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Exploring the Effects of Carbon Sources on the Metabolic Capacity for Shikimic Acid Production in *Escherichia coli* Using *In Silico* Metabolic Predictions

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Effects of various industrially important carbon sources (glucose, sucrose, xylose, gluconate, and glycerol) on shikimic acid (SA) biosynthesis in *Escherichia coli* were investigated to gain new insight into the metabolic capability for overproducing SA. At the outset, constraints-based flux analysis using the genome-scale *in silico* model of *E. coli* was conducted to quantify the theoretical maximum SA yield. The corresponding flux distributions fueled by different carbon sources under investigation were compared with respect to theoretical yield and energy utilization, thereby identifying the indispensable pathways for achieving optimal SA production on each carbon source. Subsequently, a shikimate-kinase-deficient *E. coli* mutant was developed by blocking the aromatic amino acid pathway, and the production of SA on various carbon sources was experimentally examined during 5 l batch culture. As a result, the highest production rate, 1.92 mmol SA/h, was obtained when glucose was utilized as a carbon source, whereas the efficient SA production from glycerol was obtained with the highest yield, 0.21 mol SA formed per mol carbon atom of carbon source consumed. The current strain can be further improved to satisfy the theoretically achievable SA production that was predicted by *in silico* analysis.

Keywords: Shikimic acid production, constraints-based flux analysis, genome-scale *in silico* model, *Escherichia coli*, carbon sources

Shikimic acid (SA) is a hydroaromatic intermediate in the biosynthetic pathways of aromatic amino acid. Owing to its highly functionalized chiral characteristics, SA has been recognized as an essential starting material for synthesizing neuraminidase inhibitors (marketed as Tamiflu), which are effective in the treatment of influenza [6, 17]. The current state-of-the art for isolating SA from the fruit of *Illicium* plants is cumbersome and costly, which eventually motivates the microbial production of SA from renewable resources [3]. Towards this end, many researchers have rationally designed and engineered *E. coli* strains in which the accumulation of shikimic acid can be facilitated by preventing further conversions into aromatic amino acids [8, 16, 19, 34, 35]. However, the microbial SA synthesis was substantially limited by the *in vivo* availability of both D-erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) that are direct precursors for SA biosynthesis [7, 22, 23]. Thus, various genetic strategies have been applied for enhancing both E4P and PEP productions to alleviate such bottlenecks: E4P availability was increased by overexpressing key enzymes of the pentose phosphate pathway such as transketolase [7] or transaldolase [21]; strategies for increasing the PEP availability include overexpression of PEP synthase [23], inactivation of PEP carboxylase [22] or pyruvate kinase [12], and alteration of phosphotransferase system (PTS)-mediated glucose uptake to non-PTS-mediated uptake [4, 11, 34, 35]. It has also been reported that overexpression of both transketolase for E4P and PEP synthase for PEP are effective in the biosynthesis of SA [4], whereas alteration of phosphotransferase system (PTS)-mediated sugar uptake to non-PTS-mediated uptake [4, 35] has been found to be more effective in increasing the PEP availability rather than the overexpression of PEP synthase.

Apart from genetic modification, better nutritional and environmental conditions should be taken into account as well for improving SA productivity. One of the key environmental considerations is to select suitable carbon sources and

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explore their effects on the microbial synthesis of SA. In general, various carbon sources yield different metabolic capabilities for microbially synthesized products. Moreover, using an alternative transporter for a given carbon source can significantly affect the carbon flux and the yield of desired microbial product. In this regard, the modulation of various carbon source uptake systems has been well studied for improving microbial production of useful substances [1, 4, 11, 34, 35].

Presented herein is our exploration of the effects of different carbon sources along with different transporter systems on the metabolic capacity for SA production. It can be achieved by resorting to a constraints-based flux analysis, which is one of the most widely employed techniques in metabolic engineering [10, 26, 27, 30]. Application of flux analysis to a genome-scale *E. coli* metabolic model allows us to determine the theoretical maximum yield of a cellular system [14, 25, 29] and quantify the intracellular metabolic states towards the enhanced production of desired products [18, 29]. In the current study, throughout the aforementioned *in silico* analysis, the overall capacity (*i.e.*, maximum yield) of SA in *E. coli* on each carbon source was theoretically determined, thus identifying important metabolic pathways for optimal SA production. In addition, SA production on various carbon sources was experimentally examined using a shikimate-kinase-deficient *E. coli* strain.

Materials and Methods

Genome-Scale *In silico E. coli* Metabolic Model

The current genome-scale *in silico* model describing *E. coli* metabolism is derived and slightly modified from the iJR904 model [27]. The metabolic network of the model incorporates 904 genes, 932 biochemical reactions, and 625 metabolites. Embedded in the metabolic network are the central, energy, and redox metabolisms, and many others, along with the necessary transport reactions for extracellular metabolites and important carbon sources including glucose, sucrose, xylose, gluconate, and glycerol. Necessary reactions for sucrose uptake and SA secretion were newly added, whereas transhydrogenase (introversion of NADH and NADPH) was excluded from the model so that the NADPH produced by the pentose phosphate pathway (PPP) and TCA cycle could be used for SA biosynthesis. The reactions corresponding to the genes, *aroL* and *aroK*, encoding shikimate kinase were also excluded to prevent further conversions of SA intermediate into aromatic amino acids. Added enzymes and corresponding reactions includes *cscA* (SUCR →FRU+GLC), *cscB* (SUCRxt+H→SUCR+H), *fruk2* (ATP+FRU→ ADP+F6P), and *skmt* (SKM→SKMxt).

Constraints-Based Flux Analysis

Once the relationships among all metabolites and reactions were balanced in terms of stoichiometry under the stationary hypothesis, using the LP approach, a given cellular objective was linearly optimized to evaluate the unknown fluxes within the metabolic reaction network, subject to the constraints pertaining to mass conservation, reaction thermodynamics, and capacity, as described elsewhere [20, 32]. The availability of given carbon source and oxygen supply level were specified to be less than 10 mmol/g_{DCW}/h and 20 mmol/g_{DCW}/h, respectively, which signifies aerobic condition. In this work, the quantification of metabolic fluxes on various carbon sources for the maximized production of SA and comparative analysis of the resultant metabolic flux maps have been implemented by the MetaFluxNet program [20].

