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L-Xylo-3-hexulose, a new rare sugar produced by the action of acetic acid bacteria on galactitol, an exception to Bertrand Hudson's rule

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ABSTRACT

Background: In acetic acid bacteria such as *Gluconobacter oxydans* or *Gluconobacter cerinus,* pyrroloquinoline quinone (PQQ) in the periplasm serves as the redox cofactor for several membrane-bound dehydrogenases that oxidize polyhydric alcohols to rare sugars, which can be used as a healthy alternative for traditional sugars and sweeteners. These oxidation reactions obey the generally accepted Bertrand Hudson's rule, in which only the polyhydric alcohols that possess cis D-*erythro* hydroxyl groups can be oxidized to 2-ketoses using PQQ as a cofactor, while the polyhydric alcohols excluding cis D-erythro hydroxyl groups ruled out oxidation by PQQ-dependent membrane-bound dehydrogenases.
Methods: Membrane fractions of *G. oxydans* were prepared and used as a cell-free catalyst to oxidize galactitol, with or without PQQ as a cofactor.
Results: In this study, we reported an interesting oxidation reaction that the polyhydric alcohols galactitol (dulcitol), which do not possess cis D-*erythro* hydroxyl groups, can be oxidized by PQQ-dependent membrane-bound dehydrogenase(s) of acetic acid bacteria at the C-3 and C-5 hydroxyl groups to produce rare sugars L-xylo-3-hexulose and D-tagatose.
Conclusions: This reaction may represent an exception to Bertrand Hudson's rule.
General significance: Bertrand Hudson's rule is a well-known theory in polyhydric alcohols oxidation by PQQ-dependent membrane-bound dehydrogenase in acetic acid bacteria. In this study, galactitol oxidation by a PQQ-dependent membrane-bound dehydrogenase represents an exception to the Bertrand Hudson's rule. Further identification of the associated enzymes and deciphering the explicit enzymatic mechanism will prove this theory.

1. Introduction

Both *Gluconobacter* and *Acetobacter* are acetic acid bacteria that belong to the family *Acetobacteraceae*. Most of the genera of this family are well known as vinegar producers because of their strong ability to oxidize ethanol to acetic acid by membrane-bound dehydrogenases, including alcohol dehydrogenase and aldehyde dehydrogenase. Besides ethanol, *Gluconobacter* and *Acetobacter* have a highly active respiratory chain in their membrane, which can oxidize various sugars and sugar alcohols in a stereo- and regio-selective manner to organic acids, aldehydes, and ketones by membrane-bound dehydrogenases [1]. In *Gluconobacter oxydans* 621H, eight known and two unknown membrane-bound dehydrogenases have been found [2,3]. Most of these membrane-bound dehydrogenases, i.e., glucose dehydrogenase (GOX0265), inositol dehydrogenase (GOX1857), D-sorbitol dehydrogenase (GOX0854-0855), GOX1441, and 0516 are proved to be PQQ-dependent; however, some are also FAD-dependent (i.e., D-gluconate dehydrogenase) [4]. The whole cells of *G. oxydans* have a higher specific activity of the PQQ-dependent membrane-bound polyol dehydrogenases and can be used as biocatalysts to stereo- and regiospecifically oxidize polyols, primary/secondary alcohols yielding corresponding ketoses or organic acids in high yields [1,5,6].

The stereo- and regiospecificity of *Gluconobacter* and *Acetobacter* genera bacterial oxidation in alditol series such as D-arabitol, D-sorbitol, ribitol, glycerol, meso-erythritol, D-mannitol has been expressed as a well-known Bertrand-Hudson rule [7], in which polyols having the D-*erythro* configuration is oxidized at the secondary alcohol group giving 2-ketose products within the pH range of 5.0–6.5 (Fig. S1a). Whereas alditols without D-erythro configuration, including D-threitol, L-threitol, xylitol, L-arabitol, and dulcitol, excludes oxidation by PQQ-dependent

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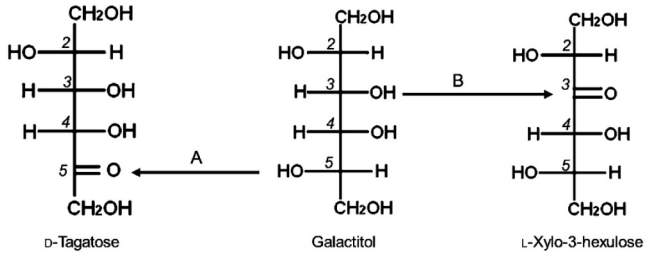

