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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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Name

学位論文題目：
Title of Dissertation

Enzymatic Production of Rare 6-Deoxyhexoses from L-Rhamnose and L-Fucose and Characterization of Rare Sugar Producing Enzymes
（L-ラムノースおよびL-フコースからの希少６ーデオキシヘキソースの酵素的生産とそれらを生産する酵素の性質に関する研究）

学位論文要約：
Dissertation Summary

Deoxy sugars are monosaccharide derivatives which their one or more hydroxyl group is substituted with a hydrogen atom, results in a variety of deoxy sugars. Some of them are defined as rare sugar due to their rarity in nature (Granström *et al*., 2004). Interestingly, some 6-deoxy monosaccharides are structural components of the naturally active compounds such as anticancer, antitumor, anticoagulant, and antimicrobial agents (Aisa *et al*., 2005; Itoh *et al*., 1993; Nishino *et al*., 1991; Yoon *et al*., 2006). On account of the attractive properties of rare sugars and rare deoxy sugars, they become compounds of considerable interest. However, their characteristic and functions have not been investigated extensively, because of their rarity. Therefore, biochemical process using enzymatic reactions is developed on the basis of a potentially and environmentally friendly method. To establish the effective methodology for production of rare sugars, our laboratory constructed an Izumoring strategy involving a combination of several microbial enzymes. It is a structural framework containing 34 six-carbon monosaccharides, 16 five-carbon monosaccharides, 9 four-carbon monosaccharides, and their alditols, linked through a series of enzymatic reactions (Granström *et al*., 2004; Izumori, 2006). The formation of rare sugars is catalyzed by (1) aldose isomerases, which catalyze the isomerization of ketoses and aldoses, (2) ketose 3-epimerases (KE)-family enzymes, which epimerize the C-3 of ketoses, (3) alditol dehydrogenases, which oxidize alditols to ketoses, and (4) aldose reductase, which oxidize alditols to aldoses.

The microbial enzymes constructing an Izumoring strategy have broad substrate specificity which one enzyme can catalyze the transformation of multiple monosaccharides. This phenomenon is due to the similarity in configuration of the substrate. This important feature leads the possibility to produce various rare sugars and rare deoxy sugars from the inexpensive monosaccharides. Noticeably, L-rhamnose isomerase (L-RhI) and L-fucose isomerase (L-FI) which their native substrates are 6-deoxy monosaccharides, L-rhamnose and L-fucose, also catalyze the interconversion of common monosaccharides (Bhuiyan *et al*. 1997a; Bhuiyan *et al*. 1997b; Leang *et al*., 2004; Usvalumpi *et al*., 2012). It was hypothesized that the enzymes involving in Izumoring strategy, which their native substrates are common monosaccharides, are able to be active toward deoxy monosaccharides. As a result, a deoxy-Izumoring strategy was constructed for production of 1- and 6-deoxy monosaccharides based on the results of previous Izumoring strategy and our experiences.

In this study, it was elucidated that aldose isomerases and KE-family enzymes involving in Izumoring strategy have a special ability to catalyze the interconversion of 6-deoxyheoses effectively. These enzymes are L-rhamnose isomerase from *Pseudomanas stutzeri* LL172 (PsLRhI), D-arabinose isomerase from *Bacillus pallidus* 14a (BpDAI), L-arabinose isomerase from *Enterobacter aerogenes* IK7 (EaLAI), L-ribose isomerase from *Acinetobacter calcoaceticus* LR7C (AcLRI), D-tagatose 3-epimerase from *Pseudomonas cichorii* ST-24 (PcDTE), D-allulose 3-epimerase from *Shinella* sp. NN-6 (SDAE), recently discovered by our laboratory (Nonogaki, 2014), and a commercial D-glucose isomerase from *Streptomyces murinus* (DGI). To fulfill the deoxy-Izumoring, these enzymes were utilized to convert commercially available substrates, L-rhamnose, into 6-deoxy-L-glucose, 6-deoxy-L-altrose, and 6-deoxy-L-allose; and to convert L-fucose into 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose. According to results in this study, they strongly suggested that aldose isomerases and KE-family enzymes have the possibility to apply in the production of various rare deoxy sugars. In addition, a new isolated SDAE was purified and characterized on its enzymatic properties. These results will be published in the future.