Microorganisms and Plasmid

E. coli DH5α was used as a host strain for the cloning and maintenance of plasmids. *E. coli* K-12 strain mutant derivative KPM SA1, developed by various random mutations using NTG (unpublished), was used as a host strain for shikimic acid production. This strain is L-tyrosine auxotrophic and has the characteristics of producing little acetate under the high glucose concentration. The mutant was further developed by inactivating chromosomal *aroL* and *aroK* genes encoding shikimate kinase using PCR primers [5]. In addition, shikimate kinase deficient *E. coli* KPM SA1 was transformed with plasmid pKPM-SA1 (derived from pET-39(b)+). Fig. 1 depicts pKPM-SA1 containing tyrosine-insensitive *aroF(fbr)* with Pro-148-to-Leu-148-substitution [33] through alignment PCR and wild-type *aroE* controlled by P_R-P_L promoter and temperature-sensitive CI_{857} repressor from bacteriophage λ and kanamycin resistance marker.

Cultivation

For the seed culture, Luria-Bertani (LB) medium (0.5% yeast extract, 1% bacto-tryptone and 1% NaCl) was used. A single colony grown on a LB agar plate was inoculated into 1,000 ml of Erlenmeyer flask containing 100 ml of LB medium. The culture was incubated overnight at 37°C and 200 rpm, and then it was used as a seed for batch cultures. During batch cultures, the seed (100 ml) was inoculated into a 5 l jar fermentor (KoBiotech, Korea) containing

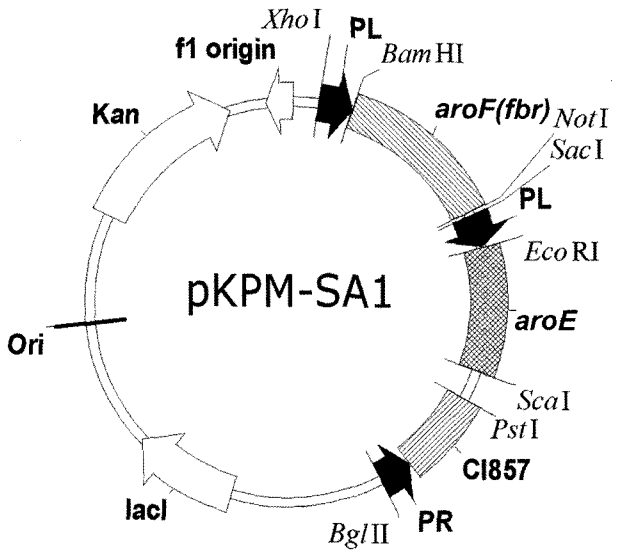


Fig. 1. Vector map of the pKPM-SA1 used in this study.
aroF(fbr): tyrosine-insensitive DAHP synthase; PR and PL: promoter from bacteriophage λ; CI857: temperature-sensitive repressor from bacteriophase λ; Kan, kanamycin resistance marker; ori, origin; lacI, lacI repressor.

1,900 ml of medium consisting of (g/l) carbon source (glucose, gluconate, glycerol, or xylose) 60, $(NH_4)_2SO_4$ 20, $MgCl_2$ 0.9, K_2SO_4 0.5, fumarate 0.8, K_2HPO_4 1.0, yeast extract 5, $C_6H_5Na_3O_7 \cdot 2H_2O$ 0.5, monosodium glutamate 0.5, $FeSO_4 \cdot 7H_2O$ 0.002, $MnCl_2 \cdot 4H_2O$ 0.005, $CoCl_2 \cdot 6H_2O$ 0.001, L-tyrosine 0.6, L-tryptophane 0.9, L-phenylalanine 0.9, *p*-aminobenzoic acid 0.002, 2,3-dihydroxybenzoic acid 0.002, *p*-hydroxybenzoic acid 0.002, and 1 ml of trace element. The temperature was maintained at 37°C; pH 6.5 was maintained by the addition of 24% (v/v) ammonia water. The dissolved oxygen concentration was kept above 20% of air saturation by controlling the agitation speed to 1,000 rpm.

Analytical Methods

The growth of *E. coli* cells was monitored by measuring the optical density at 600 nm (OD_{600}) (UVICON930, Switzerland). The dry cell weight was estimated by a predetermined conversion factor of 0.34 g dry cell weight/l/OD. To determine the concentrations of residual carbon source, 1 ml of culture broth was centrifuged and the concentration of carbon source in the supernatant was measured by HPLC (Gilson) analysis using an HPX 87H column and RI detector. In addition, shikimic acids were quantified, by HPLC analysis using an HPX 87H column and UV detector set at 210 nm, from a standard calibration curve.

Results and Discussion

E. coli Metabolic Network for Shikimic Acid Production

The central metabolic pathway for synthesizing SA on various carbon sources (glucose, sucrose, xylose, gluconate, and glycerol) in *E. coli* is illustrated in Fig. 2. These carbon sources are supplied using transporters through different entrance sites into the carbon metabolism. Alternative carbon uptake systems exist in glucose, sucrose, and xylose. Both glucose and sucrose are transported and phosphorylated by PTS or non-PTS. Glucose uptake with non-PTS is catalyzed by the glucose H^+-symporter (Glf) and glucokinase

