5 g/l L-isoleucine). The temperature was controlled at 33 °C and the pH was set at 6.5 using ammonia gas. The agitation rate was set at 1,100 rpm. Airflow was kept constant at 0.4 l/min. The feed was started when the dissolved O_2 tension increased abruptly, and it consisted of 600.0 g/l sucrose, 8.0 g/l KH_2PO_4, 0.5 g/l L-methionine, 0.5 g/l L-threonine, and 0.5 g/l L-isoleucine. Feed media were fed in to maintain the sucrose concentration in the bioreactors between 0.5 and 5.0 g/l.

Hydrolysis of SH

Crystal of SH was treated with HCl, 1.1–13.5 eq at 50–100 °C for 3 h in 500-ml flask. After that, the mixture was chilled and SA was filtrated by filter paper (Whatman™ Cat No. 1541-110). HSL·HCl was concentrated and dried in vacuum oven at 70 °C overnight.

Conversion of GBL derivatives from HSL·HCl

HSL·HCl (120 g), distilled water (70 g), and HCl (10 cc) were reacted in 3-neck round-bottom flask at room temperature under normal pressure. NO gas and air, especially NO_2, were supplied into the HSL·HCL solution using the dip tube to form NOx species. The molar ratio of HSL·HCl:NO_2 was maintained as 1:1. Both gases were supplied enough to implement gas–liquid contact progress while the reaction solution was mixed with magnetic bar. The leakage of the unreacted and product gas can be prevented by the installed ventilation line. After the reaction, the result solution was followed to be extracted with chloroform for Cl-GBL or water for HO-GBL due to their different hydrophilicity and hydrophobicity so that the products can be selectively separated into Cl-GBL and HO-GBL with the reduced pressure distillation. The reacted samples were analyzed by gas chromatography (GC) with FID detector. DB-200 column (30 m × 0.32 mm, 0.5 μm, PN 123-2033, Agilent) was used. The injected volume was 0.2 μL and the sample was separated by the gradient from 100 to 250 °C condition [16].

Production of GBL from Cl-GBL

The obtained Cl-GBL after the prior reaction was reacted with a catalyst in continuous fixed-bed flow system for dechlorization. $Pd(NO_3)_2$, the catalyst precursor, was prepared and loaded on activated carbon support by impregnation method. Pd catalyst was loaded on the support with the amount of 5 wt%. Before performing a catalytic reaction, the catalyst went through H_2 reduction at 250 °C for 6 h. The catalysis reaction was performed at 200 °C, 1 atm in a fixed-bed reactor. A thermocouple was installed in 1 g catalyst for monitoring the reaction temperature. Weight hourly space velocity (WHSV) was 1.0 h^{-1} while the Cl-GBL solution was fed into the catalytic reactor at 43:1 of the molar ratio H_2/Cl to GBL. The 95 % yield of GBL was maintained over 450 h.

HO-GBL to GBL

The obtained HO-GBL after the prior reaction was reacted with a catalyst in continuous fixed-bed flow system for the dehydration reaction. A 25/75 volume ratio of HO-GBL/1,4-dioxane was fed into the reactor. 5 wt%Rh–5 wt%Re was loaded on an activated carbon support at 5 wt% by impregnation method. Then, the catalyst is reduced by hydrogen for 6 h at 250 °C. The catalysis reaction was performed at 250 °C, 5 atm in a fixed-bed reactor. The amount of used catalyst was 2 g. A thermocouple was fixed in an actual catalyst bed to measure the reaction temperature. WHSV was 0.15 h^{-1} and the molar ratio H2 to Cl-GBL was 110:1. The GBL yield was kept about 69 % over 57 h.

Conversion of GBL to 1,4-BDO

Hydrogenation of GBL to produce 1,4-BDO in continuous fixed-bed flow system was performed. GBL was reacted with a silica-supported copper-modified catalyst [18]. The catalyst is reduced by hydrogen for 6 h at 250 °C. The catalysis reaction was performed at 170 °C, 40 atm in a fixed-bed reactor. The amount of used catalyst was 2 g. A

thermocouple was fixed in an actual catalyst bed to measure the reaction temperature. WHSV was 0.6 h^{-1} and the molar ratio H_2 to GBL was 30:1. The reaction was finished by 60 h. The yield of 1,4-BDO was reached to 98.1 %.

