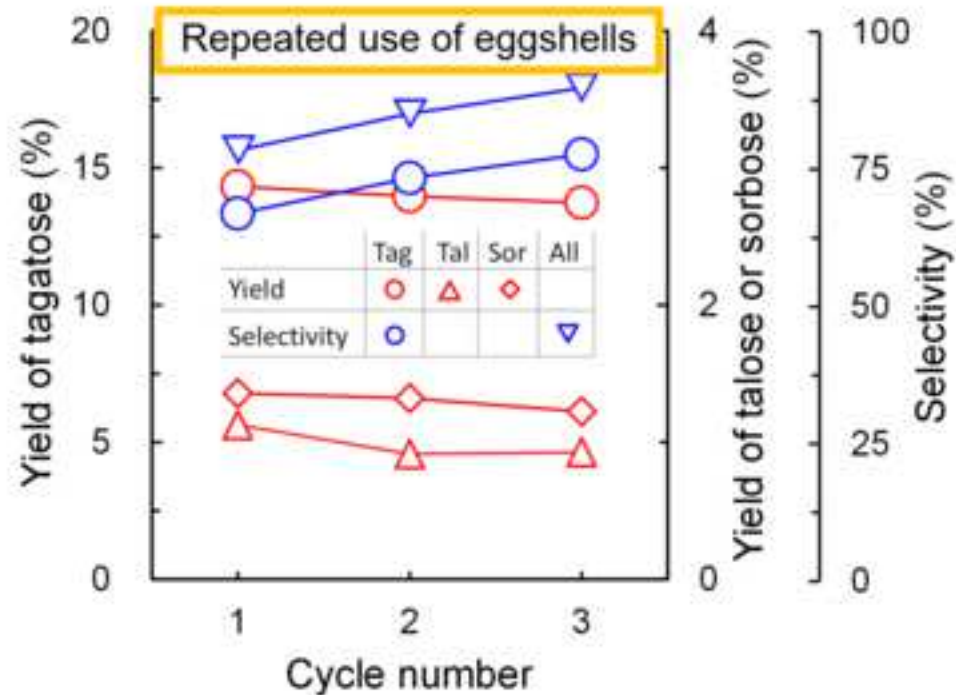
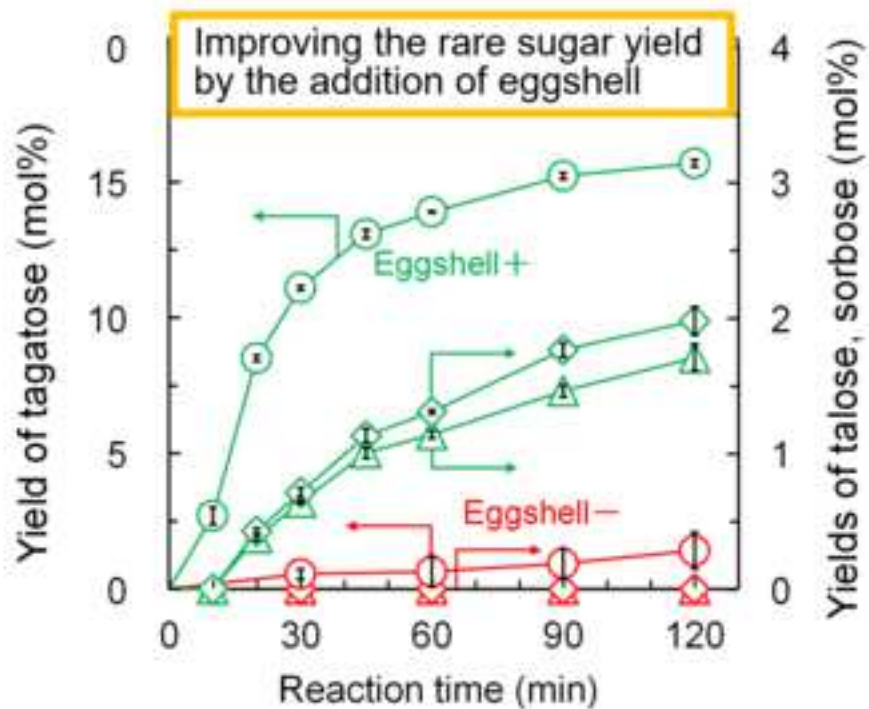
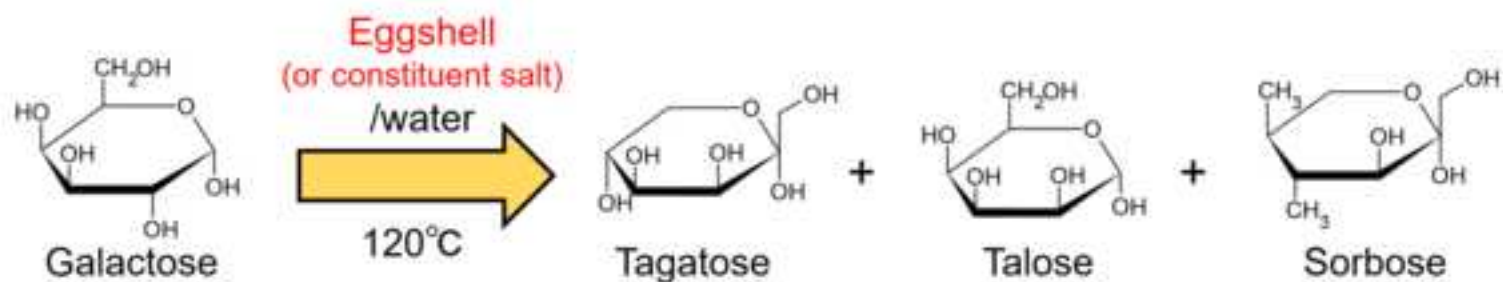




Highlights

- Eggshells promoted the isomerization of galactose.
- Tagatose, talose, and sorbose were formed in hot compressed water.
- Calcium ion concentration increased with the formation of organic acids.
- Repeated use of eggshells improved the selectivity of rare sugars.