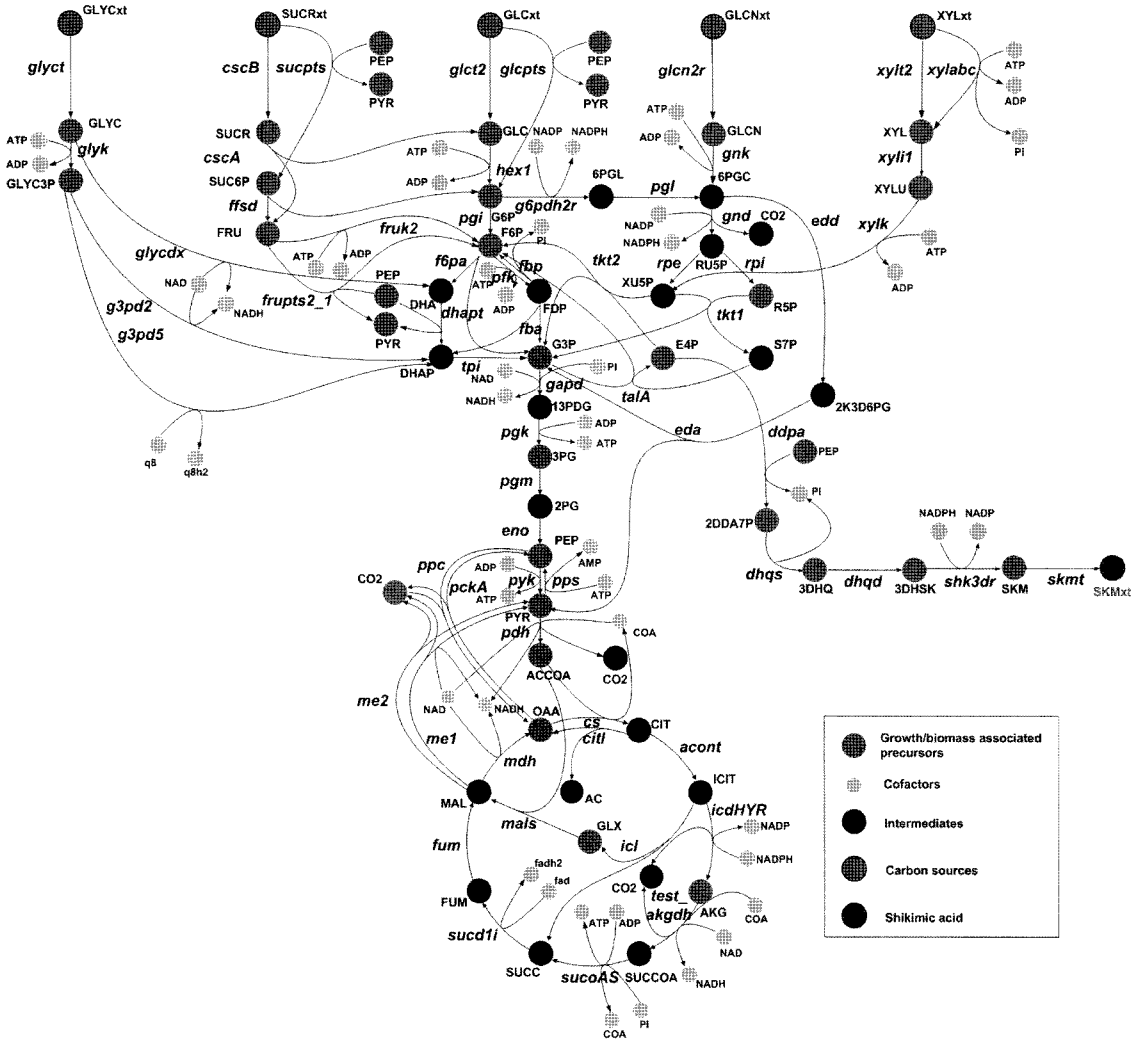


Fig. 2. Metabolic network map for the production of SA on different carbon sources.
The glycolytic pathway, pentose phosphate pathway, TCA cycle, and shikimate pathway are included.

(Gif). For sucrose uptake, there exist the Scr (Sucrose) system [28] where sucrose is phosphorylated by PTS, followed by invertase-catalyzed hydrolysis into G6P and fructose, and the Csc-(Chromosomally encoded *sucrose*) system [2] where sucrose is transported by an H^+-symporter and subsequently converted into glucose and fructose *via* invertase-catalyzed hydrolysis. Xylose is transported either by a XylFGH-encoded ATP-dependent ABC-type transporter [31] or by a XylE-encoded xylose/H^+ symporter [13].

For the synthesis of SA in *E. coli*, an equivalent amount of E4P, PEP, and NADPH productions are required. The availability of these precursors is highly correlated to the activities of the pentose phosphate pathway (PPP) and Embden-Meyerhof-Parnas (EMP) pathway. Furthermore, E4P can be synthesized by tkt-encoded transketolase, and PEP by *pps*-encoded PEP synthase or *pckA*-encoded PEP carboxykinase in the metabolic network of *E. coli*. Thus, increased flux, through EMP for transketolase and PPP for PEP synthase as well as PEP carboxykinase, will lead to the equivalent amount of E4P and PEP productions for the biosynthesis of SA.

Theoretically Maximized Metabolic Pathway for SA Production on Different Carbon Sources

The effect of carbon substrate on the SA production and intracellular metabolic fluxes in *E. coli* was explored by