Fig. 1. The regiospecific oxidation of galactitol by the genera of *Acetobacter* and *Gluconobacter*.
Galactitol can be simultaneously oxidized by PQQ-dependent membrane-bound dehydrogenase(s) to D-tagatose and L-xylo-3-hexulose at the C-5 and C-3, respectively.

membrane-bound dehydrogenases of *A. suboxydans* (nowadays as *G. suboxydans*) [8–11] (Fig. S1b). However, an anomalous oxidation pattern was observed five decades ago in the case of ω-deoxy sugar alcohols such as L-fucitol, a kind of 6-methyl substituted hexitol (Fig. S2a) [8,12]. This reaction to ω-deoxy alcohols was considered as the extension to Bertrand-Hudson rule, in which the terminal methyl group was simply as an elongated CH_2OH group, i.e., the methyl group can be seen as the substitution of H atom covalently linked to a carbon atom. Thus the 6-deoxy-L-galactitol has the D-*erythro* configuration, and it's corresponding –OH group can be oxidized to keto group by *A. suboxydans* [8] (Fig. S2b).

In this study, we found that the hexitol galactitol (also named as dulcitol) does not possess the *cis* D-erythro, but D-lyxo configuration can also be acted by genera of *Acetobacter* sp. and *Gluconobacter* sp. strains. The enzyme was evidenced to be a PQQ-dependent membrane-bound dehydrogenase. Both of the hydroxyl groups on C-3 and C-5 can be oxidized simultaneously by PQQ-dependent membrane-bound dehydrogenase from *Gluconobacter* and *Acetobacter* genera, with L-xylo-3-hexulose (PubChem CID: 18392540, also alternative as D-lyxo-4-hexulose) and D-tagatose as products (Fig. 1). Though D-threitol, xylitol, and D-iditol possess D-lyxo configuration as that of galactitol, however, all these three sugar alcohols cannot be oxidized by PQQ-dependent membrane-bound dehydrogenase from *Gluconobacter* and *Acetobacter* genera (Fig. S1b).This galactitol to 3-ketose L-xylo-3-hexulose conversion by PQQ-dependent membrane-bound dehydrogenases may represent an exception to Bertrand Hudson's rule, instead of the extension.

2. Materials and methods

2.1. Materials

Sugars or sugar alcohols including galactitol, erythritol, L-erythrulose, D-threitol, xylitol, D-xylulose, D-tagatose, L-tagatose, D-sorbitol, D-arabitol, L-arabitol, L-fucitol, D-mannitol, and nuclease Benzonase were purchased from Merck & Co Inc. or Tokyo Chemical Industry (TCI, Japan). NAD, NADH, NADP, NADPH, PQQ, coomassie brilliant blue R-250, and antibiotics were provided by Sangon Biotech (Shanghai, China). All other reagents or chemicals used were of high-purity grade.

2.2. Strains, media, and culture conditions

Microorganisms used in this work are listed in Table 1. *E. coli* strains were cultured at 37 °C in Luria-Bertani (LB) media supplemented with ampicillin (100 $mg{\cdot}L^{-1}$) or kanamycin (50 $mg{\cdot}L^{-1}$). *Acetobacter* or *Gluconobacter* strains were aerobically grown at 30 °C and 200 rpm in a complex medium comprising 15 $g{\cdot}L^{-1}$ yeast extract, 3 $g{\cdot}L^{-1}$ tryptone, and 50 $g{\cdot}L^{-1}$ glucose (15 $g{\cdot}L^{-1}$ calcium carbonate was added when necessary). When required, sugar alcohols such as galactitol, L-fucitol, D-sorbitol, or mannitol were used as carbon and energy sources. Yeast was cultivated in YPD medium (pH 5.5) containing 10 $g{\cdot}L^{-1}$ yeast extract, 5 $g{\cdot}L^{-1}$ tryptone, 20 $g{\cdot}L^{-1}$ glucose, and 15 $g{\cdot}L^{-1}$ agar (only for solid media).