The biocatalysts used in this study were two difference forms, immobilized form (IE-enzyme) and toluene-treated form (TT-enzyme). 6-Deoxy-L-glucose, 6-deoxy-L-altrose, and 6-deoxy-L-allose were produced from L-rhamnose *via* the intermediates, 6-deoxy-L-allulose and L-rhamnulose (Figure 1A). 6-Deoxy-L-allulose was prepared directly from L-rhamnose by coupling reaction of TT-PsLRhI and TT-PcDTE. Subsequently, purified 6-deoxy-L-allulose was epimerized into L-rhamnulose by IE-PcDTE. 6-Deoxy-L-glucose was produced from L-rhamnulose by TT-BpDAI. In contrast, 6-deoxy-L-altrose and 6-deoxy-L-allose were produced from 6-deoxy-L-allulose by TT-EaLAI and TT-AcLRI, respectively. On the other hand, 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose were produced from L-fucose, by coupling and sequential enzymatic reactions (Figure 1B). 6-Deoxy-L-talose was directly produced from L-fucose by coupling reaction of IE-BpDAI and IE-PsLRhI. Using sequential enzymatic reactions of IE-BpDAI and TT-SDAE, L-fucose was converted into L-fuculose and 6-deoxy-L-sorbose. Furthermore, purified 6-deoxy-L-sorbose was isomerized into 6-deoxy-L-gulose and 6-deoxy-L-idose by TT-AcLRI and DGI, individually. In addition, the enzymatic condition for production of each 6-deoxyhexose is presented in Table 1. The equilibrium ratios of substrate and product of the particular enzymatic reaction was analyzed by high performance liquid chromatography (HPLC). In exception, the L-rhamnulose concentration in the reaction catalyzed by TT-BpDAI was determined by cysteine–carbazole method (Dishe and Borenfreund, 1951), providing a calculated 6-deoxy-L-glucose concentration.

