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Basidiomycotic Yeast *Cryptococcus diffluens* Converts L-Galactonic Acid to the Compound on the Similar Metabolic Pathway in Ascomycetes

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Abstract: (1) Background: It has been shown that D-galacturonic acid is converted to L-galactonic acid by the basidiomycotic yeast, *Cryptococcus diffluens*. However, two pathways are hypothesized for the L-galactonic acid conversion process in *C. diffluens*. One is similar to the conversion process of the filamentous fungi in D-galacturonic acid metabolism and another is the conversion process to L-ascorbic acid, reported in the related yeast, *C. laurentii*. It is necessary to determine which, if either, process occurs in *C. diffluens* in order to produce novel value-added products from D-galacturonic acid using yeast strains. (2) Methods: The diethylaminoethy (DEAE)-fractionated enzyme was prepared from the cell-free extract of *C. diffluens* by the DEAE column chromatography. The L-galactonic acid conversion activity was assayed using DEAE-fractionated enzyme and the converted product was detected and fractionated by high-performance anion-exchange chromatography. Then, the molecular structure was identified by nuclear magnetic resonance analysis. (3) Results: The product showed similar chemical properties to 2-keto-3-deoxy-L-galactonic acid (L-threo-3-deoxy-hexulosonic acid). (4) Conclusions: It is suggested that L-galactonic acid is converted to 2-keto-3-deoxy-L-galactonic acid by dehydratase in *C. diffluens*. The L-galactonic acid conversion process of *C. diffluens* is a prioritized pathway, similar to the pathway of ascomycetes.

Keywords: *Cryptococcus diffluens*; basidiomycotic yeast; L-galactonic acid; dehydratase; 2-keto-3-deoxy-L-galactonic acid; D-galacturonic acid; NMR

1. Introduction

Pectin-rich substrates, which are present in large amounts in citrus peel and sugar beet pulp, are generated in the waste products that result from processing citrus or sugar beet in the food industry (for example, following juice extraction) [1–3]. Pectin residues left in peels after squeezing citrus make up approximately 25% of the dry weight [1]. Approximately 200 million tons of sugar beet pulp is produced from sugar beet in Europe [2], and approximately 25% of the sugar constituents is pectin [3]. Pectin-containing wastes are usually used in animal feed. However, pectin-containing wastes are sometimes simply discarded, because processing animal feed from them is relatively expensive due to the costs incurred in drying and milling the waste [4]. To convert pectin-rich residues into novel value-added products, we previously isolated the yeast strain *Cryptococcus diffluens*, which utilizes D-galacturonic acid (D-GalUA) (which is abundant in pectin [5]), and determined that the enzyme galacturonate reductase converts D-GalUA to L-galactonic acid (L-GalA) [6]. This, in turn, enhanced the production of L-GalA using recombinant *Saccharomyces cerevisiae* containing the gene Cd-*GAR1*, which encodes galacturonate reductase of *C. diffluens* [7]. However, it is not known what is produced following L-GalA conversion in the D-GalUA metabolic pathway of *C. diffluens*. It has been shown in

ascomycetes that one metabolic pathway from L-GalA processes a dehydratase reaction that converts L-GalA to L-threo-3-deoxy-hexulosonic acid/2-keto-3-deoxy-L-galactonic acid (L-2KDGal) and then, an aldolase reaction converts L-2KDGal to L-glycerol and pyruvic acid [8–10]. Another pathway from L-GalA results in the conversion to L-ascorbic acid, which has been shown in *Cryptococcus laurentii*, which is closely related to *C. diffluens* [11]. To elucidate the L-GalA conversion pathway in *C. diffluens*, we analyzed the product converted from L-GalA using an extract of *C. diffluens* cells. Here, we describe the determination of a conversion pathway from L-GalA that is similar to the pathway shown in ascomycetes.

2. Materials and Methods

2.1. Strain, Media, and Culture Condition

The yeast strain used was *C. diffluens* OPU-FC11 [6]. The media used were GYP (2% glucose, 0.5% yeast extract, and 1% tryptone; pH 6.0) [12] and GalUA-YP, in which the 2% glucose is replaced with 0.1% D-GalUA.