2.3. General molecular biology methods

Restriction endonucleases, DNA polymerases, and ligases were purchased from Thermo Fisher Scientific. Genomic DNA was prepared as reported earlier [13]. High fidelity Taq DNA polymerase (KOD plus, Toyobo) was used for DNA cloning, whereas genotype verification was carried out by using ExTaq DNA polymerase (Takara). The PCR-amplified products were purified from the agarose gels using a GeneJet Gel Extraction Kit (Thermo Scientific). Sangon Biotech (Shanghai, China) performed the primers synthesis and DNA sequencing, while the synthetic DNA was provided by Genewiz (Suzhou, China).

2.4. Biotransformations of galactitol with whole resting cells

The *Acetobacter* or *Gluconobacter* strains in Table 1 were first cultivated in 250 mL baffled shake flasks containing 50 mL complex medium (15 g/L yeast extract, 3 g/L tryptone, supplemented with 50 g/L glucose and 15 g/L calcium carbonate) for 60 h at 30 °C and 150 rpm. The cells were harvested by centrifugation at 10,000 *g* for 10 min and washed twice with sterilized potassium phosphate buffer (10 mM PBS, pH 6.0). These cells were used for the biotransformation of galactitol (40 g/L) in 20 mL reaction mixture at 30 °C, and 150 rpm with a final cell density of OD_{600} 5.0 (1 mL 1 OD_{600} is approximate 0.32 mg dry cell weight, thus 20 mL OD_{600} is equal to 32 mg DCW). Samples were taken regularly for HPLC analysis. The conversion rate of the product was calculated as the ratio of product to the substrate, while the production rate was calculated as the amounts of product (g) produced by per g dry cell biomass per hour per liter (g/L·h·g biomass).

2.5. Bioremoval of residual galactitol and D-tagatose by yeast strains

The *G. cerinus* X512 strains were first cultured in a 250 mL baffled shake flask containing 20 g/L yeast extract, 5 g/L tryptone, 50 g/L glucose, and 15 g/L calcium carbonate for 72 h at 30 °C and 200 rpm. After cultivation, the cells were harvested by centrifugation at 10,000 *g* for 10 min, washed twice with sterilized PBS buffer (10 mM, pH 6.0), and used for the biotransformation of galactitol (20 g/L) in 50 mL reaction mixture at 30 °C, and 200 rpm for 120 h with a final cell density of OD_{600} 5.0. Then the biotransformation liquid was centrifuged, and the clear supernatant was filtered (0.22 μm membrane) to eliminate residual cells. Different yeast strains were separately inoculated into the test tubes containing 5 mL clear biotransformation liquid and cultivated at 30 °C. Samples were collected regularly to analyze using the HPLC to determine which yeast strain can completely exhaust galactitol and tagatose but not the unknown product. Once galactitol and tagatose but not the unknown product was completely exhausted, the yeast strains were employed to biodegrade galactitol and tagatose in 250 mL flasks containing 50 mL biotransformation liquid and cultivated at 30 °C. When galactitol and tagatose were completely exhausted, the yeast cells were eliminated by centrifugation, and the clear supernatant was decolored and subjected to ion-exchange chromatography by activated carbon absorption and anion and cation exchange resins. The unknown product was then collected by HPLC and concentrated by rotary evaporation at 50 °C. The resulting purified compound was identified by the sub-sequential methods.

2.6. Identification of the unknown product

Various methods were applied to identify the unknown compound produced from galactitol by acetic acid bacteria. First, we excluded the unknown compound L-galactose, D-galactose, L-tagatose, or D-tagatose, which C1, C6, C2, or C5 position was dehydrogenated separately.

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Characteristics	sources
E.coli DH5a	*supE44 _lacU169(_80lacZ_M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Stratagene
E.coli BL21(DE3)	*F-, ompT, hsdS(rBB-mB-), gal, dcm(DE3)*	Stratagene
Gluconobacter cerinus X512		This study
Gluconobacter oxydans CGMCC 1.110		CGMCC
Gluconobacter oxydans 621H		ATCC
Gluconobacter frateurii DSM7146		DSM
Acetobacter aceti DSM2002		DSM
Acetobacter pasteurianus DSM 3509		DSM
Gluconobacter japonicus CGMCC 1.49		CGMCC
Yeast X535 (*Candida* sp. CGMCC3268)	Biodegradation of D-tagatose and galactitol, but not L-xylo-3-hexulose	This study
plasmid: pET28a-AnLadB	*AnLadB* gene inserted into *NcoI*/*XhoI* in pET28a	This study
pUC57-pqqA-knockout	Containing the upstream region of *pqqA* (500 bp), kanamycin resistance cassette and 700 bp downstream of *pqqA* (2.2 kb)	This study

ATCC: American Type Culture Collection.
DSM: Deutsche Sammlung von Mikroorganismen;
CGMCC: China General Microbiological Culture Collection Center.