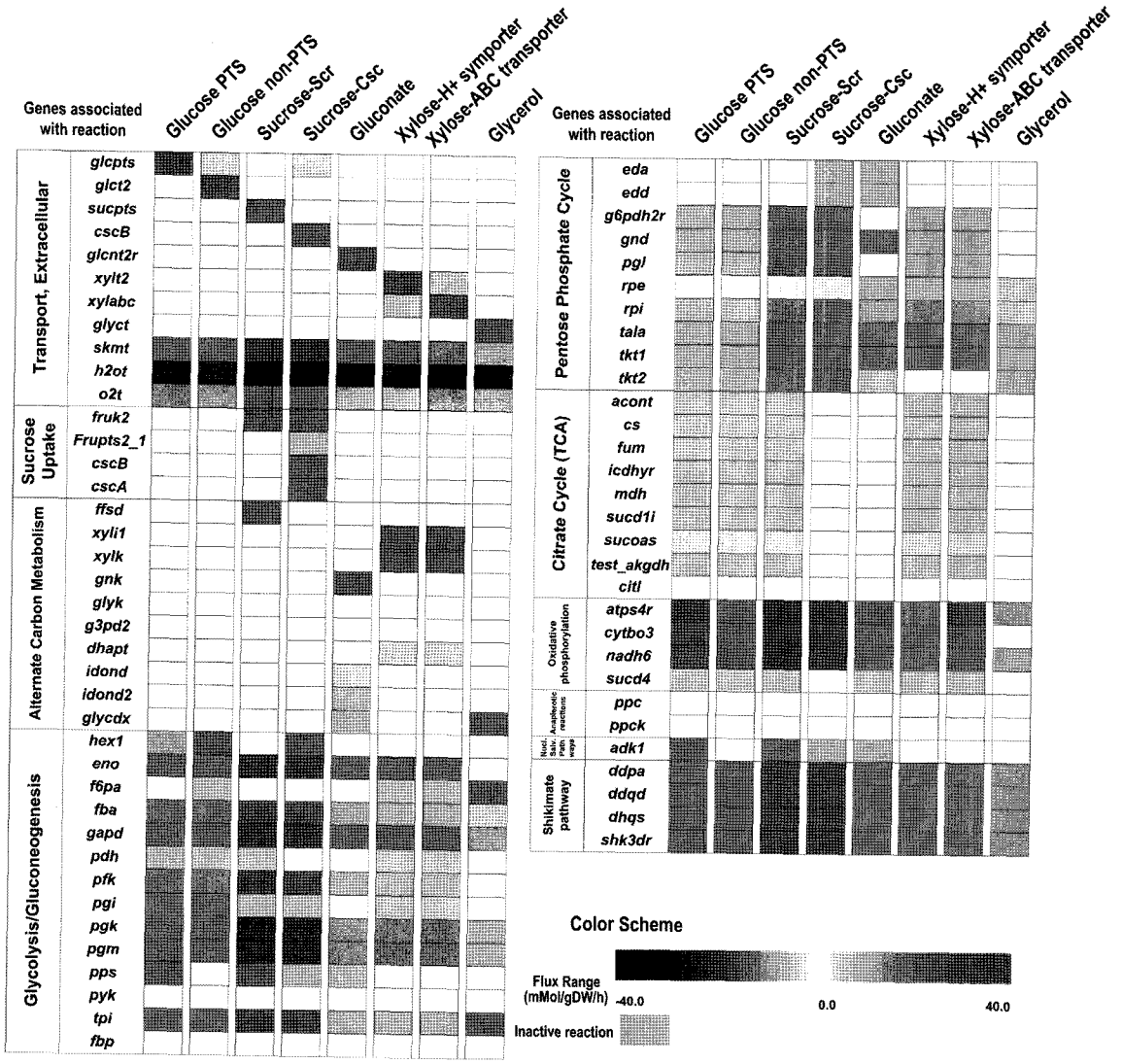


Fig. 3. Summary of the predicted flux distributions of *E. coli* metabolism attaining the optimal shikimic acid production on various carbon sources with different transporter systems.

Ten subsystems are included and relevant enzymatic reactions in each subsystem are clustered accordingly. The abbreviations of the uptake systems and enzymes are given in notation section. The resultant fluxes range from −40 mmol/gDCW/h to +40 mmol/gDCW/h, numerical values of which correspond to blue and red colors, respectively. In addition, grey-colored reaction flux means the inactive state for a given uptake system.

means of a constraints-based metabolic flux analysis of the genome-scale *in silico E. coli* model. The flux distributions of *E. coli* for eight different carbon source routes (glucose with PTS or non-PTS, sucrose with Scr or Csc, xylose with ABC-type transport system or xylose/H^+ symporter, gluconate, and glycerol) were evaluated and summarized for the comparison in Fig. 3, thus allowing us to gain new insight into the characterization of the metabolic pathway for SA production. It should be noted that production of SA was set as the objective function in the analysis, and neither growth nor maintenance requirement was considered for maximum SA yield. Fig. 4 depicts the optimal metabolic utilization on each carbon source and corresponding flux distribution from which the indispensable pathways for theoretically achieving maximum SA yield can be identified. The availabilities of E4P and PEP as precursors and NADPH as cofactor for SA biosynthesis can be increased *via* the following pathways. Amounts of NADPH required for SA synthesis are mostly synthesized by PPP in all carbon source cases. For E4P, relatively huge amounts of fluxes catalyzed by transketolase were found for all carbon sources except xylose. In the case of PEP, enormous amounts of fluxes (above 90%) through PYR recycling to PEP catalyzed by PEP synthase were required for PTS-mediated sugar uptakes (glucose with PTS and sucrose with Scr) with a stoichiometric consumption of PEP and coproduction of PYR. In contrast, a low value of this flux in all the non-PTS-mediated sugar uptakes except glycerol uptake [with a relative high flux (30.3%) due to large amounts of PYR produced by EDP] indicated its lower significance to the enhanced SA production.

Analysis of Metabolic Capacity for SA Production on Different Carbon Sources

Various carbon sources were compared with respect to their metabolic capabilities and energy demands for attaining optimal SA production, which correspond to the carbon yield and resultant ATP demand for SA biosynthesis, respectively (Table 1). On average, sucrose demonstrated the highest yield of 1.598 mol of SA per mol of carbon source consumed ($Y_{SA/Carbon\ source}$) owing to its disaccharide. When comparing them in terms of the carbon yield ($Y_{SA/C1}$), per carbon atom, a maximum of 0.143 mol of SA was found for glycerol, whereas the other carbon sources showed approximately 13% yields, which were slightly different depending on the transport system. We also compared the resultant ATP demand attaining the maximum SA production on each carbon source. When glycerol is utilized as a carbon source, 3.00 mol of ATP should be generated to synthesize one mol of SA from the substrate within the biochemical reaction network of *E. coli*, whereas ATP demand for 1 mol SA production is the lowest on xylose with H^+-symporter. This indicates that energetically less efficient pathways are utilized for glycerol compared with other carbon sources. Interestingly, ATP demand significantly depended on the transporter system unlike the carbon yield. In the cases of glucose and sucrose, the ATP efficiency in PTS was less effective than the non-PTS one, although the carbon uptake in PTS is known to be more effective than the non-PTS one [9]. Such contradictory result might be attributable to the pyruvate metabolism, where pyruvate produced via PTS must be further metabolized into PEP or organic acid and CO_2. However, a high energy cost is required to increase the PEP availability from the pyruvate for SA production; once PEP is converted to pyruvate by either PTS or PYR kinase, it is difficult to return into PEP [24]. As a consequence, a large amount of carbon flux in a real cell can be channeled through PYR and eventually to organic acid, carbon dioxide, and cell mass. Furthermore, it has also been observed that the overexpression of only PEP synthase led to growth inhibition of *E. coli* and excretion of pyruvate and acetate [24]. Therefore, from the bioenergetic point of view, the H^+-symporter, non-PTS, and Csc systems seem to be more suitable transporters in xylose, glucose, and sucrose for SA production, respectively, demonstrating higher yield and less energy requirement than the other carbon transport alternative.