Results and discussion

Observation of SH production on constructing methionine producing strain

O-Succinyl-L-homoserine (SH) is the first metabolite in the de novo methionine biosynthesis pathway of *E. coli* (Fig. 1). It is generated by transferring a succinyl group from succinyl-CoA to HS through homoserine *O*-Succinyltransferase (MetA). The accumulation of SH has been detected on the research of methionine fermentation [15]. Although methionine production by fermentation has been developed in the academia and industry for several decades, commercial-scale bio-production of methionine has been still limited by our lack of understanding of metabolic flux regulation and the challenges involved in sulfur incorporation in de novo methionine biosynthesis [5]. Only a few grams per liter of fermented methionine production have been reported as a maximum titer [5]. In our previous study, we constructed methionine producing strains; however, interestingly several strains produced more SH than methionine [15]. Those strains had high copy numbers of mutated *thrA* and *metA*, which were engineered to be released from feedback regulation by threonine and methionine, respectively [6, 15]. Among the metabolites, there would be relatively easily accumulated ones than the others. Those may be not involved in other reactions as an inhibitor or simply transported out of the cell. It is not clear which property of SH determines the ease of accumulation. Furthermore, two substrates of MetA (HS and succinyl-CoA) have been known not to be accumulated individually. Succinyl-CoA is quite an unstable compound, and also intracellular HS has been reported as a toxic metabolite for the *E. coli* cell growth [14]. However, SH is relatively easily produced without growth retardation. To screen out the metabolites which are accumulated well than the others and develop further, bioconversion steps could be one of the strategies for expanding available biochemicals.

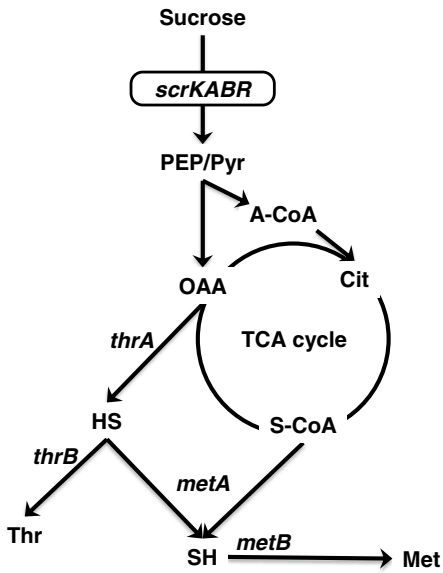

Fig. 1 Sucrose-based SH production biosynthetic pathway in *Escherichia coli*. SH is generated by transferring succinyl group from succinyl-CoA to HS catalyzed by *metA*. *scrKABR scr* regulon of *Streptococcus* species (ATCC700610D-5), *PEP/Pyr* phosphoenolpyruvate/pyruvate, *A-CoA* acetyl-coA, *OAA* oxaloacetate, *Cit* citrate, *S-CoA* succinyl-CoA, *HS* homoserine, *SH O*-Succinyl-L-homoserine, *Met* methionine, *thrA* aspartate kinase/homoserine dehydrogenase, *thrB* homoserine kinase, *metA* homoserine *O*-Succinyltransferase, *metB O*-Succinylhomoserine lyase