Efficient synthesis of rare sugars from galactose in hot
compressed water using eggshells as an environmentally
friendly catalyst

Shin Futamata ^a, Yuichiro Onishi ^a, Shuji Adachi ^b, Pramote Khuwijitjaru ^c, Yoshiyuki
Watanabe ^d, Fumito Tani ^a, Takashi Kobayashi ^{a,*}

^a *Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto
University, Sakyo-ku, Kyoto 606-8502, Japan*

^b *Department of Agriculture and Food Technology, Faculty of Bioenvironmental Sciences,
Kyoto University of Advanced Science, Kameoka, Kyoto 621-8555, Japan*

^c *Department of Food Technology, Faculty of Engineering and Industrial Technology,
Silpakorn University, Nakhon Pathom 73000, Thailand*

^d *Department of Applied Biological Chemistry, Graduate School of Agriculture, Osaka
Metropolitan University, Naka-ku, Sakai, Osaka 599-8531, Japan*

*Corresponding author:

E-mail: kobayashi.takashi.6w@kyoto-u.ac.jp (T. Kobayashi)

Fax: +81-75-753-6285

Abstract

Aqueous galactose solutions containing eggshell was heated at 120°C to produce calcium supplements containing rare sugars. Galactose was isomerized to rare sugars with improving rare sugar yields compared to those without eggshell. Organic acids were also formed as byproducts during the reaction. These acids were neutralized by dissolving eggshells with increasing the calcium ion concentration in the solution.

When eggshell components (calcium carbonate, magnesium carbonate, or calcium phosphate) were used for the treatment, rare sugars were also formed. Especially, addition of magnesium carbonate improved rare sugar yield, but byproduct formation became more pronounced.

Eggshells used in the treatment were used for repeated treatments. When eggshells were used three times, rare sugar yield changed only slightly but the selectivity of rare sugars improved significantly. By these processes, we obtained an aqueous solution of rare sugars containing calcium ion at 295 mg/L, which has potential as ingredients for dietary supplements.

Keywords: Calcium supplement, eggshell, hot compressed water, isomerization, rare sugar

1. Introduction

The term rare sugars refers to sugars that are rarely found in nature. Some of them are low in calories and have a sweet taste (Beerens et al., 2012; Levin et al., 1995), some have useful physiological activities such as anti-inflammatory effects and suppression of hypertension (Beerens et al., 2012), and some are effective in the prevention of obesity and diabetes (Itoh et al., 2015; Lu et al., 2008; Roy et al., 2018). Tagatose is a rare sugar that is low in calories (1.5 kcal/g) (Lu et al., 2008) but has 92% of the sweetness of sucrose (Levin et al., 1995); it has also been shown to help prevent obesity and diabetes (Lu et al., 2008; Roy et al., 2018). There are various production methods for rare sugars, such as enzymatic methods (Cheetham & Wootton, 1993; Izumori, 2002; Kim, 2004), alkaline isomerization (Mendicino, 1960; Sowden & Schaffer, 1952), and isomerization in hot compressed fluids (Gao et al., 2015; Onishi et al., 2021; Onishi et al., 2020; Usuki et al., 2007). Enzymatic methods show high selectivity, but the costs associated with this type of production are high. On the other hand, the alkaline isomerization method has the problem of low selectivity.

In this study, we examined the production of rare sugars in hot compressed water, which has been attracting attention in recent years. Hot compressed water is water maintained in a liquid state by pressurization at 100°C or higher and which promotes various reactions due to its high ion product (Alenezi et al., 2009; Holliday et al., 1998; Rogalinski et al., 2008). The isomerization of reducing sugars is one such reaction, which proceeds via the Lobry de Bruyn-Alberda van Ekenstein (LBAE) transformation in the presence of hydroxide ions, a reaction mechanism similar to alkaline isomerization. For example, glucose isomerizes to fructose and mannose (Khuwijitjaru et al., 2018; Kishida et al., 2006; Onishi et al., 2022). It has been reported that treatment of galactose in hot compressed water can produce the rare sugars tagatose, talose, and sorbose (Onishi et

al., 2021; Onishi et al., 2020). However, organic acids and other byproducts are simultaneously formed by side reactions alongside the formation of the rare sugars (Onishi et al., 2020). Furthermore, the progress of isomerization is inhibited by the decrease in pH resulting from the formed organic acids. Therefore, if this decrease in pH can be suppressed, the isomerization yield could be improved. A previous study showed that tagatose yield could be improved in hot compressed phosphate buffer to avoid the pH decrease (Onishi et al., 2020). Thus, the suppression of pH decrease leads to the more efficient production of rare sugars.

In addition to the use of buffer solution, another means of suppressing pH decrease is the neutralization of the formed organic acids. In this study, we used eggshells as a neutralizer. According to data from the FAO, worldwide egg production was 76.7 million tons in 2018. Eggshells are mostly discarded as waste during the process of egg utilization and are therefore available at a low price (Tsai et al., 2008; Wei et al., 2009). The main component of eggshells is calcium carbonate, which can neutralize organic acids and promote isomerization due to its basic properties. In addition to calcium carbonate, calcium phosphate and magnesium carbonate are minor constituents of eggshells. The typical composition of eggshells is 96 wt% calcium carbonate, 1 wt% magnesium carbonate, 1 wt% calcium phosphate, and 2 wt% proteins (Murakami et al., 2007; Tsai et al., 2008).