2.2. Preparation of Enzymes

2.2.1. Preparation of Cell-Free Extract

Preparation of cell-free extract (CFE) was done using ultrasonication as described in Nishimoto et al. [12], with minor modifications, as follows. *C. diffluens* OPU-FC11 was precultured in GYP for 48 h at 25 °C and then cultured in GalUA-YP for 48 h with shaking at 25 °C. Yeast cells were harvested by centrifugation at 5000 × g and suspended in 25 mM sodium acetate buffer (NaAB) at pH 6.0 with a proteinase inhibitor. Yeast cell suspensions were then mixed with the same volume of 0.5-mm diameter glass beads in microfuge tubes and sonicated for 7 min at 30 s intervals at 4 °C using a Bioruptor ultrasonic fragmentation device with a cooling pump (Cosmo Bio Co., Tokyo, Japan). The supernatant obtained by centrifuging the disrupted cells at 15,000 × *g* for 10 min and dialyzing in NaAB (pH 6.0) was used as the CFE. The protein content of each solution was measured by absorbance at 280 nm [7].

2.2.2. Preparation of Diethylaminoethy (DEAE)-Fractionated Enzyme

Preparation of DEAE-fractionated enzyme (DFE) was performed by chromatography using an anion-exchange column with DEAE-TOYOPEARL 650 M (Tosoh Co., Tokyo, Japan), as follows. Desalted-CFE was loaded onto the DEAE-TOYOPEARL 650 M column (diameter 1.5 × 80 cm), which was equilibrated with NaAB (pH 6.0), and fractions with enzyme activity were eluted with a linear gradient from 0 to 1 M sodium chloride (NaCl) in NaAB (pH 6.0). The elution rate of this chromatography was 3.0 mL/min. Eluted fractions (NaCl-concentration was about 75 to 225 mM) with enzyme activity were collected as DFE and concentrated 5-fold by freeze-drying using FD-1000 (EYELA Japan, Tokyo, Japan) and solubilized in NaAB (pH 6.0).

2.3. Assay for Sugar Acids by High-Performance Anion-Exchange Chromatography

The analysis of the substrates and products of the enzyme reaction was performed by high-performance anion-exchange chromatography and pulsed amperometric detection system (HPAEC-PAD) using HPAEC system Dionex ICS-5000 (Thermo Fisher Scientific K. K., Tokyo, Japan) with a Dionex CarboPac PA1 column (φ 4 mm × 250 mm: Thermo Fisher Scientific K. K., Tokyo, Japan). The analytic conditions of the HPAEC-PAD were as follows: the column temperature was 35 °C and the flow rate was 1 mL/ min. The composition of the elutes was only 50 mM NaOH for 0–5 min, the sodium acetate (NaOAc) linear gradient from 0 to 250 mM in 50 mM NaOH for 5–15 min, 250 mM NaOAc in 50 mM NaOH for 15–20 min, and 50 mM NaOH for 20–25 min.

2.4. Assay for Dehydratase Activity

The L-GalA dehydratase activity was assayed using a reaction mixture composed of 3.5 mg/mL L-GalA, 25 mM NaAB (pH 5.5), 3 mM cobalt chloride, and the protein solution (5 mg/mL) containing an enzyme (*C. diffluens* CFE and DFE). The substrate L-GalA was prepared by decyclizing L-galactono-1, 4-lactone (Carbosynth, Tokyo, Japan) as follows: L-galactono-1, 4-lactone solution (5 mg/mL) was mixed with one-fifth of a volume of 0.3 m sodium hydroxide and vigorously agitated by vortexing for 20 s. Then, the pH was adjusted to 6.0 after treatment with basic conditions for 30 min using 0.3 m hydrochloric acid. The enzyme reaction was carried out at 30 °C for 48 h and was terminated by heating at 98 °C for 15 min. L-GalA dehydratase activity was measured by the generation of the reaction product L-2KDGal, which is easily detected by thiobarbituric acid (TBA) assay [13]. The concentration of L-2KDGal was calculated by its molar extinction coefficient (67.8×10^3 M^{-1}) and the absorbance was measured at 549 nm. One unit of activity is defined by an increase of 1 mM L-2KDGal for 1 min.