Authentic L-galactose, D-galactose, L-tagatose, or D-tagatose were applied to HPLC, and their retention times were compared to that of the unknown compound. To further determine whether the unknown product is aldose or ketose, 5 μL of bromine water (brown) was added into 100 μL of the purified compound, mixed for 1 min, and then incubated at 65 °C. The unknown compound was also hydrogenated in the presence of sodium borohydride ($NaBH_4$) according to the strategy of Mizanur et al. [14]. HPLC separated the reduced products (galactitol and another isomeric alcohol). The optical rotation of the isomeric alcohol was recorded by a polarimeter (P-2000, Jasco, Japan) at 28 °C using sodium light and water as a control to determine the D or L configuration. The isomeric alcohol was also confirmed by ^{1}H and ^{13}C NMR (500 MHz Bruker Advance III NMR spectrometer).

2.7. Galactitol catalysis by NAD-dependent galactitol dehydrogenase of Aspergillus niger (AnLadB)

It was reported that AnLadB of *A. niger* can oxidize 3-OH of galactitol to L-xylo-3-hexulose [15], which was supposed to be the same oxidation product of galactitol by *G. oxydans* 621H or *G.cerinus* X512. Thus, the NAD-dependent galactitol dehydrogenase gene (*LadB*, NT_166531, ORF: 1077 bp) of *Aspergillus niger* was in-vitro synthesized and ligated to the *Nco*I-*Xho*I site yielding plasmid pET28a-AnladB. The resultant plasmid was transformed into *E. coli* BL21 (DE3) for expression of *A. niger LadB* gene. The AnLadB was induced, purified, and used as an enzyme to catalyze galactitol oxidation. A 500 μL reaction mixture comprising 50 μL purified AnLadB (5 mg/mL), 250 μL 100 mM galactitol, 50 μL 50 mM NAD, and 150 μL 30 mM Na-phosphate buffer (pH 8.0), was mixed and incubated at 30 °C for 24 h. The reaction product was collected by HPLC preparation, concentrated, and further applied to GC/MS analysis, comparing with that of the reaction product of galactitol oxidized by *G. cerinus* X512.

2.8. GC/MS comparison analysis of reaction products produced by AnLadB and G. cerinus

The galactitol oxidation product by purified AnLadB and the unknown product produced by *G. cerinus* X512 were dissolved in pyridine and then converted into trimethylsilyl derivatives by treatment with derivatizing reagent BSTFA (N, O-Bis (trimethylsilyl) trifluofoacetamine) and heated at 80 °C for 50 min. Then 1 μL of derivatized aliquot was injected into the GC/MS. All GC/MS experiments were performed on Agilent gas chromatography coupled with an Agilent ion trap mass spectrometer (Agilent 6850/5975C). The analytical column was an HP5-MS column (30-m capillary column, 0.25 μm film thickness) with highly pure helium as the carrier gas at a flow of 1 mL/min. After separation by the column, the ionized sample was analyzed by a mass selective detector (MSD). The temperature program was as follows: initially, 60 °C held for 1 min, increased to 280 °C at 60 °C/min and held for 5 min, and then increased to 30 °C at 20 °C/min and held for 2.5 min. Peak identification was achieved by comparing the retention times with the internal standard in the SIM mode (Selected Ion Monitoring).

2.9. Preparation of Gluconobacter cells membrane fractions

The *G. oxydans* 621H strains were grown in complex medium. Cells were collected by centrifugation (at 10,000 *g* for 10 min), washed twice with potassium phosphate buffer (PBS, 10 mM, pH 6.0), and resuspended in the same buffer. The cells of *G. oxydans* 621H strains were disrupted through a continuous high-pressure cell disrupter. After centrifugation at 10,000 *g* for 20 min at 4 °C to eliminate intact cells or cell debris, the supernatants (crude extract) were centrifuged at 200,000 *g* for 1 h, yielding soluble cytoplasmic supernatant and pellets (membrane fraction). The resulting pellets were washed in the same buffer but containing 1% Triton X-100 and 1 mM PMSF and centrifuged again (at 200,000 *g*), then were resuspended in the same buffer containing 1% Triton X-100 and 1 mM PMSF, and used as the membrane fractions to catalyze D-sorbitol, galactitol, or L-fucitol oxidation. The protein concentrations were quantified by Bradford assay using bovine serum albumin (BSA) as a standard.