Experimental Observation of the Shikimic Acid Production on Various Carbon Sources

In order to experimentally observe the SA production, an *E. coli* mutant producing SA was developed through the

Table 1. Theoretical metabolic capacities and energy demands for SA production on various carbon sources with different transporters.

Carbon source	$Y_{SA/carbon\ source}$ (mol (mol)$^{-1}$)	$Y_{SA/C1}$ (mol (mol)$^{-1}$)	ATP demand for SA production[a] (mol (mol)$^{-1}$)
Glucose with PTS	0.772	0.129	1.97
Glucose with Glf	0.798	0.133	0.99
Sucrose with Scr	1.588	0.132	1.56
Sucrose with Csc	1.608	0.134	1.14
Gluconate	0.731	0.122	1.32
Xylose with H^+-symporter	0.663	0.133	0.97
Xylose with ABC-type transporter	0.634	0.127	2.27
Glycerol	0.428	0.143	3.00

[a]Overall mol ATP demand for the production of 1 mol SA.

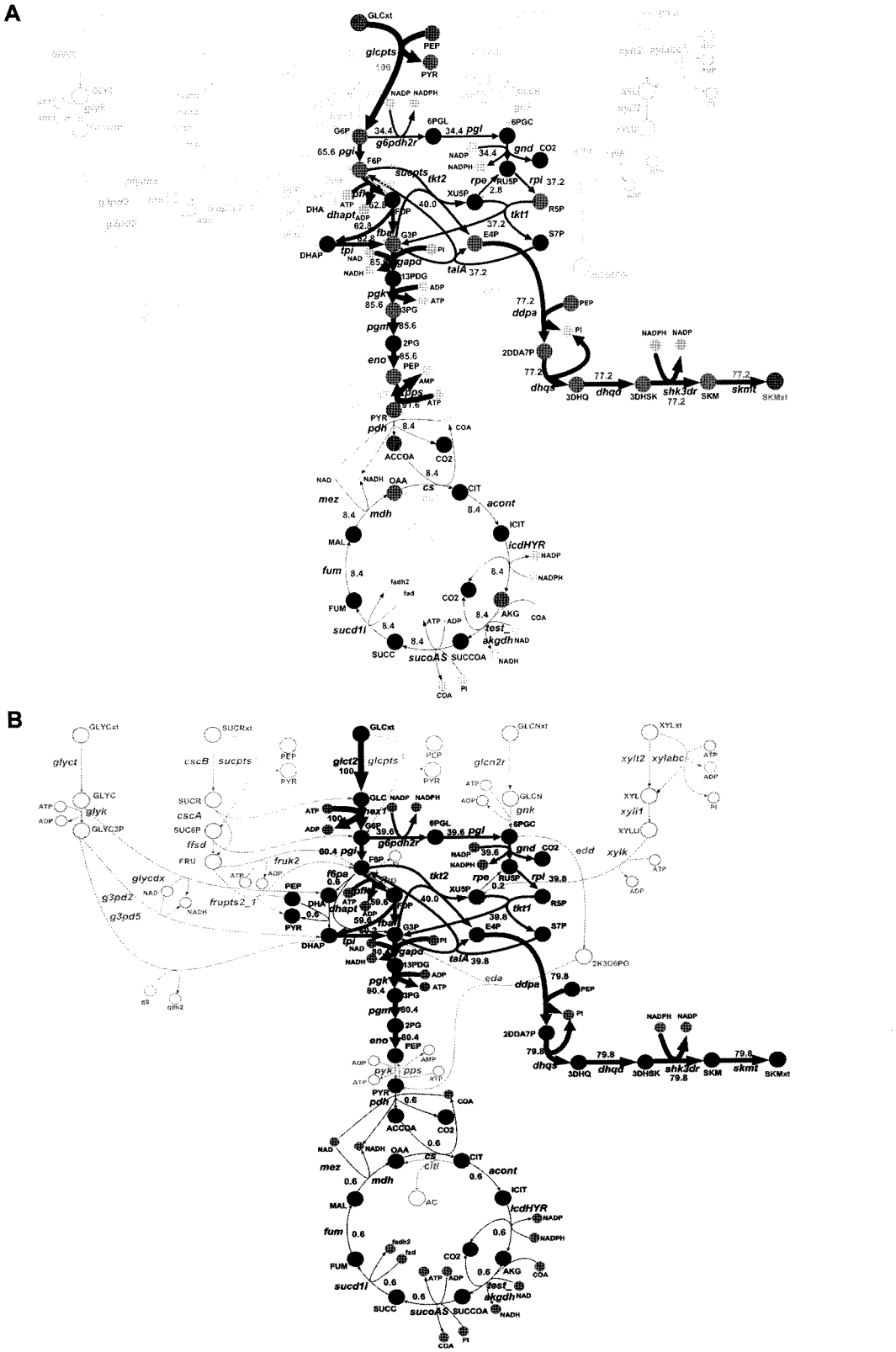
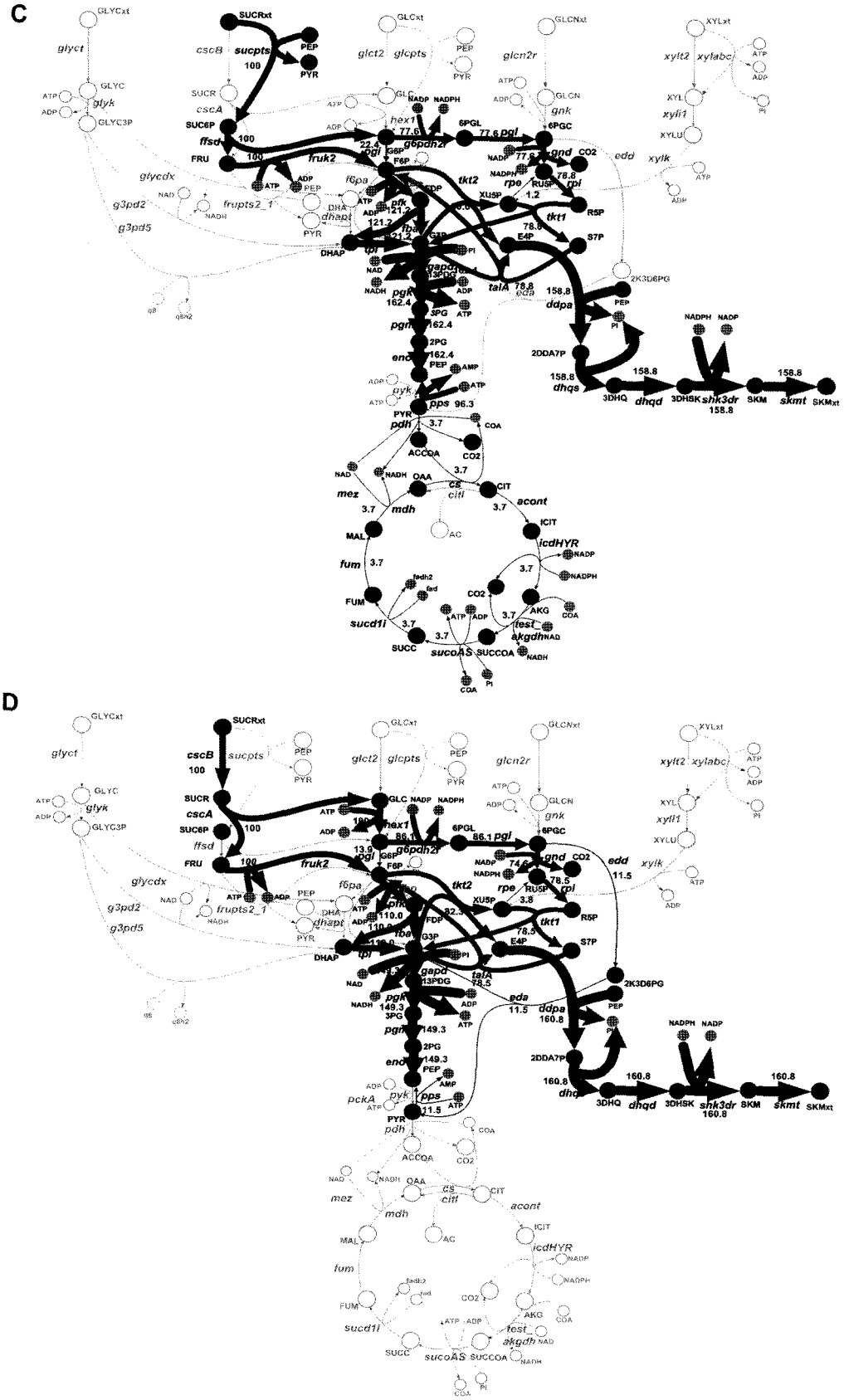