2.5. Identification of L-2KDGal

2.5.1. Determination of Molecular Mass

The molecular mass of L-2KDGal was determined by mass spectrometry (MS) and liquid chromatography–MS/MS (LC–MS/MS). MS and LC–MS/MS were entrusted to Hokkaido Global Facility Center (Sapporo, Japan), using ESI-MS extraction equipment (Thermo Fisher Scientific K. K., Tokyo, Japan) and LTQ-Orbitrap XL (Thermo Fisher Scientific K. K., Tokyo, Japan), respectively.

2.5.2. Nuclear Magnetic Resonance

The chemical formula of L-2KDGal was determined by nuclear magnetic resonance (NMR) analysis. In NMR, JNM-ECZ 500R (JEOL, Tokyo, Japan) was used. The proton and carbon NMR analyses were carried out with the use of heavy water and heavy acetone, respectively, as solvents.

3. Results

3.1. Detection of the Product Converted from L-GalA

When CFE of *C. diffluens* OPU-FC11 was reacted with L-GalA, two novel peaks were detected as reaction products (Figure 1). The elution time of one peak was the same as that of pyruvic acid, but the other peak did not correspond to commercial reagents including D-GalUA, L-GalA, glycerol, acetate, ascorbic acid, and other various compounds related to D-GalUA metabolism. These results suggest that enzymes converted L-GalA to pyruvic acid and the new compound (named Compound-X), which is different from commercial reagents involved in D-GalUA metabolism. When testing the conversion activity from L-GalA to Compound-X and pyruvic acid using DFE, the production activity of Compound-X was only recognized in DFE (Figure 2). These results suggest that the enzyme that converts L-GalA to Compound-X is present in DFE. When TBA assay was performed for Compound-X-generated samples, the production of keto acid was recognized and the specific activity in DFE was 272 units/mg protein.