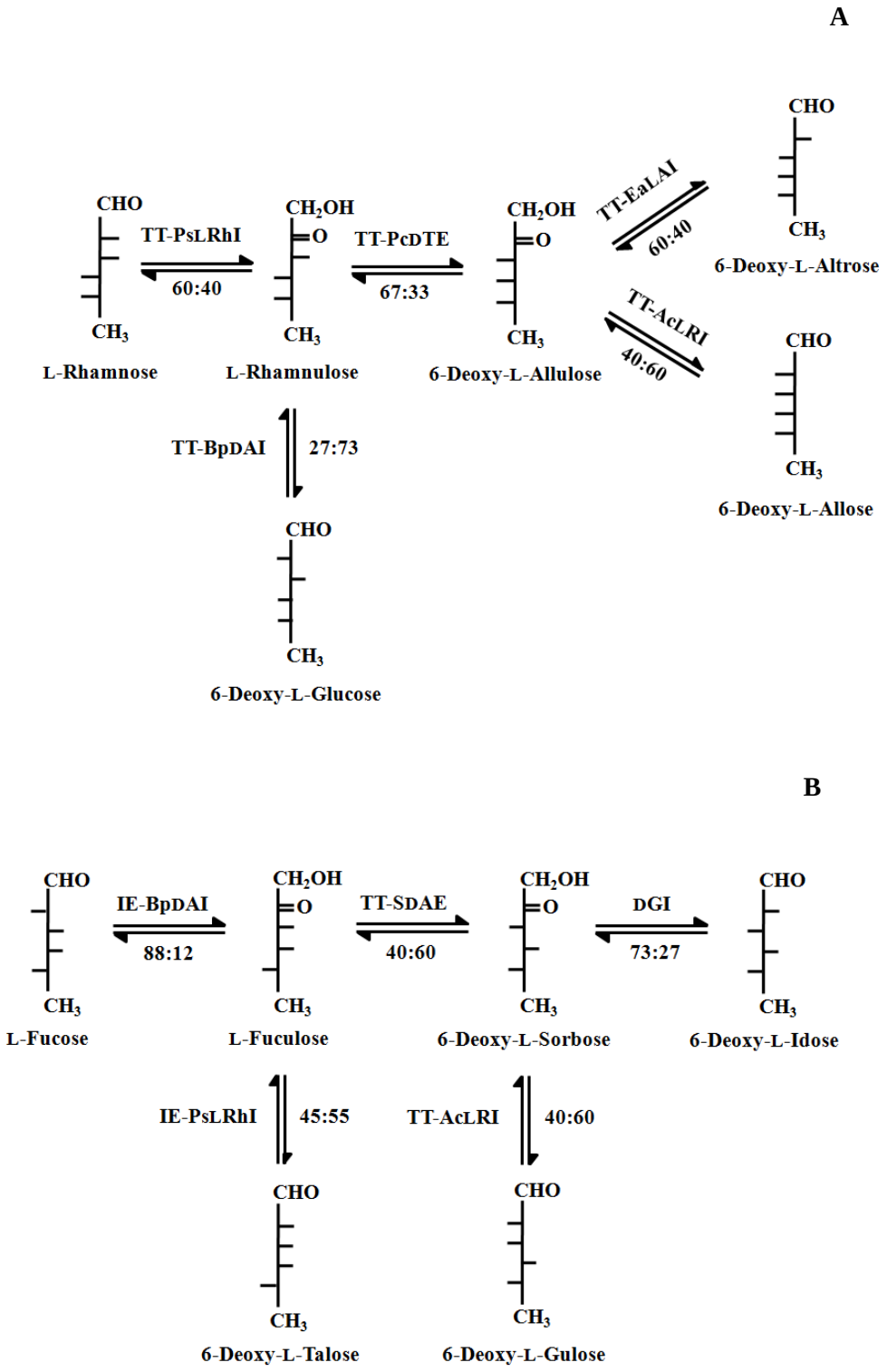

Figure 1 Schematics of the production of 6-deoxy-L-glucose, 6-deoxy-L-altrose, and 6-deoxy-L-allose from L-rhamnose (A) and production of 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose from L-fucose (B) by coupling and sequential enzymatic reactions.

Table 1 Condition of enzymatic reaction for production of 6-deoxyhexoses.