It has been reported that lactulose can be produced from lactose in milk ultrafiltrate using eggshells (Montilla et al., 2005). Aldo-disaccharides can generally be converted to keto-disaccharides in higher yields than aldo-monosaccharides, such as galactose, in phosphate buffer (Onishi et al., 2022). However, the reason for the difference between mono- and di-saccharides has not been elucidated. Information about how organic acids affect isomerization behavior remains insufficient. In addition, the effects of eggshells on

the isomerization behavior of galactose are not clear. Therefore, we sought to clarify the role of eggshells and find a way to improve the isomerization yield.

The reaction of organic acids with calcium carbonate yields water-soluble calcium salts. Originally, eggshells also served as a source of calcium, and some of them have been used as food additives (Bartter et al., 2018; Ray et al., 2017). Therefore, if water-soluble calcium salts can be obtained from eggshells at the same time as rare sugars, they can be used in dietary supplements, thereby expanding the use of eggshells.

In this study, we investigated the effects of the presence of eggshells on the formation of rare sugars from galactose, an aldo-monosaccharide, in hot compressed water. We evaluated the formation of the rare sugars tagatose, talose, and sorbose. The formation of organic acids and other byproducts was also evaluated, as well as the effects of these byproducts on isomerization. The effects of the minor components of eggshells (calcium phosphate and magnesium carbonate) on the formation of rare sugars were also examined. In addition, we explored the potential for repeated use of eggshells in hot compressed water, aiming for the most efficient use of eggshells.

2. Materials and Methods

2.1. Materials

Galactose, calcium carbonate, calcium phosphate, and magnesium carbonate were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan). Potassium hydroxide was purchased from Nacalai Tesque (Kyoto, Japan), EDTA (ethylenediaminetetraacetic acid disodium dihydrate) was purchased from Sigma-Aldrich Japan LLC (Tokyo, Japan), and NN reagent (2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid) was purchased from Dojin Chemical Laboratory (Kumamoto, Japan). Chicken eggshells were purchased from Leaf Corporation (Gunma, Japan).

121
122 *2.2. Reaction of galactose in hot compressed water in the presence of eggshells or*
123 *constituent salts*

124 Galactose was dissolved in distilled water to a final concentration of 5% (g/g-solution).
125 To a screw-top test tube (15-mm internal diameter, I.D.), 5 mL of the galactose solution
126 and 500 mg of eggshell powder or constituent salts of eggshells (calcium carbonate,
127 magnesium carbonate, or calcium phosphate) were added and the tube was sealed. The
128 test tubes were placed in a heat block, heated to 120°C, and allowed to react for 120 min.
129 During the reaction, the pressure inside the test tube was maintained at about 0.198
130 MPa. Dependence of ion product of water on the pressure near the experimental
131 temperature is very small; for example, ion product of water at 100°C is $10^{-12.27}$ (mol/kg)²
132 at 0.1 MPa and $10^{-12.18}$ (mol/kg)² at 25 MPa (Marshall & Franck, 1981). Hence, the
133 pressure dependence of rare sugar yields was not examined in this study. The test tubes
134 were removed from the heat block every 5 min and shaken by hand until the powder was
135 dispersed in the solution. After the reaction, the test tubes were quenched by immersing
136 them in an ice bath. The suspension was then filtered through filter paper (No. 1, TOYO
137 ROSHI, Tokyo), and the filtrate was obtained as the treated solution. Experiments using
138 eggshells were performed in triplicate, while the others were performed once.

139 Yields of rare sugars (tagatose, talose, and sorbose) and organic acids were calculated
140 based on their concentrations determined as described later. Selectivity of rare sugar
141 was defined as follows:

$$\text{Selectivity (\%)} = \frac{\text{Molar yield of rare sugar}}{\text{Molar conversion of galactose}} \times 100$$

142
143
144 *2.3. Reaction of galactose in hot compressed water using model eggshells*

145 Model eggshells were prepared by mixing each component of eggshells (98 wt%

calcium carbonate, 1 wt% magnesium carbonate, and 1 wt% calcium phosphate). The 2 wt% protein in eggshells was substituted with calcium carbonate, as the main ingredient. The model eggshells were mixed with the galactose solution, and reaction was performed using the same method as described above. The reaction conditions were 120°C for 120 min.

2.4. Repeated reactions

The reusability of eggshells was evaluated by conducting repeated reactions. The reaction conditions were set at 120°C for 60 min. Galactose was treated in hot compressed water using the method described above, and the treated eggshells were collected by filtration. The eggshells were dried under reduced pressure in a desiccator at room temperature and subjected to further treatment again. This process was repeated three times.

2.5. Analysis

2.5.1. Sugar analysis:

The concentration of sugars in the treated solution was determined by high-performance liquid chromatography (HPLC). The analysis was performed using an HPLC system composed of a pump (LC-10ADVP, Shimadzu, Kyoto), a COSMOIL Sugar-D column (3.0 mm I.D. × 250 mm, Nacalai Tesque), and a refractive index detector (RID-20A, Shimadzu). The mobile phase was 80% (v/v) acetonitrile at a flow rate of 0.4 mL/min. The column temperature was controlled at 40°C using a column oven (CTO-10AVP, Shimadzu).