Fig. 4. Metabolic flux maps for optimal SA production on (**A**) glucose with PTS, (**B**) glucose with non-PTS, (**C**) sucrose with Scr, (**D**) sucrose with Csc, (**E**) gluconate, (**F**) xylose with H^+-symporter, (**G**) xylose with ABC-type transporter, and (**H**) glycerol.
Herein, all the fluxes are expressed as a percentage of the carbon flux entering the system as substrate. The thickness of the arrow is proportional to the percentage value.



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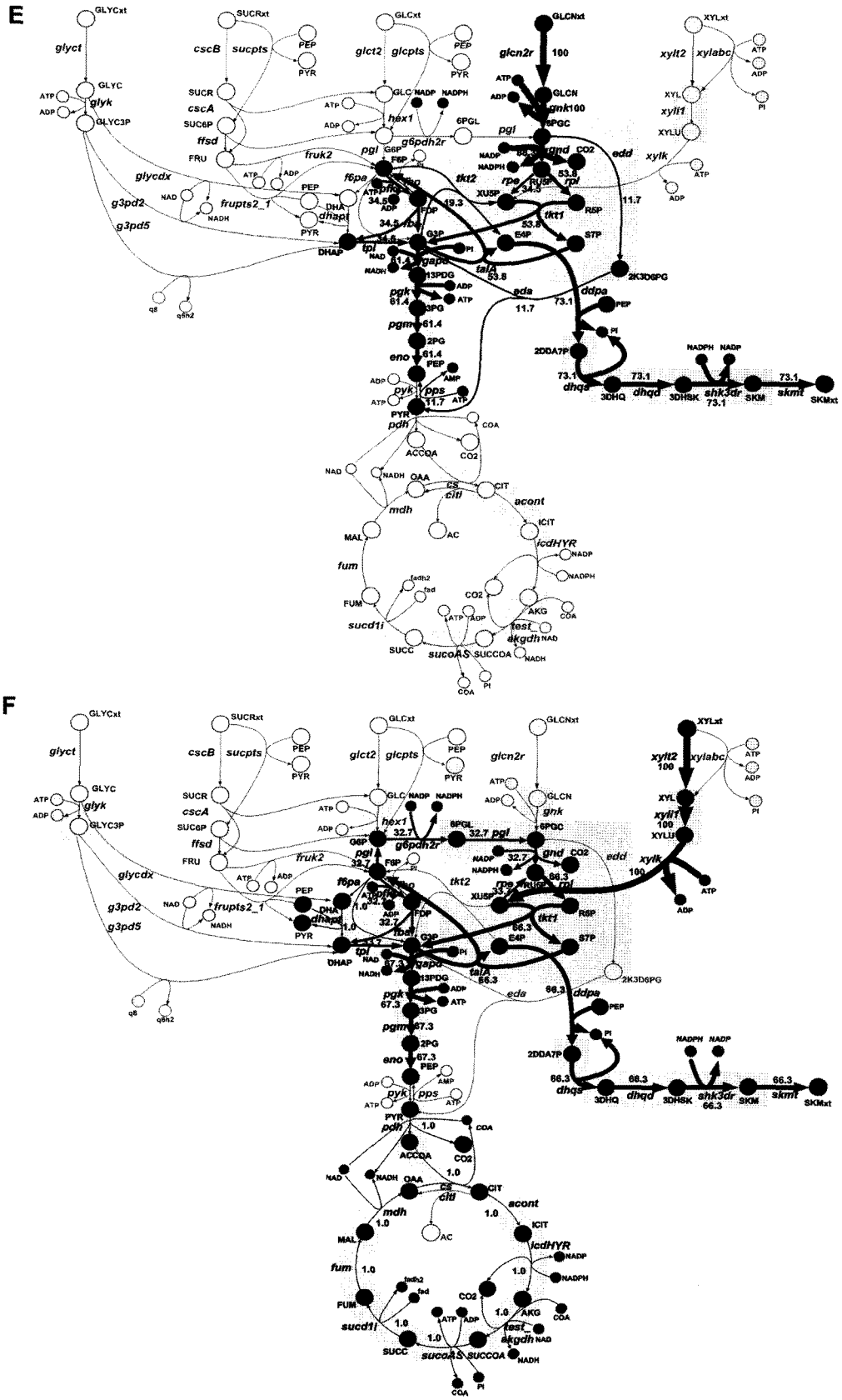