Substrate	Product	Microbial enzyme [a]	Metal ion	Buffer	Incubation temperature
5% L-Rhamnose [b]	L-Rhamnulose	TT-PsLRhI (64 U/mL)	1 mM $MnCl_2$	50 mM Sodium phosphate buffer (pH 7.5)	37°C
10% L-Rhamnose	6-Deoxy-L-allulose	TT-PsLRhI (64 U/mL) and TT-PcDTE (80 U/mL)	1 mM $MnCl_2$	50 mM Sodium phosphate buffer (pH 7.5)	37°C
10% 6-Deoxy-L-allulose	L-Rhamnulose	IE-PcDTE (450 U/mL)	None	10 mM Tris–HCl buffer (pH 7.5)	45°C
1% L-Rhamnulose	6-Deoxy-L-glucose	TT-BpDAI (2 U/mL)	1 mM $MnCl_2$	50 mM Glycine–NaOH buffer (pH 9.0)	55°C
10% 6-Deoxy-L-allulose	6-Deoxy-L-altrose	TT-EaLAI (3.5 U/mL)	1 mM $MnCl_2$	50 mM Glycine–NaOH buffer (pH 10.0)	37°C
10% 6-Deoxy-L-allulose	6-Deoxy-L-allose	TT-EaLAI (1.4 U/mL)	1 mM $MnCl_2$	50 mM Glycine–NaOH buffer (pH 9.0)	30°C
10% L-Fucose	L-Fuculose	IE-BpDAI (5 U/mL)	1 mM $MnCl_2$	50 mM Glycine–NaOH buffer (pH 9.0)	55°C
0.5% L-Fuculose [b]	6-Deoxy-L-talose	IE-PsLRhI (5 U/mL)	1 mM $MnCl_2$	50 mM Glycine–NaOH buffer (pH 9.0)	45°C
10% L-Fucose	6-Deoxy-L-talose	IE-BpDAI (5 U/mL) and IE-PsLRhI (5 U/mL)	1 mM $MnCl_2$	50 mM Glycine–NaOH buffer (pH 9.0)	45°C
2% L-Fuculose	6-Deoxy-L-sorbose	TT-SDAE (2.5 U/mL)	1 mM $CoCl_2$	50 mM Tris–HCl buffer (pH 8.0)	60°C
1% 6-Deoxy-L-sorbose	6-Deoxy-L-gulose	TT-AcLRI (6 U/mL)	1 mM $MnCl_2$	50 mM Glycine–NaOH buffer (pH 9.0)	30°C
1% 6-Deoxy-L-sorbose	6-Deoxy-L-idose	DGI (3.5 U/mL)	1 mM $MgCl_2$	50 mM Sodium phosphate buffer (pH 7.5)	60°C

[a] Enzyme concentration means unit of enzyme per milliliter of the reaction mixture.

[b] Investigation of an equilibrium ratio only.

Production of three rare 6-deoxyhexoses from L-rhamnose

Three rare 6-deoxyhexoses including 6-deoxy-L-glucose, 6-deoxy-L-altrose, and 6-deoxy-L-allose were produced from a commercially available substrate L-rhamnose *via* the intermediates 6-deoxy-L-allulose and L-rhamnulose. Initially, L-rhamnose was isomerized to L-rhamnulose by TT-PsLRhI with an equilibrium ratio of 60:40 (Figure 2A). However, L-rhamnose and L-rhamnulose were not able to be separated by chromatography system in our laboratory due to their close retention times. As a result, 6-deoxy-L-allulose was produced directly from L-rhamnose in one-pot reaction of TT-PsLRhI and TT-PcDTE under the same condition. The coupling reaction exhibited an equilibrium ratio of L-rhamnose, L-rhamnulose, and 6-deoxy-L-alllulose of 50:35:15 (Figure 2B). Subsequent purification by DOWEX 50W-X2 (Ca^{2+} form) column chromatography gave 6-deoxy-L-allulose at a production yield of 14%, on the basis of L-rhamnose. Furthermore, purified 6-deoxy-L-allulose was converted into L-rhamnulose by IE-PcDTE. An equilibrium ratio of L-rhamnulose and 6-deoxy-L-allulose was found at 67:33 as showed in Figure 2C. Pure L-rhamnulose separated by DOWEX 50W-X2 column chromatography was obtained at 0.62 g from 10 g of L-rhamnose without any byproduct contamination. The procedure used in this study provided a production yield of L-rhamnulose at 6.2% on the basis of L-rhamnose utilized as a starting material.

6-Deoxy-L-glucose was produced from pure L-rhamnulose by TT-BpDAI reactivity. An equilibrium ratio between L-rhamnulose and 6-deoxy-L-glucose was found at 27:73 (Figure 2D). However, L-rhamnulose and 6-deoxy-L-glucose were presented at the same retention time of chromatography system which was unable to be separated. Therefore a new methodology was developed to purify 6-deoxy-L-glucose using a degrading activity of *E. coli*, followed by preparative HPLC. Finally, 0.14 g of pure 6-deoxy-L-glucose was derived from 1 g of L-rhamnulose, representing a production yield of 14%. The entire procedure from L-rhamnose to 6-deoxy-L-glucose achieved a production yield of 0.9%.