2.5.2. Organic acid analysis:

Determination of organic acids was performed by HPLC in the same manner as for the sugar analysis. However, an RSpak KC-811 (8.0 mm I.D. × 300 mm, Rezonac, Tokyo) and an RSpak KC-G8B guard column (8.0 mm I.D. × 50 mm, Rezonac) were used as columns and detected by the refractive index detector. An aqueous phosphoric acid solution (0.1% v/v) was used as the mobile phase at a flow rate of 0.8 mL/min.

2.5.3. pH measurement:

The pH of each treated solution was measured at room temperature using a pH meter (D-71, HORIBA, Kyoto).

2.5.4. Chelatometric titration:

To determine the calcium ion, chelatometric titration was conducted. The treated solution (200 µL) was diluted 30-fold with distilled water. Potassium hydroxide solution (60 µL, 6 mol/L) and NN indicator (20 µL, 2.3 mg/mL) were then added, and the solution turned reddish purple. EDTA solution (0.010 mol/L) was added dropwise, and the endpoint was set to be the point when the solution turned blue. Measurements were made in triplicate for each treated solution.

2.5.5. Scanning electron microscopy:

The surface morphology of eggshell particles was observed using an analytical scanning electron microscope (SEM, SU-6600, Hitachi High-Tech, Tokyo, Japan).

3. Results and Discussion

3.1. Effect of addition of eggshells and constituent salts

Reaction of galactose in hot compressed water was performed by adding eggshells to

an aqueous galactose solution. The reaction without eggshells was used as a control. Reaction conditions were set at 120°C and the reaction was allowed to run for 120 min. Figure 1 shows the time courses of the yields of rare sugars. When eggshells were added, galactose was isomerized; after 120 min, the remaining galactose was 70.1 mol%. In addition to the formation of tagatose, talose and sorbose were formed, and the yields at 120 min of tagatose, talose, and sorbose were 15.7 mol%, 1.6 mol%, and 1.9 mol%, respectively. The yield of tagatose obtained in this reaction was somewhat higher than that obtained in phosphate buffer (14 mol%) (Onishi et al., 2024). When no eggshells were added, 94.4 mol% of galactose remained at 120 min. Although tagatose was formed, it was at most 3.4 mol%, much lower than in the presence of eggshells. No formation of talose or sorbose was observed. These results suggest that eggshells are effective for isomerizing galactose.

Turning our attention to the rare sugar selectivity, the selectivity ranged from 65 to 80% in the presence of eggshells and was always significantly higher than in the absence of eggshells (25 to 43%). These results suggest that eggshell suppresses side reactions. However, regardless of whether eggshells were present or not, the selectivity showed a decreasing trend with reaction time. It is suggested that organic acids were formed due to the gradual side reaction, and their catalytic activity probably promoted the decomposition of rare sugars.

Figure 2 shows the time courses of calcium ion concentration, organic acid yield, and pH. When eggshells were added, the pH gradually decreased from slightly alkaline (pH 9) to neutral (pH 7). When no eggshells were added, the pH value shifted from neutral (pH 7) to acidic (pH 4). This was due to the formation of various organic acids, which are byproducts of the treatment of galactose. We confirmed the formation of formic acid, acetic acid, and glycolic acid, with the formation of acetic acid being particularly

significant. These organic acids were then neutralized by calcium carbonate from the eggshells, forming soluble calcium salts. As a result, the pH decrease of the treated solution was suppressed, and the calcium ion concentration increased, reaching 295 mg/L after treatment for 120 min. On the other hand, only a slight formation of glycolic acid (yield=0.05 wt%) was observed in the absence of eggshells, resulting in lower pH. This would be due to the suppression of the reaction by lower pH. We previously found that LBAE transformation progressed significantly when pH>6.3 (Onishi et al., 2020). The LBAE transformation, which readily progresses under alkaline conditions, was thus facilitated when the pH of the treatment solution was maintained above neutral by neutralization, resulting in the accelerated isomerization of galactose to various rare sugars.

Here, the sum of the yields of rare monosaccharides by LBAE transformation was 19.2 mol% at 120 min, which was lower than the yield of lactulose, a keto-disaccharide (26.2 mol%) reported in a previous study (Montilla et al., 2005). Although there could be several possible reasons behind these results, the stability of the intermediate product, enediol, formed during LBAE transformation, may be a key factor. This would be because in the reaction of disaccharides an intermediate, enediol substituted with a sugar moiety at C4-position, would be stable; for monosaccharides, however, there is no substituent and the intermediate would be unstable.

3.2. Effect of constituent salts of eggshells on reaction behavior

We next investigated the effect of the major and minor constituent salts of eggshells. For this, the treatment was applied to the galactose solution after adding a constituent salt (calcium carbonate, calcium phosphate, or magnesium carbonate) at a concentration of 500 mg/5 mL. Figure 3 shows the composition of the treated solution after treatment

for 120 min at 120°C. There was no significant difference between the composition of the treated solution when eggshells and calcium carbonate were added. When calcium phosphate was added the yield of talose and sorbose was slightly higher, and the yield of byproducts was also higher. On the other hand, when magnesium carbonate was added, the yields of tagatose, talose, and sorbose were 16.4 wt%, 5.7 wt%, and 5.6 wt%, respectively, indicating that the formation of talose and sorbose was particularly enhanced. At the same time, however, the formation of byproducts became more pronounced, with a very high yield of 39.3 wt%. Thus, both isomerization of galactose to rare sugars and side reactions were promoted when magnesium carbonate was added.