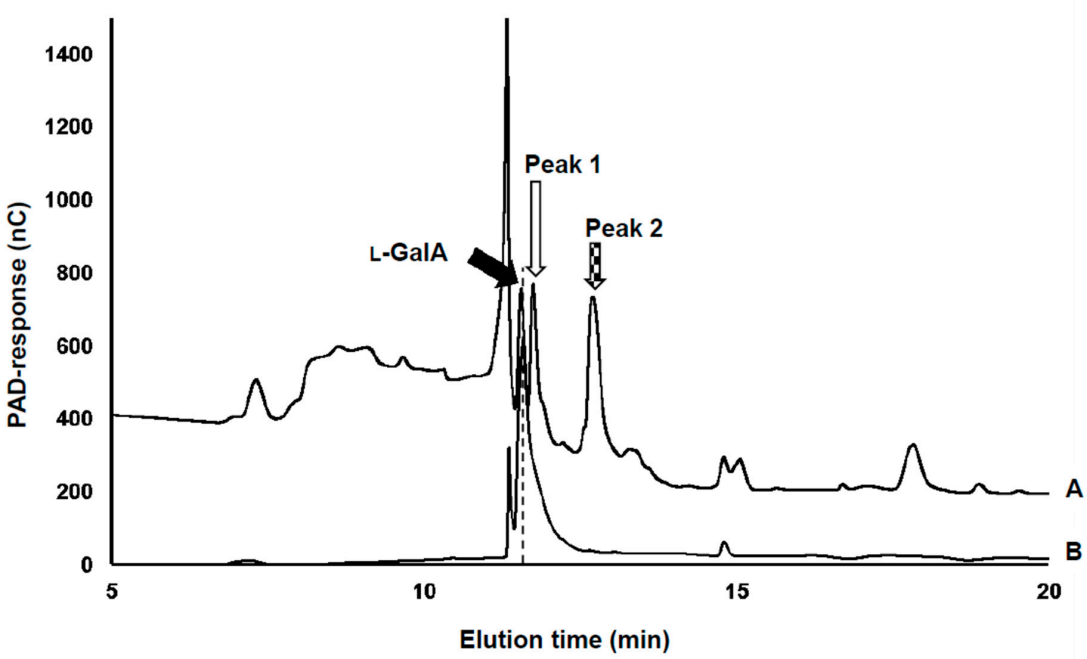

Figure 1. Elution profile of the L-galactonic acid conversion product using a cell-free extract. Profiles A and B show the reaction product and pure L-galactonic acid, respectively. The peak shown by a black arrow in Profile A is at the same position as the peak derived from L-galactonic acid in Profile B (indicated by a dashed line). Peak 1 and Peak 2 (shown by white and checked arrows, respectively) are new peaks generated by the conversion of L-galactonic acid to the reaction product. PAD: pulsed amperometric detection.

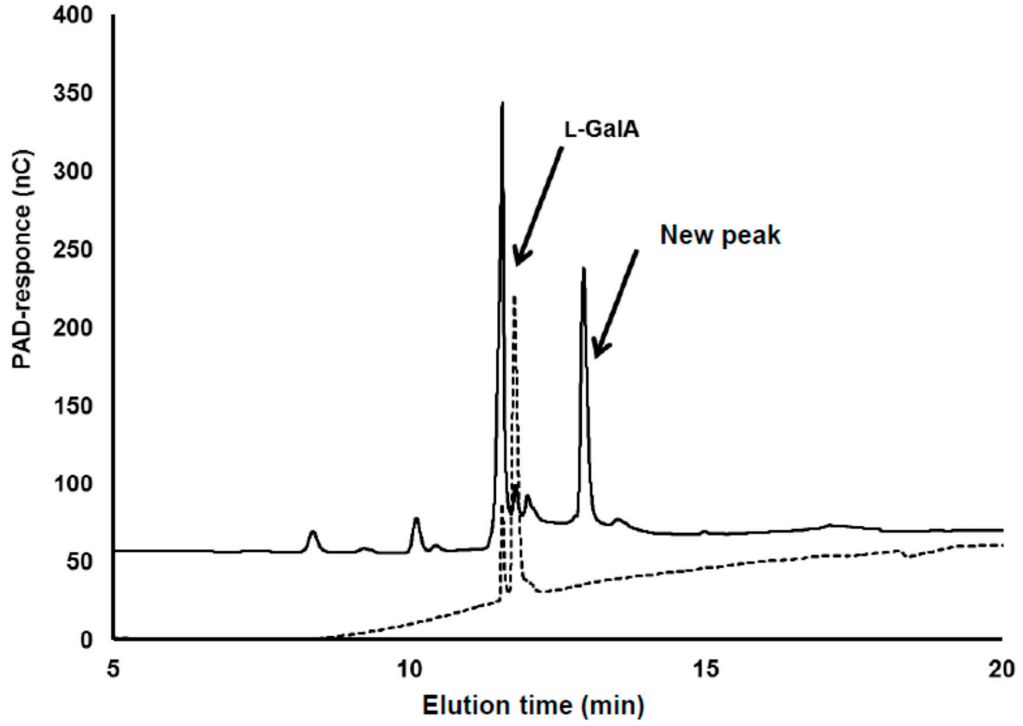

Figure 2. Elution profile of the L-galactonic acid conversion product by the diethylaminoethy (DEAE)-fractionated enzyme. Profiles A (solid line) and B (dotted line) show the reaction product and pure L-galactonic acid, respectively. PAD: pulsed amperometric detection.

3.2. Identification of Compound-X

3.2.1. MS Analysis

The precise molecular mass of Compound-X was calculated by LC–MS/MS and the calculated value was 177.04. The molecular formula of Compound-X was predicted to be $C_6H_{10}O_6$, based on the molecular mass of 177.04. The predicted molecular formula indicated the possibility that Compound-X is 3-deoxy-L-threo-hex-2-ulosonic acid/2-keto-3-deoxyl-L-galactonic acid (L-2KDGalA), which has the same molecular mass and molecular formula that were previously reported in ascomycetes [9,14].