6-Deoxy-L-altrose was synthesized from purified 6-deoxy-L-allulose by the activity of TT-EaLAI. The reaction had an equilibrium ratio between 6-deoxy-L-allulose and 6-deoxy-L-altrose of 60:40 (Figure 2E). On the other hand, 6-deoxy-L-allose was isomerized from pure 6-deoxy-L-allulose using TT-AcLRI. The isomerizing reaction exhibited an equilibrium ratio between 6-deoxy-L-allulose and 6-deoxy-L-allose of 40:60 (Figure 2F). Using DOWEX 50W-X2 column chromatography, 6-deoxy-L-altrose and 6-deoxy-L-allose were obtained at 0.17 g and 0.29 g from 1 g of 6-deoxy-L-allulose, representing 17% and 29% of production yields. On the basis of L-rhamnose consumed, the entire procedure provided total production yields of 6-deoxy-L-altrose and 6-deoxy-L-allose of 2.3% and 4.0%, respectively.

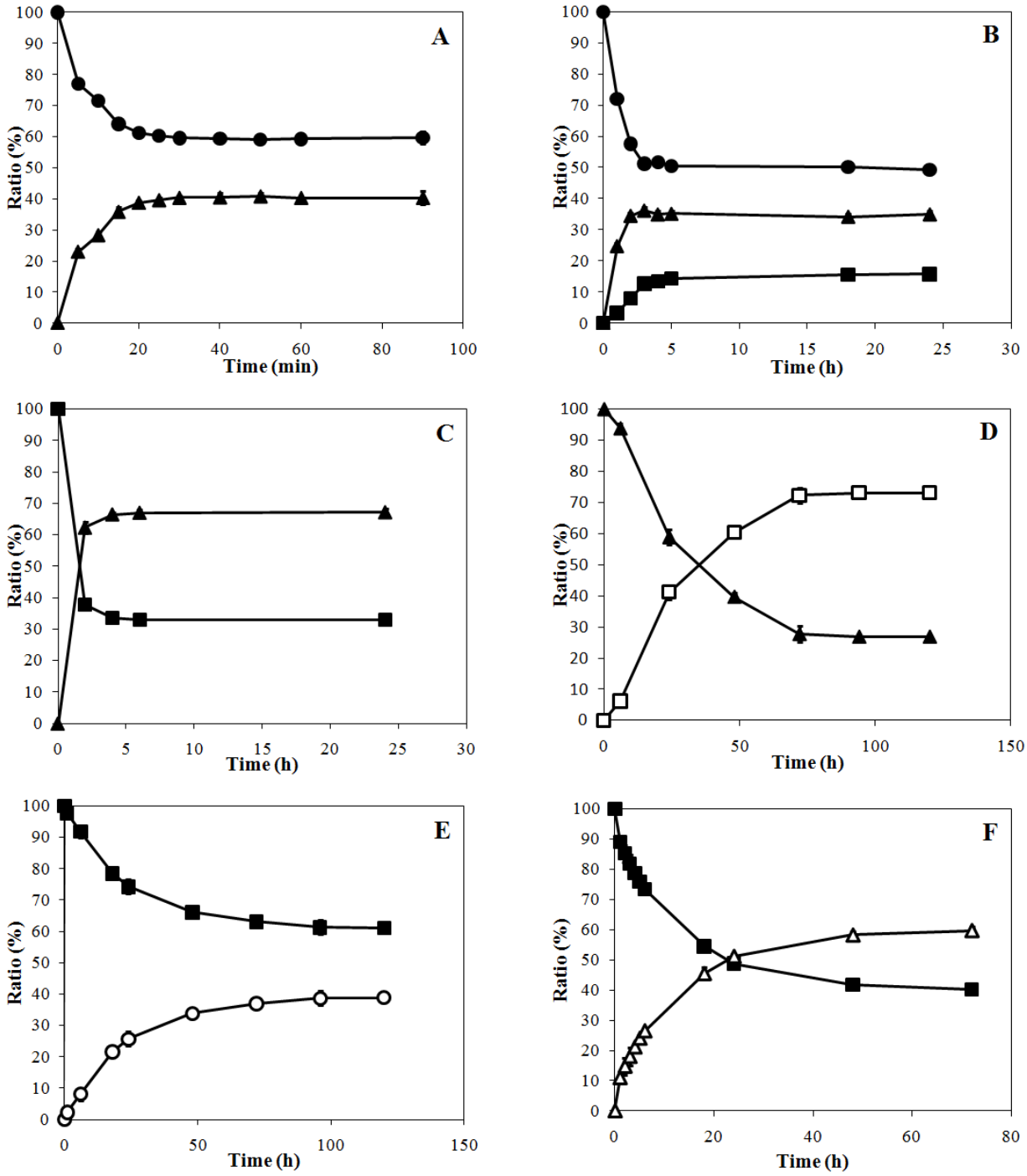

Figure 2 Production of three rare 6-deoxyhexoses from L-rhamnose. The figure represents conversion of L-rhamnose into L-rhamnulose by TT-PsLRhI (A), L-rhamnose into L-rhamnulose and 6-deoxy-L-allulose by mixed enzymes of TT-PsLRhI and TT-PcDTE (B), 6-deoxy-L-allulose into L-rhamnulose by IE-PcDTE (C), L-rhamnulose into 6-deoxy-L-glucose by TT-BpDAI (D), 6-deoxy-L-allulose into 6-deoxy-L-altrose by TT-EaLAI (E), and 6-deoxy-L-allulose into 6-deoxy-L-allose by TT-AcLRI (F). Symbol: L-rhamnose (●), L-rhamnulose (▲), 6-deoxy-L-allulose (■), 6-deoxy-L-glucose (□), 6-deoxy-L-altrose (○), and 6-deoxy-L-allose (△).

Production of four rare 6-deoxyhexoses from L-fucose