As shown above, the yields of rare sugars and byproducts differed depending on the salt added. One of the reasons for these differences may be the different solubilities of salts in water. The solubilities of calcium carbonate and calcium phosphate in water are 13 mg/L and 25 mg/L, respectively. On the other hand, magnesium carbonate is readily soluble in water (ca. 140 mg/L) and causes the pH to increase, suggesting that isomerization was accelerated by alkali. Meanwhile, it is also suggested that a high pH also accelerates side reactions, and therefore a strong alkali is not necessarily advantageous for isomerization.

It was reported that formation of tagatose by similar reactions mechanism occurred in the presence of basic compounds, such as hydrotalcite containing aluminum and magnesium (yield=9-11 mol%) (Drabo & Delidovich, 2018), sodium phosphate (10-16 mol%) (Drabo & Delidovich, 2018), sodium hydroxide (15 mol%) (El Khadem et al., 1987), arginine (17 mol%) (Milasing et al., 2023), or in the presence of acidic Sn-BEA zeolite (yield=9-26 mol%) (Drabo & Delidovich, 2018). Some of these results are higher than the tagatose yield in this study (16 mol%). On the other hand, selectivity of tagatose was comparable, on in some cases, higher in this study. In addition, the catalysts and

reagents used are not always edible, or although arginine is edible, there are problems with Maillard reactions. The present study reduces these drawbacks and suggests that rare sugars can be synthesized using food byproducts.

3.3. Comparison of real and model eggshells

In the above study, the treatment was conducted using the same concentration of each constituent salt. However, the calcium phosphate and magnesium carbonate content in eggshells is actually low. Therefore, we next conducted the treatment using model eggshells comprising a mixture of each salt, similar to the composition of real eggshells, and investigated the differences between the model and real eggshells.

Figure 4 shows the composition of the treated solutions. When model eggshells were added, the yields of rare sugars and byproducts were both higher than those of eggshells. The composition somewhat resembled those obtained with the addition of magnesium carbonate or calcium phosphate (Fig. 3). A reason for this may be as follows. In the model eggshells, particles of magnesium carbonate are present as a pure substance and are easily dissolved in water. On the other hand, magnesium carbonate in eggshells forms part of a mixed crystal with calcium carbonate. In other words, magnesium carbonate in eggshells is embedded in the mixed crystal and is not fully exposed on the crystal surface, which probably makes it less soluble in water.

3.4. Repeated use of eggshells

The treatment in hot compressed water dissolves a proportion of the eggshells, leaving the undissolved eggshells behind at the end. As there is a potential to reuse these undissolved eggshells for further treatment, we investigated the repeated use of the spent eggshells. Figure 5(A) shows the yields of tagatose, talose, and sorbose, and the

selectivity of rare sugars at 60 min in each cycle. Figure 5(B) shows the remaining galactose, pH, and yield of organic acids at 60 min for each cycle. The yields of each rare sugar were almost the same regardless of the repeated reactions (Fig. 5(A)). On the other hand, selectivity of both tagatose and all rare sugars increased with repeated reactions. In the third cycle, the selectivity of rare sugars reached 90%. This is explained as follows. The fraction of remaining galactose increased with increasing cycles (Fig. 5(B)). Nevertheless, the yield of rare sugars remained unchanged, resulting in an increase in the selectivity of rare sugars. This also suggests that side reactions were less likely to occur. However, the yield of organic acids increased with increasing cycles, indicating that byproducts other than organic acids could be formed less readily. Our results suggest that eggshells change into a more suitable catalyst for rare sugar production following their repeated use.

The factor responsible for this change was examined in terms of changes in the surface structure of eggshells. The surface structure of eggshell powder after each treatment cycle was observed by SEM (Fig. 6). Numerous pores were observed on the surface of eggshells, but no obvious changes to the surface structure caused by the treatment were observed. Thus, further investigation is required to clarify the change in the selectivity of rare sugars during the repeated use of eggshells.

4. Conclusion

Rare sugars with various potentially useful bioactivities exist in nature only in very small quantities, and most of them have not yet been mass-produced. Here, we aimed to produce rare sugars from galactose in hot compressed water, one of the methods used for rare sugar production. When eggshells were added to galactose solution and treated in hot compressed water, the isomerization of galactose was accelerated and the rare sugars

tagatose, talose, and sorbose were formed. Organic acids were simultaneously formed by side reactions, but isomerization continued due to the neutralizing effect of the eggshells. The calcium concentration in the treated solution increased, also due to the neutralization effect of the eggshells. Consequently, the treated solution could be used as a raw material for supplements containing calcium and rare sugars.

However, for the practical application of supplement production, there are many other points that must be verified in addition to the facts revealed in this study. For example, it is necessary to evaluate the dissolution behavior of eggshells and changes in eggshell components due to neutralization and repeated reactions. Furthermore, in scale-up, it is also necessary to evaluate the effects of thermal history, energy balance, heat balance, etc. In addition, techno-economic analysis upon scale-up is also necessary, which is an issue that will be clarified for industrialization in the future.

CRediT authorship contribution statement

T. Kobayashi: Conceptualization, Methodology, Writing - Original draft preparation, Supervision; S. Futamata: Investigation, Data curation; Y. Onishi: Data curation; S. Adachi: Validation, Reviewing & Editing; P. Khwijtjaru: Validation, Reviewing & Editing; Y. Watanabe: Validation, Reviewing & Editing; F. Tani: Validation - Reviewing & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

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 433
 434

Table 1. Composition of the treated solution obtained by the treatment in the presence of constituent salts of eggshells.