Development of SH producing bioprocess with high productivity

To remodel the strain for producing SH, FTR2533 strain used was generated by random mutagenesis and amino acid analogue-based screening process to produce higher amount of threonine [12]. Firstly, *metB* and *thrA* were inactivated for the blocking of metabolic fluxes into methionine and threonine in the FTR2533 strain (Table 1; Fig. 1). Secondly, mutant forms of *metA* and *thrA* that had resistance to feedback inhibition from methionine and threonine, respectively, were amplified [6, 15]. And then *scrKABR* operon was overexpressed for the utilization of sucrose as a carbon source [4]. The constructed strain was named as FTR-SH (Table 1). The ancestor strain FTR2533 could not produce SH at all, since there were still the fluxes of threonine and methionine from HS. FTR-SH strain showed relatively high SH yield, 65.5 % (g/g sucrose) (Fig. 2a). This value is quite close to commercialized amino acid production yield such as threonine and lysine, which means that the cost of SH could be possible in the range of commodity chemicals. In addition, there is space for improving yield since the maximum theoretical yield of SH based on sucrose is over 80 % (g/g). And reduction of expensive nutrients such as amino acids could be possible by engineering cells that require the minimum amount of amino acid or have very weak fluxes from HS to each amino acid. The more important feature is monitored in SH productivity. The SH accumulated to over 100 g/l in 25 h, in 1-l fed-batch fermenter (Fig. 2b), translating into a high SH productivity of over 4 g/l/h. This aspect of the process is extremely beneficial for commercial deployment. SH is composed of SA and HS, which implies that SH production bioprocess can be an alternative way to produce SA,

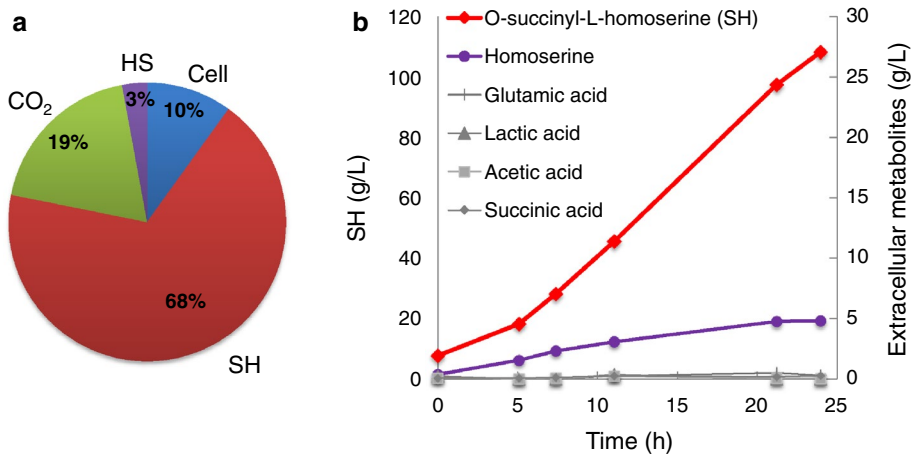

Fig. 2 Total carbon distribution of SH fermentation (g cell or product carbon/g sucrose carbon). Cell and fermentation products such as SH, CO_2, and HS (g) were taken into account for carbon distribution calculation in terms of sucrose carbon gram equivalent. (**a**) Fermentation profile of SH production. SH and extracellular metabolites such as organic acids including homoserine were presented (**b**)

Table 2 Hydrolysis of SH and products

Entry	Reaction condition	SA yield (mol%)	HSL·HCl yield (mol%)
1	75 % SH 60 g, 20 % HCl 137 ml (3.23 eq), 50 °C, 2 h	67.6	86.0
2	75 % SH 60 g, 20 % HCl 137 ml (3.23 eq), 60 °C, 2 h	64.8	81.8
3	75 % SH 60 g, 20 % HCl 137 ml (3.23 eq), 70 °C, 2 h	66.9	85.8
4	75 % SH 60 g, 20 % HCl 137 ml (3.23 eq), 80 °C, 2 h	65.4	85.3
5	90 % SH 60 g, Conc. HCl 73 ml (3.23 eq) + H_2O (18 ml), 80 °C, 3 h	75.3	89.9
6	92 % SH 60 g, Conc. HCl 73 ml (3.23 eq) + H_2O (18 ml), 80 °C, 3 h	63.0	89.6
7	98 % SH 60 g, Conc. HCl 73 ml (3.23 eq) + H_2O (64 ml), 80 °C, 3 h	74.0	91.3
8	98 % SH 60 g, Conc. HCl 73 ml (3.23 eq) + H_2O (18 ml), 80 °C, 3 h	82.0	85.9