L-Fucose was another one commercially available substrate used in the production of rare 6-deoxyhexoses including 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose. L-Fucose was isomerized into L-fuculose by IE-BpDAI with equilibrium ratio of 88:12 (Figure 3A). Considering the conversion rate of L-fucose to L-fuculose at low level of 12%, the production of L-fuculose was improved using purified L-fucose as a substrate of IE-BpDAI for 6 cycles. The production yield was significantly increased from 9% to 27% (data not shown). Following the purification by DOWEX 50W-X2 column chromatography, 2.7 g of pure L-fuculose was eventually obtained from 10 g L-fucose. Subsequently, 6-deoxy-L-talose was produced from purified L-fuculose by isomerization activity of IE-PsLRhI. The equilibrium ratio between L-fuculose and 6-deoxy-L-talose was 45:55 (Figure 3B). Due to the isomerization of L-fucose into L-fuculose was found at low level (Figure 3A) which consequently provided a low production yield of 6-deoxy-L-talose; hence, a one-pot reaction method was selected for direct production of 6-deoxy-L-talose from L-fucose. It was found that the coupling reaction of mixed IE-BpDAI and IE-PsLRhI exhibited an equilibrium ratio of L-fucose, L-fuculose, and 6-deoxy-L-talose at 80:9:11 (Figure 3C). Eventually, 6-deoxy-L-talose purified by DOWEX 50W-X2 column chromatography was obtained at 0.71 g from 10 g L-fucose, representing a production yield of 7.1%.