Additive	Composition of the reaction mixture (wt%)				
	Galactose	Tagatose	Talose	Sorbose	Byproducts
Without eggshell	94.4	1.4	0	0	4.2
Eggshell	70.1	15.7	1.7	2.0	10.5
CaCO ₃	73.8	15.0	1.2	1.7	8.3
Ca ₃ (PO ₄) ₂	60.9	16.1	5.3	2.7	15.0
MgCO ₃	32.9	16.4	5.7	5.6	39.3

The reaction conditions were 120°C for 120 min.

Table 2. Composition of the treated solution obtained by the treatment in the presence of eggshells, model eggshells, or calcium carbonate.

Composition of the reaction mixture (wt%)					
Additive	Galactose	Tagatose	Talose	Sorbose	Byproducts
CaCO ₃	73.8	15.0	1.2	1.7	8.3
Eggshell	70.1	15.7	1.7	2.0	10.5
Model eggshell	58.8	18.7	3.5	3.6	15.5

The reaction conditions were 120°C for 120 min.

Figure legends

Fig. 1. Time courses for the isomerization with and without addition of eggshells: (A) Fraction of remaining galactose and selectivity of rare sugars, and (B) yield of each rare sugar. Lines between the experimental points are for improving readability.

Fig. 2. Time courses for pH, concentration of Ca^{2+} , and yields of various organic acids during isomerization: (A) With and (B) without eggshells. Lines between the experimental points are for improving readability.

Fig. 3. Changes in (A) the yield of each rare sugar and the selectivities of total rare sugars and tagatose, and (B) the fraction of remaining galactose, yield of organic acids, and pH after treatment for 60 min at each reaction cycle. Lines between the experimental points are for improving readability.

Fig. 4. Scanning electron microscopic (SEM) images of the eggshells obtained during each reaction cycle.