3.2.2. NMR Analysis

The ^{13}C NMR spectrum of Compound-X is shown in Figure 3. Compound-X is composed of six carbons. One chemical shift value indicated a carboxyl group (δ_C 174.72), one (δ_C 96.80) indicated a typical quaternary carbon in a hemiketal structure such as C2 signals in sialic acids, and the other four were attributed to two CH_2- and two CH-type carbon atoms (δ_C 71.58, 69.66, 64.24, and 39.77). These carbons corresponded to the carbon structure from C3 to C6 of L-2KDGalA. The ^{13}C NMR results are similar to previous reports [9,14] (Table 1).

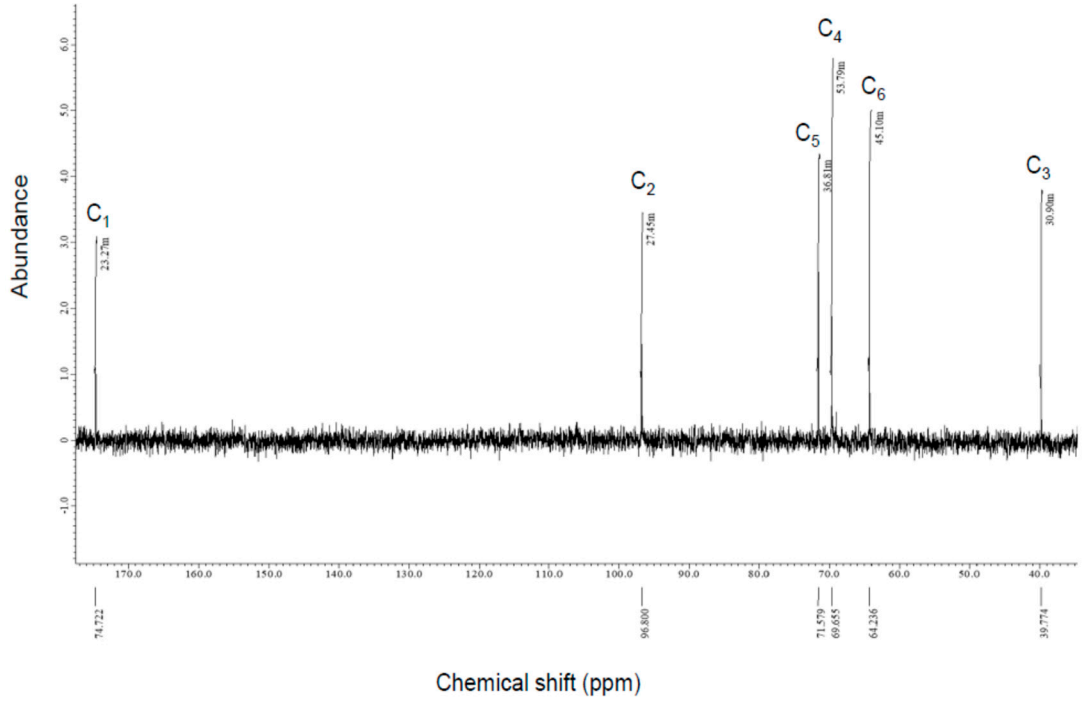

Figure 3. ^{13}C nuclear magnetic resonance (NMR) spectrum of the purified Compound-X. The product signals are numbered according to Figure 5. The product has two CH_2 groups and the position of the signal of C2 is characteristic of a hemiketal structure, indicating that the molecule exists predominately in ring form.

The ^{1}H NMR spectrum of Compound-X is also shown, although unclear signals are clearly visible (Figure 4). However, it is predicted that Compound-X has the proton spin system, CH_2-CH-CH-CH_2, which is similar to the proton spin system from the previously reported values of L-2KDGalA [9,14], as follows: one of the CH_2 functions that indicated typical chemical shifts (δ_H 3.58 and 3.76) of a hydroxymethyl group indicated similar chemical shifts bound to C6 (H6 and H6′, respectively), and the second CH_2 function indicated unique proton chemical shifts (δ_H 1.74 and 2.18), typical of a CH_2 group situated close to a keto group or a hemiketal similar to those bound to C3 (H3 and H3′, respectively). The other two CHs (δ_H 3.54 and δ_H 3.82) were also similar to the chemical shifts from H5 and H4,

respectively, of reported L-2KDGalA [9,14]. The comparison of the ^{1}H and ^{13}C chemical shifts of Compound-X to those of previous data [9,14] are shown in Table 1.