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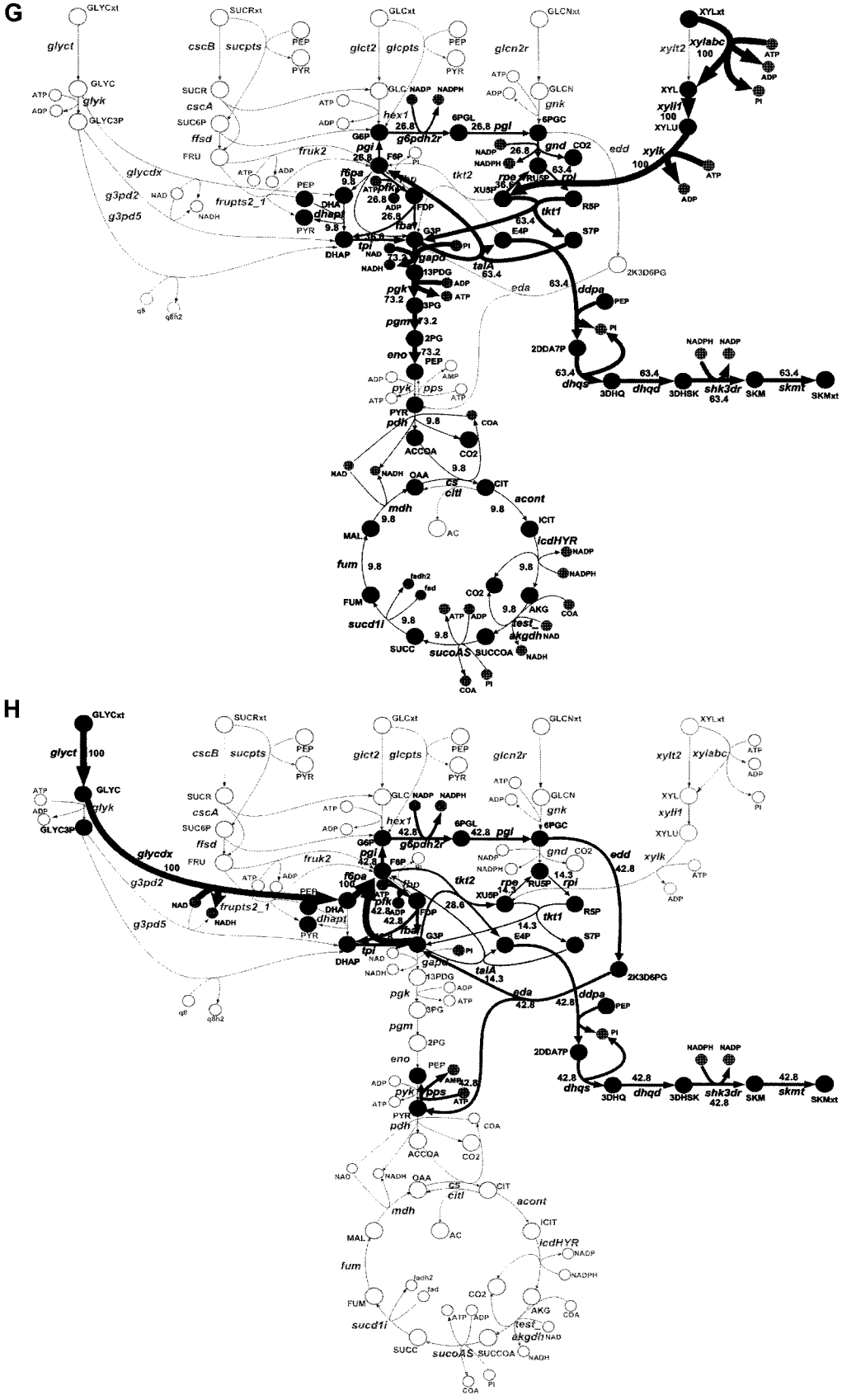