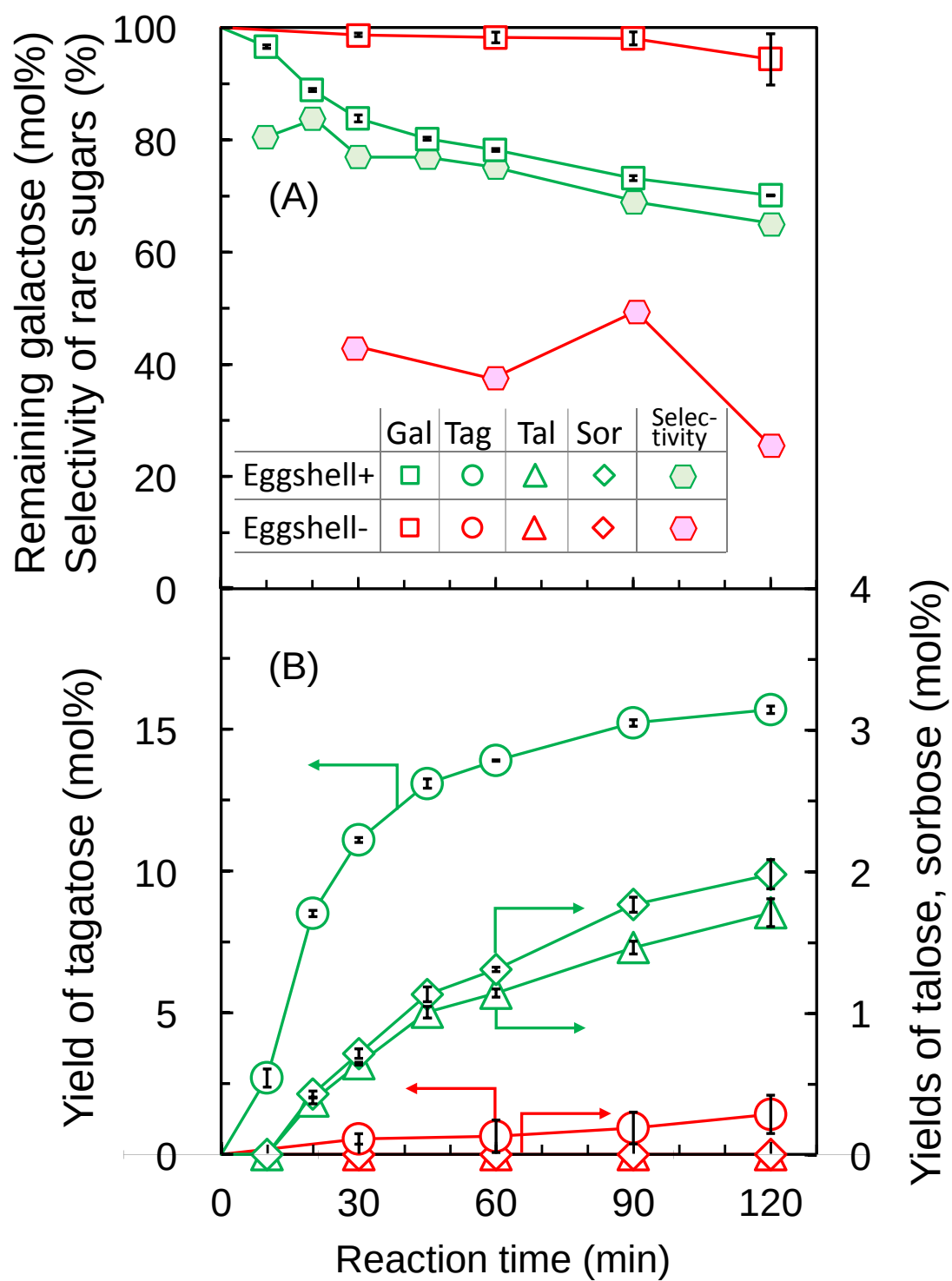
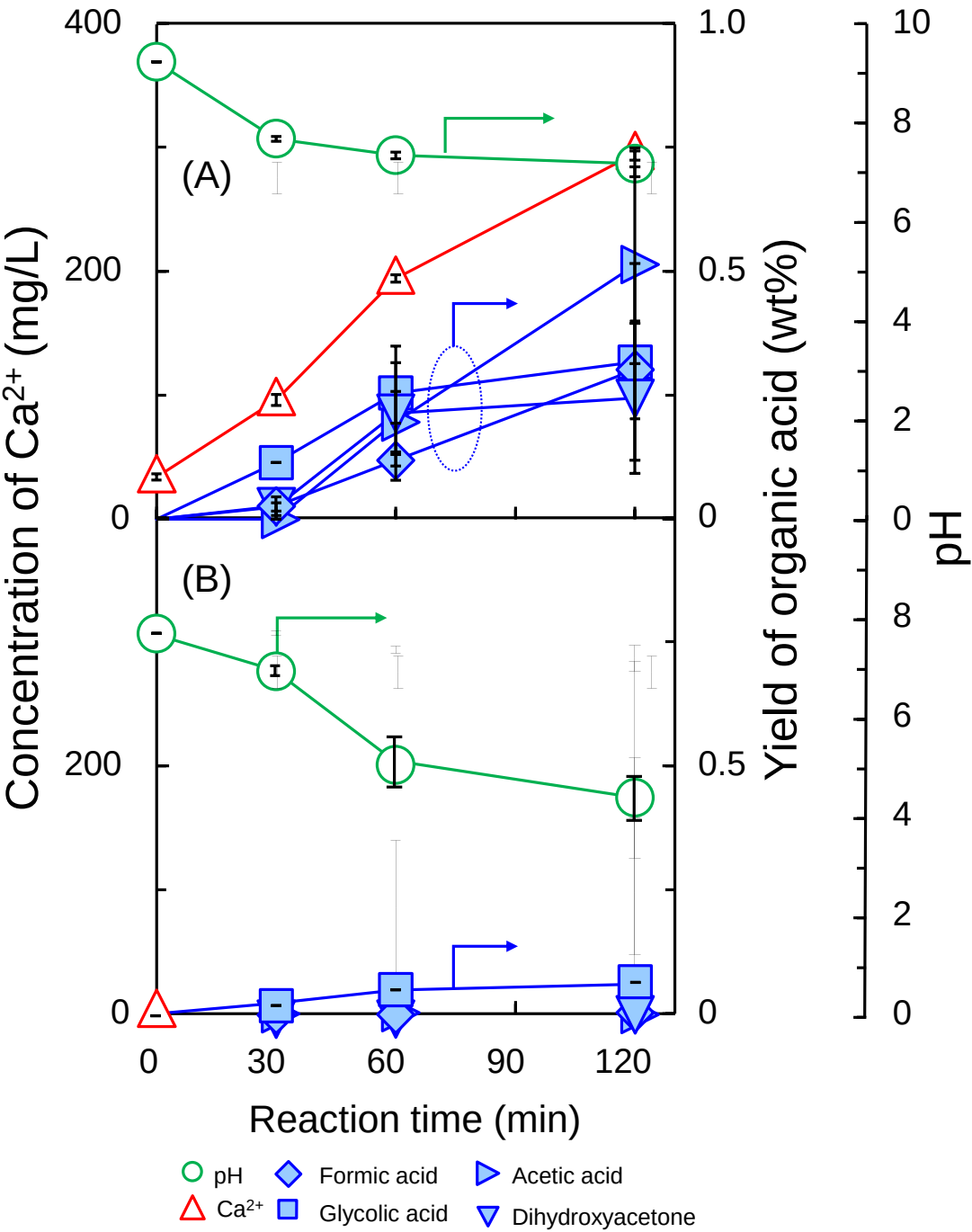


Fig. 1. Futamata et al.