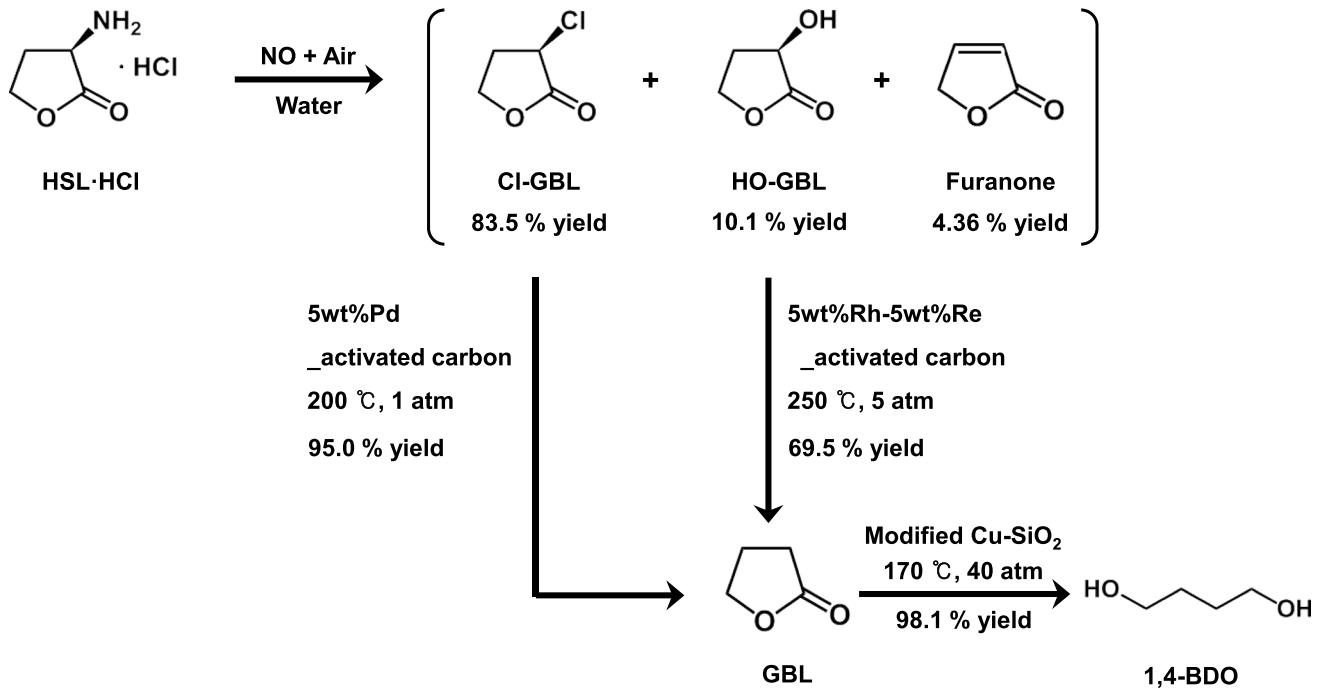

Fig. 3 Chemical conversion from HSL·HCl to 1,4-BDO through passing GBL derivatives and GBL. Only part of whole reactions is depicted

one of the key platform biochemicals [17]. Based on public reports, even though the yield of SA fermentation is very high, it has low productivity, around under 2–3 g/l/h [1]. To overcome low productivity, dual-phase production, and even aerobic condition, has been suggested because of poor carbon uptake and growth rates in anaerobic state. For the commercial-scale production of biochemical, the manufacturing cost is one of the most important things. While the product yield from carbon is a critical factor that determines the cost of production, scaling the size of a fermenter could offset the lower yield. This situation presents a scenario where SH-based SA production could be competitive way compared to direct SA fermentation in large scale. Furthermore, the other by-product, HS or HSL·HCl, is also a valuable product which contributes to the profitability.