Table 1. ^{13}C and ^{1}H NMR chemical shifts of Compound-X and reported L-2KDGal.

	Compound-X [A]	l-2KDGal [B]	l-2KDGal [C]
Atom	**s (ppm)**	**s (ppm)**	**s (ppm)**
C1	174.72	177.53	176.28
C2	96.8	97.84	96.63
C3	39.77	40.22	38.92
C4	69.66	70.13	68.93
C5	71.58	71.92	70.72
C6	64.24	64.18	62.98
H3	1.74	1.79	1.75
H3′	2.18	2.16	2.14
H4	3.82	3.86	3.85
H5	3.54	3.6	3.58
H6	3.59	3.61	3.57
H6′	3.77	3.8	3.78

A: Values of C and H were referenced to D_2O (4.79 ppm) and acetone (31.5 ppm), respectively. B and C: Values were referenced from [14] and [9], respectively.

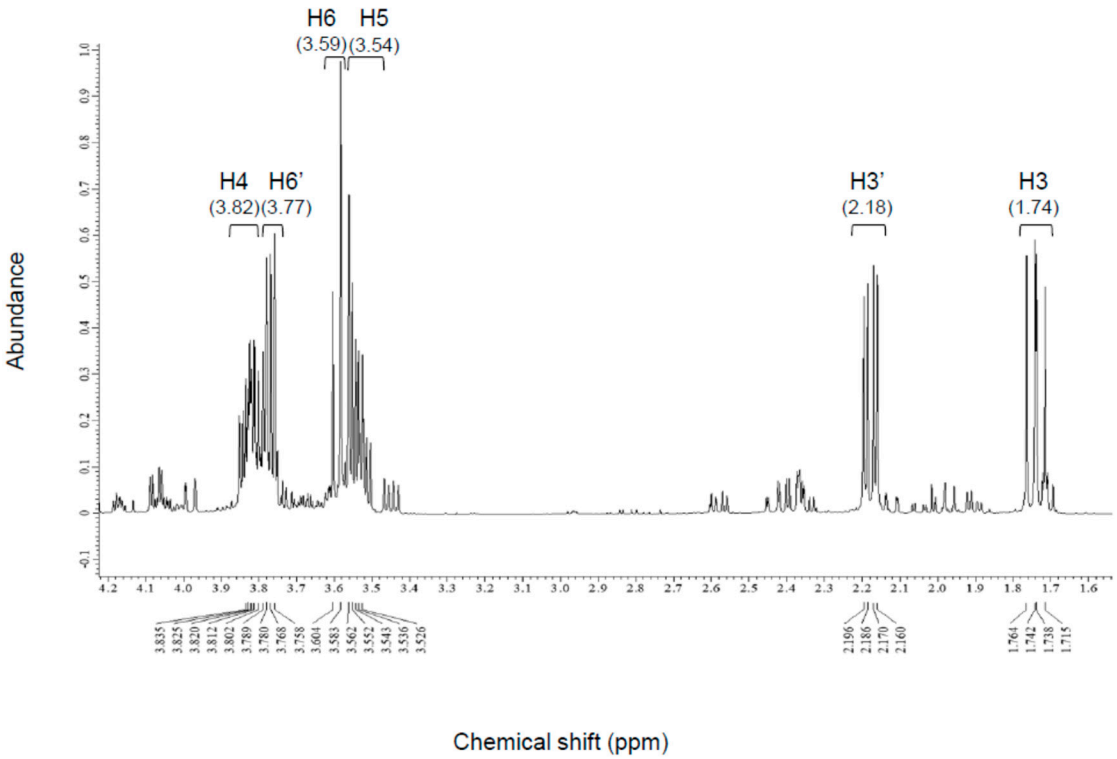

Figure 4. ^{1}H NMR spectrum of the purified Compound-X. The product signals are numbered carbons according to Figure 5. The products derived from C1 and C2 are not shown in this analytic condition. Values in parentheses show the calculated chemical shift.