Moreover, purified L-fuculose was converted into 6-deoxy-L-sorbose by TT-SDAE with an equilibrium ratio of 40:60 (Figure 3D). Purification procedure using DOWEX 50W-X2 column chromatography provided 1 g of pure 6-deoxy-L-sorbose from 2 g of L-fuculose, representing a production yield of 50%. On the basis of L-fucose used, the entire process provided a production yield of 6-deoxy-L-sorbose of 14%. Furthermore, pure 6-deoxy-L-sorbose was subsequently used in the production of 6-deoxy-L-gulose and 6-deoxy-L-idose. 6-Deoxy-L-sorbose was transformed into 6-deoxy-L-gulose by TT-AcLRI with an equilibrium ratio of 40:60 (Figure 3E). The resulting 6-deoxy-L-gulose purified by recycling preparative HPLC was obtained at 0.14 g from 1 g 6-deoxy-L-sorbose, representing a production yield of 2.0% on the basis of L-fucose. On the other hand, 6-deoxy-L-idose was isomerized from purified 6-deoxy-L-sorbose using a commercial DGI. The equilibrium ratio between 6-deoxy-L-sorbose and 6-deoxy-L-idose was observed at 73:27 (Figure 3F). The preparative HPLC was used for purification of 6-deoxy-L-idose. Finally, the entire procedure provided 0.17 g pure 6-deoxy-L-idose from 1 g 6-deoxy-L-sorbose, representing a production yield of 17%. The total production yield of 6-deoxy-L-idose was 2.4%, based on L-fucose consumed.