Solubility of SH and hydrolysis of SH into SA and HSL·HCl

SH has much lower solubility than SA in water, 8.7 g/l, while SA is reported to have a solubility of 56.0 g/l. It means that the crystallization of SH is much easier than SA, and this results in higher recovery percentage of SH. Hence, there is a clear opportunity to reduce the cost of overall purification process. The way to break ester bond of SH was tested by controlling the concentration of HCl, reaction time, and temperature (Table 2). The max yield of SA was 80 mol%, and the one of HSL·HCl was 91 mol%. It is confirmed that SH was simply hydrolyzed into SA and homoserine lactone·HCl (HSL·HCl) by acid treatment.

Chemical conversion of HSL·HCl to GBL, GBL derivatives and BDO

HSL·HCl was converted to diverse γ-butyrolactone (GBL) derivatives and GBL by catalytic and non-catalytic deamination, and then GBL was converted to 1,4-butanediol (BDO) by hydrogenation based on metal catalyst (Fig. 3). The deamination using the mixture of NO gas and air was achieved, and resulted in 83.5 % molar yield of chloro-γ-butyrolactone (Cl-GBL), a 10.1 % yield of hydroxy-γ-butyrolactone (HO-GBL), and 4.36 % yield of furanone.

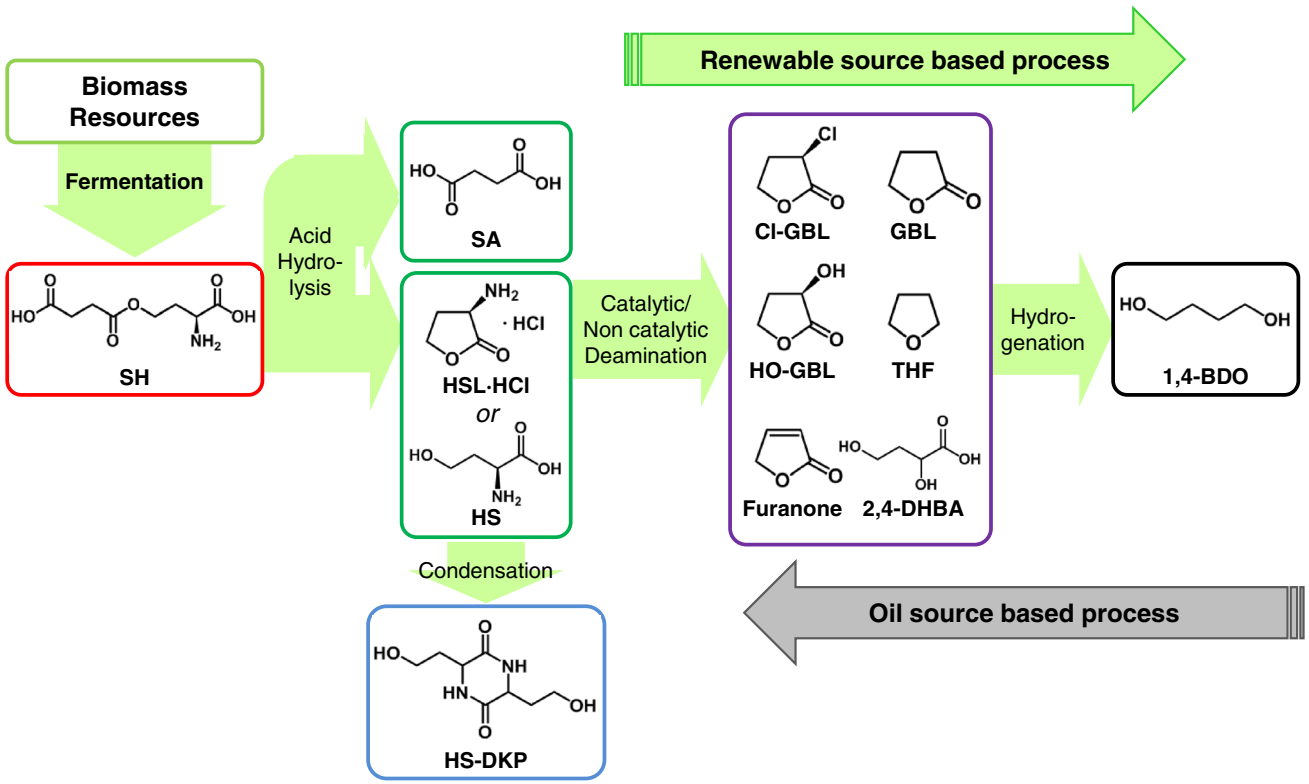

Fig. 4 Overall process of SH-based C4 biochemical production. Production of homoserine–DKP is only proposed, based on previous patent applications about other amino acids–DKP. The chemicals in the *purple color box* are generated experimentally depending on reaction conditions. The direction of process between proposed one in this study and oil source-based one is compared. *SH O*-Succinyl-L-homoserine, *SA* succinic acid, *HSL·HCl* homoserine lactone hydrochloride, *HS* homoserine, *DKP* diketopiperazine, *Cl-GBL* choride-γ-butyrolactone, *HO-GBL* hydroxy-γ-butyrolactone, *GBL* γ-butyrolactone, *THF* tetrahydrofuran, *2, 4-DHBA* 2, 4-dihydroxybutanoic acid, *1,4-BDO* 1,4-butanediol

Those compounds, Cl-GBL and HO-GBL, were converted to GBL through catalyst-based process resulting in a yield of 95.0 and 69.5 %, respectively. This GBL was converted to BDO, a 98.1 % yield in the condition of 170 °C, 40 bar with modified Cu-SiO_2 (Cu-M_SiO_2) catalyst. The overall yield of BDO from HSL·HCl was 84.74 %. These results clearly demonstrate the conversion of HS into drop-in chemicals such as GBL and BDO. Even though further development in conversion yield and optimization of reaction condition for the specific chemical production is necessary, our technology holds the promise of a new way to produce a diverse portfolio of C4 chemicals simultaneously. Reductive