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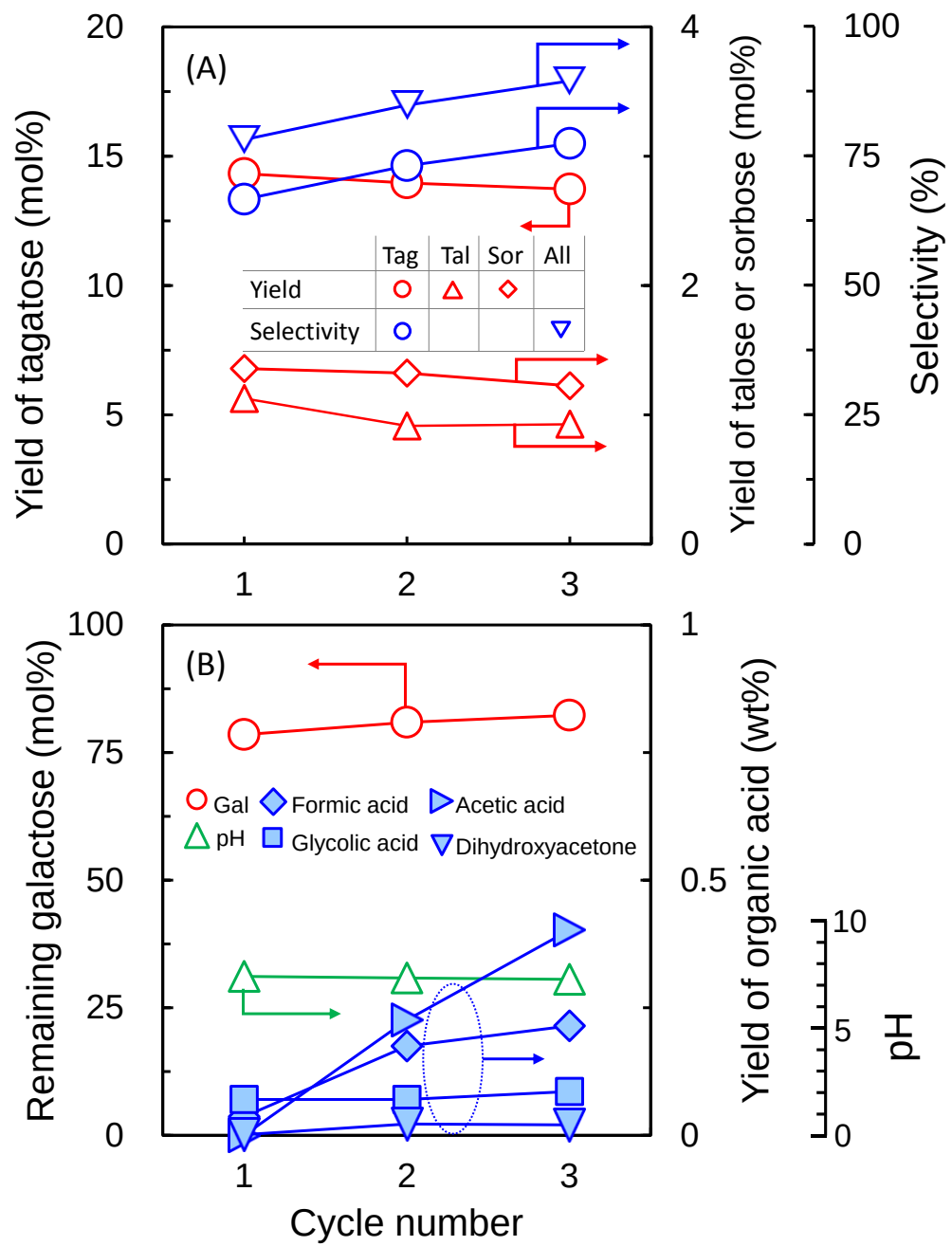
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467 Fig. 2. Futamata et al.

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474 Fig. 3. Futamata et al.

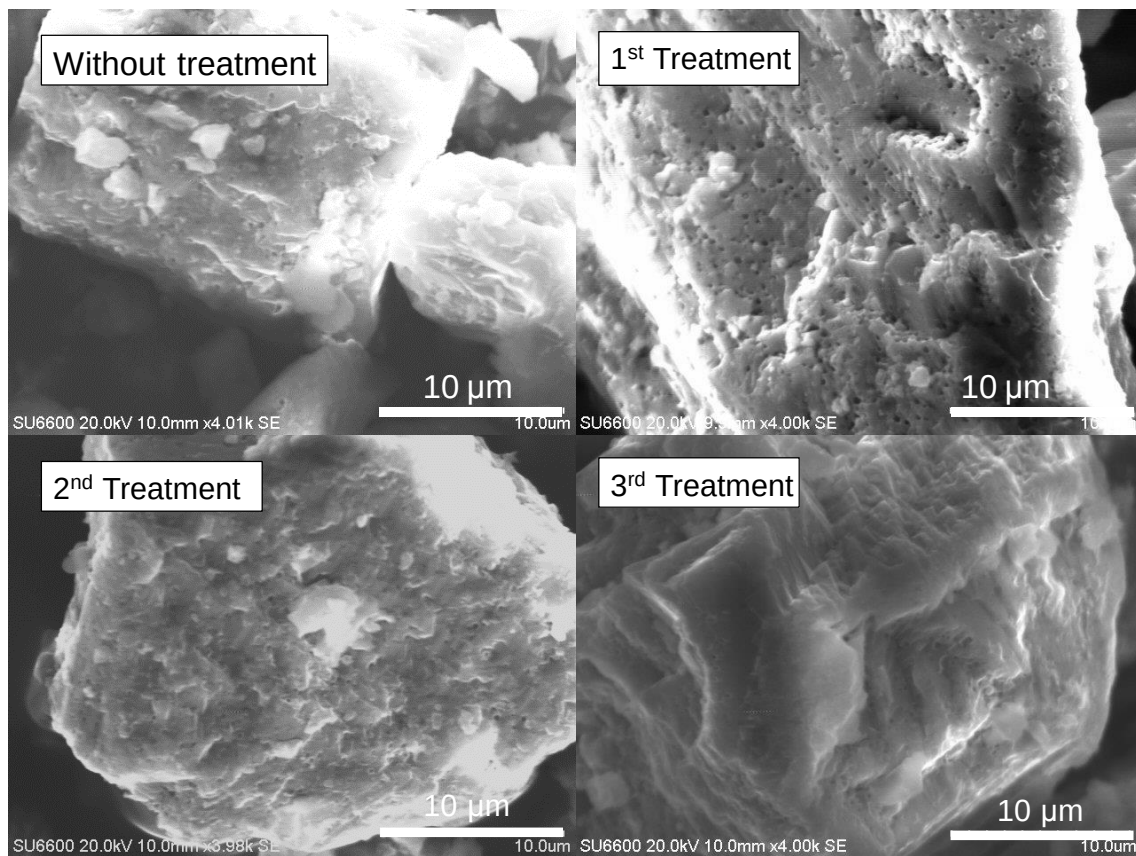


Fig. 4. Futamata et al.