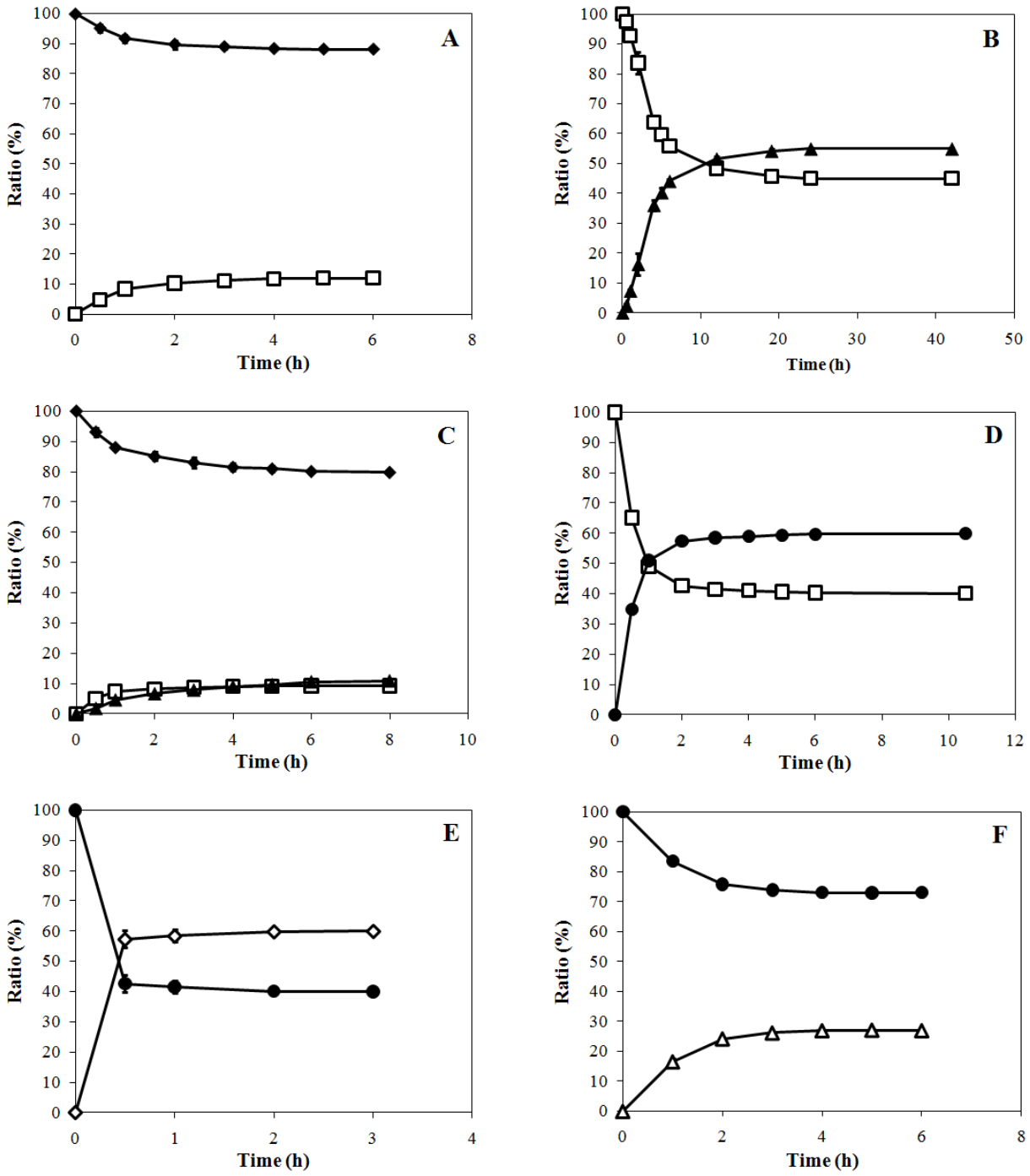

Figure 3 Production of four rare 6-deoxyhexoses from L-fucose. The figure represents conversion of L-fucose into L-fuculose by IE-BpDAI (A), L-fuculose into 6-deoxy-L-talose by IE-PsLRhI (B), L-fucose into L-fuculose and 6-deoxy-L-talose by mixed enzymes of IE-BpDAI and IE-PsLRhI (C), L-fuculose into 6-deoxy-L-sorbose by TT-SDAE (D), 6-deoxy-L-sorbose into 6-deoxy-L-gulose by TT-AcLRI (E), and 6-deoxy-L-sorbose into 6-deoxy-L-idose by DGI (F). Symbol: L-fucose (♦), L-fuculose (□), 6-deoxy-L-talose (▲), 6-deoxy-L-sorbose (●), 6-deoxy-L-gulose (◇), and 6-deoxy-L-idose (△).

Identification of products

Purified 6-deoxy-L-glucose, 6-deoxy-L-altrose, 6-deoxy-L-allose, 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose were identified on the basis of their ^{13}C NMR spectra. In comparing with the authentic and the data of the previous reports (Haverkamp *et al*., 1975; Shashkov *et al*., 1983; Wong *et al*., 1983; Katsuyama *et al*., 2005; Booth *et al*., 2008; Araya *et al*., 2012), it was found that the resulting products exhibited similar patterns of spectra to the corresponding 6-deoxyhexoses. In addition, specific optical rotation of 6-deoxy-L-glucose, 6-deoxy-L-altrose, 6-deoxy-L-allose, 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose were found at –27°, –16.6°, –2.1°, –17.1°, –49.3°, +37.8°, and –23.1°. Comparing with the previous reports, the absolute values of specific optical rotation of the resulting rare 6-doxyhexoses were not contradicted (Ireland and Wilcox, 1980; Kaufmann and Reischstein, 1967; Pazur *et al*., 1972; Haverkamp *et al*., 1975; Izumori *et al*., 2010; Tako *et al*., 2012; Wanaska and Kur, 2012). The results confirmed that seven of rare 6-deoxyhexoses were successfully produced from L-rhamnose and L-fucose by the procedures in this study.

Conclusion

Microbial enzymatic reaction is a potential procedure in the production of rare sugars. The results in this study strongly indicated that PsLRhI, BpDAI, EaLAI, AcLRI, DGI, PcDTE, and SDAE were able to catalyze the transformation of 6-deoxyhexoses which contain the coincident configuration with their native substrates. This knowledge would give us designs to produce various derivatives from monosaccharides. Moreover, seven rare 6-deoxyhexoses were successfully produced from L-rhamnose and L-fucose following the procedures in this study. Equilibrium ratio between substrate and product of each particular reaction was also investigated and summarized in Figure 1. Hereby, application research of rare deoxy sugars would be accelerated.

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