deamination is the key step for the conversion of HSL·HCl directly to GBL and BDO. In this study, NO gas-mediated deamination was used to remove amine group, which replaced it with chloro or hydroxy residue. Further development of other deamination methodologies could make one-step conversion of HSL·HCl to GBL and BDO, and reduce the chemical conversion cost. Since GBL derivatives could be used as fine chemicals and pharmaceutical intermediates, it is important to understand this reaction. There is a lot of scope for understanding the parameters that control the reactions and their effect on different derivatives; there could be significant space to design a more effective process.

Conclusion

Biochemicals from biobased SH are summarized in Fig. 4. These are produced using renewable resources. This technology has the potential to produce SA with very high productivity and simultaneously other C4 chemicals. The ester bond in SH is easily broken into SA and HSL·HCl by acid hydrolysis. Vast amount of information about the application and importance of SA as a platform biochemical has been reported [17]. While the yield of bio-SA in commercial production has almost reached to theoretical maximum, the practical issue that is impeding progress is the rate, which dictates how much of bio-SA can be produced in a year. The SH-based bio-SA production may be one of the solutions for that, since it may have the highest productivity. In addition, the HS portion of SH can be used to produce new bio-C4 chemicals. HSL·HCl or HS can be a source for GBL and BDO. Different point compared to petroleum-based C4 chemical production is opposite direction between GBL and BDO. Therefore, the HSL·HCl-based process can skip the step from BDO to GBL. It may be an advantage for GBL market. GBL derivatives generated in this manner require further optimization of the reaction conditions, and could be the basis for fine chemical production. Especially, it is possible to make homoserine–diketopiperazine (HS-DKP) from HSL·HCl or HS. The formation of DKP from amino acids has been reported [10]. The DKP has di-acid from aspartic acid, di-ol from serine, and di-amine from lysine. HS-based DKP can generate di-alcohols; it may be useful as a third monomer for improving the performance of polyester.

Development of new technology that can produce renewable biochemicals and biofuel has been a constant challenge facing our generation. A more concerted effort is needed to be beyond petroleum dependence for sustainable future.

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