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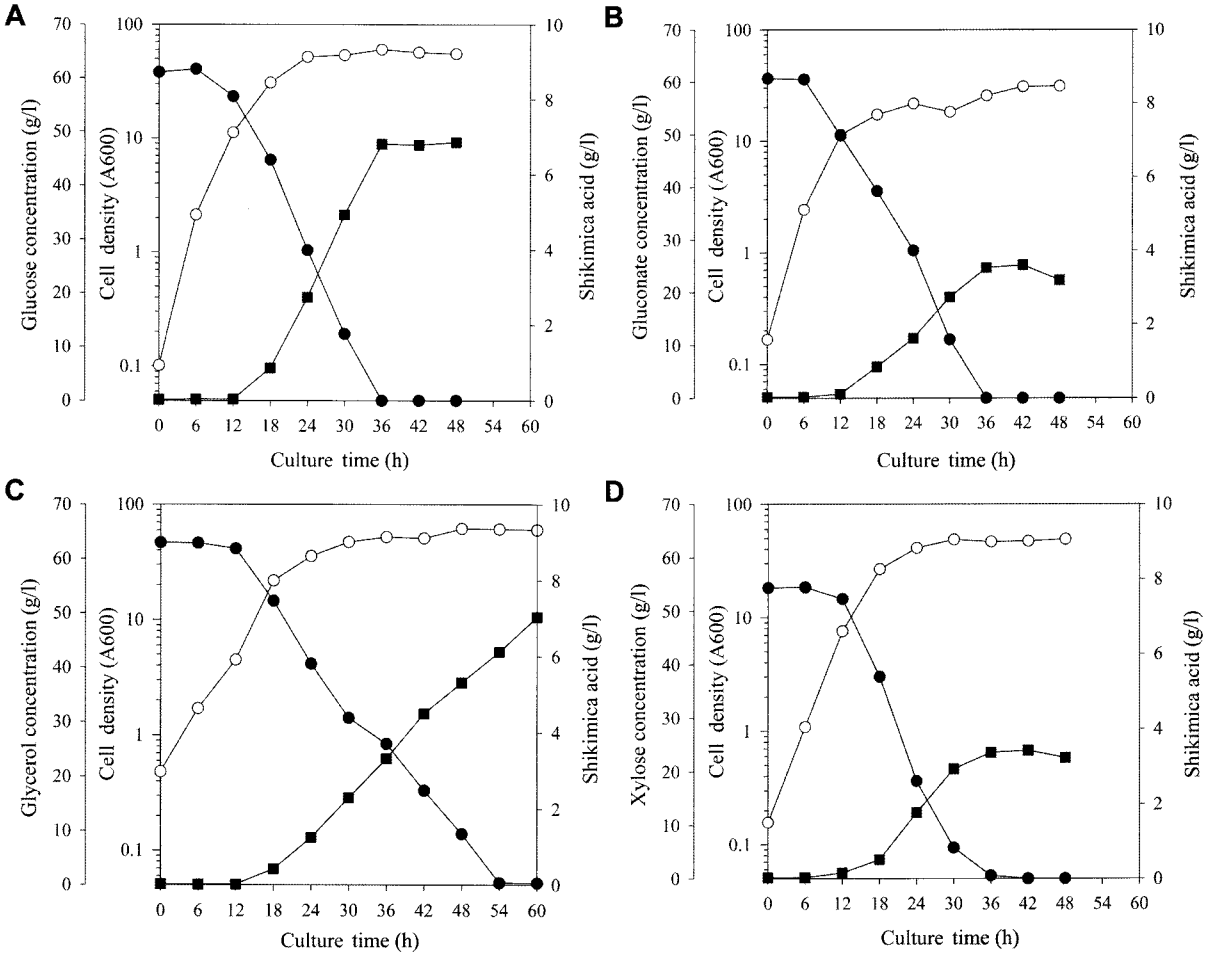

Fig. 5. Batch cultures of shikimate-kinase-deficient *E. coli* KPM SA1 harboring pKPM-SA1 on (**A**) glucose, (**B**) gluconate, (**C**) glycerol, and (**D**) xylose.
Symbols: closed circles, the concentration of carbon source (g/l); open circles, cell density (A_{600}); and closed square, the produced shikimic acid (g/l).

inactivation of both the *aroL* and *aroK* genes encoding shikimate kinase, which further catalyze SA in the aromatic amino acid pathway. Subsequently, batch cultures of the shikimate-kinase-deficient *E. coli* mutant harboring pKPM-SA1 were performed on various carbon sources including glucose, gluconate, glycerol, and xylose. Note that sucrose was not considered in the experimental observation because of the inability of the present strain to utilize it. As shown in Fig. 5, the exponential cell growth was observed until about 18 h after inoculation, and then the SA production phase started and continued until the depletion of carbon source in all cultures. Glucose, gluconate, and xylose were almost consumed within 36 h of the cultures, whereas glycerol did at about 54 h. The highest cell yield (20.4 g_{DCW}/l) was observed when glycerol was utilized as a carbon source, whereas the lowest cell growth (6.1 g_{DCW}/l) was observed

Table 2. Comparison of the results of batch cultures on various carbon sources using shikimate-kinase-deficient *E. coli* KPM SA1/pKPM-SA1.

Carbon source	Culture time[a] (h)	Cell concentration[a] (g_{DCW}/l)	Shikimic acid[a] (mmol)	SA production rate[b] (mmol/h)	$Y_{SA/C1}$ (mol (mol)$^{-1}$)
Glucose	36	18.4	39.0	1.92	0.020
Gluconate	36	6.1	20.1	0.90	0.012
Glycerol	60	20.4	40.2	0.95	0.021
Xylose	36	16.7	29.3	1.11	0.015

[a]The values obtained at the end of batch culture.
[b]The values were calculated by linear regression analysis of the data from 18–36 h (for 36–48 h on glycerol) in the production phase.

for gluconate (Table 2). Regarding SA production, as predicted by *in silico* analysis, the highest SA yield ($Y_{SA/C1}$: 0.21) was found for glycerol, whereas the highest SA production rate (1.92 mmol SA/h) in the production phase was obtained when glucose was consumed. These results suggest that utilization of not only currently used glucose but glycerol as an alternative carbon source is effective for the production of SA in this *E. coli* mutant. Nevertheless, much work remains to be done to improve the strain for achieving the optimal SA production that was predicted by the *in silico* analysis. In addition, adaptive evolution of the present strain needs to be considered for increasing the glycerol uptake rate [15], thereby leading to the enhanced SA production rate.

Concluding Remarks

In conclusion, the current work provides valuable information on the metabolic capacity and energy requirement for the production of SA on various industrially important carbon sources. Moreover, guided by constraint-based flux analysis, the theoretically achievable SA production on each carbon source and indispensable metabolic pathways for optimal production of SA were identified, and thus this experimental observation combined with the *in silico* analysis results will hereafter play an essential role in designing a desirable *E. coli* strain for biosynthesizing SA in future.

Acknowledgments

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