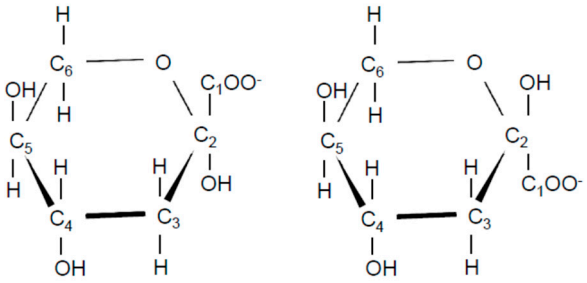

Figure 5. Fischer projection of L-2KDGalA. NMR analysis revealed that it predominantly exists in the pyranose form. Although the pyranose form can have two possible anomers, it was not determined which of the two anomers was formed.

Both chemical shifts of Compound-X were similar to the previous data of L-2KDGalA. These results strongly suggest the possibility that Compound-X is L-2KDGalA, forming predominantly as a pyranose ring, but it could not be determined which of the two pyranose ring anomers was formed (Figure 5). The result that Compound-X is L-2KDGalA suggests that L-GalA dehydratase is present in *C. diffluens*, similar to *Hypocrea jecorina* and *Aspergillus niger* [9,14].

4. Discussion

The present study indicates that L-GalA is converted to L-2KDGalA by a dehydratase in *C. diffluens*. To the best of our knowledge, this is the first case that demonstrated L-2KDGalA production in basidiomycotic yeast such as *C. diffluens*. Our preliminary research has shown the conversion activity from Compound-X to glyceraldehyde and pyruvic acid in CFE of *C. diffluens* (in preparation). This result indicates that Compound-X is L-2KDGalA produced by L-GalA dehydratase of *C. diffluens*, similarly to *H. jecorina* and *A. niger*, and also strongly suggests that the D-GalUA metabolic pathway of *C. diffluens* is a prioritized pathway similar to the metabolic pathway of ascomycetes. However, it is unknown whether *C. diffluens* also converts L-GalA to L-ascorbic acid by a similar metabolic pathway in *C. laurentii*.

5. Conclusions

Previously, we successfully synthesized a large amount of the rare acidic sugar L-GalA, using a *S. cerevisiae* recombinant [7]. If the gene encoding L-GalA hydratase involved in D-GalUA metabolism is indeed isolated from *C. diffluens*, it may be possible to produce a large amount of L-2KDGalA using a recombinant containing that gene. It has been reported that acidic keto-deoxy sugars such as 2-keto-3-deoxy-D-gluconate and 2-keto-3-deoxy-6-phosphogluconate, which are produced from alginate by the enzymes involved in alginate metabolism, have been considered as potential leading compounds for antibiotics, antiviral agents, and other drugs and medicines [15]. The similar acidic keto-deoxy sugar L-2KDGalA may also become a novel leading compound for bioactive reagents. The purification of hydratase and aldolase and the cloning of the encoding genes are necessary for breeding *S. cerevisiae* recombinants to be used in large-scale production of L-2KDGalA. Since the mutagenesis of yeast is no more difficult than the mutagenesis of fungi, the screening of mutants that produce large amounts of L-2KDGalA can be easily carried out. We also plan to isolate a mutant of *C. diffluens* that generates large quantities of L-2KDGalA. If L-2KDGalA is produced in large amounts by the fermentation of pectin, it is expected that the investigation and industrial use of L-2KDGalA will increase due to its potential as a novel leading compound for bioactive reagents.

Author Contributions: M.K. (Masao Kishida) conceived of the idea and the concept. Resources: M.K. (Masao Kishida); Data curation: T.M.; Writing—original draft preparation: T.M.; Writing—review and editing: M.K. (Michihiko Kataoka) and M.K. (Masao Kishida); M.K. (Masao Kishida) supervised the development of this work. All authors discussed the experimental results and methodology, and contributed to writing the final draft.

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Conflicts of Interest: The authors declare no conflict of interest.

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