2.10. Effects of EDTA and PQQ on the activity of membrane-bound galactitol dehydrogenase

To evaluate the effect of EDTA on membrane-bound galactitol dehydrogenase activities, the native membrane fraction prepared at 200,000 g was treated with 10 mM EDTA for 5 h at 4 °C. Excess EDTA was removed by centrifugation at 200,000 *g* for 60 min, and the enzyme activity was measured. The reaction mixture (100 μL) contains 20 μL 10 mM PBS buffer (pH 6.0), 40 μL 50 $g{\cdot}L^{-1}$ galactitol (the final concentration was 20 $g{\cdot}L^{-1}$), 10 μL 10 mg/mL EDTA treated or non-treated membrane proteins, 10 μL 1 mM DCPIP, 10 μL 6.7 mM PMS and 10 μL 40 mM sodium azide, or in the presence of PQQ, Ca^{2+}, and PQQ plus Ca^{2+}, each at final concentration of 0.5 mM and 1 mM, and reacted at 30 °C for 48 h. The dehydrogenase activities were measured at 600 nm, as described by Hölscher et al. [16]. One unit of membrane-bound dehydrogenase activity was defined as the quantity of enzyme that reduces 1 μmol DCPIP min^{-1}, corresponding to the oxidation of 1 μmol substrate min^{-1}. The reaction products were also analyzed by HPLC.

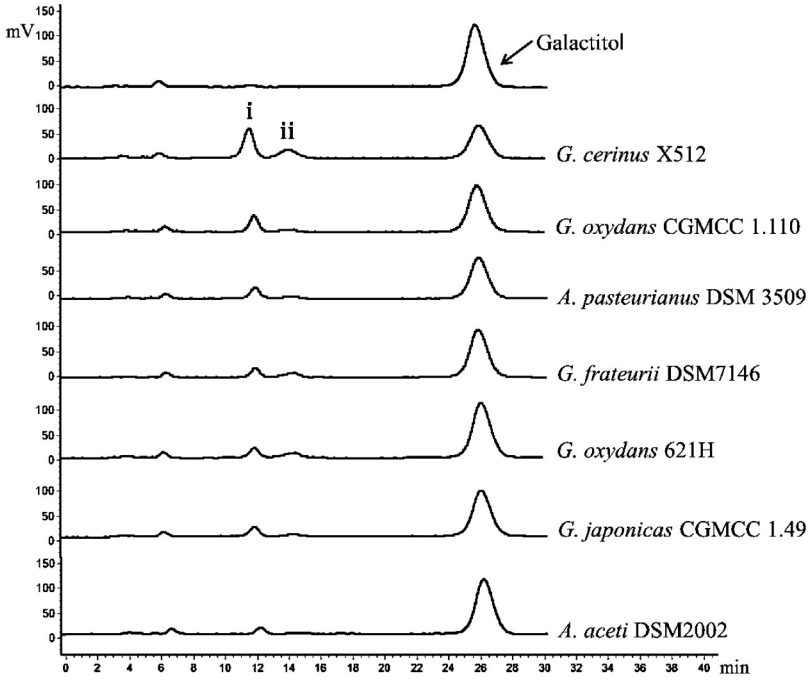

Fig. 2. The HPLC profiles of galactitol biotransformation by various acetic acid bacteria.
All acetic acid bacteria tested produced two kinds of products with various efficiency (indicated with i and ii). The seven bacterial strains were first cultivated for 60 h at 30 °C and 150 rpm in complex medium containing 15 g/L yeast extract, 3 g/L tryptone, supplemented with 50 g/L glucose and 15 g/L calcium carbonate. After cultivation, the washed cells were used for the biotransformation of galactitol (40 g/L) in 20 mL reaction mixture at 30 °C, and 150 rpm with a final cell density of OD_{600} 5.0 (1 mL 1 OD_{600} is approximate 0.32 g dry cell weight, thus 20 mL OD_{600} is equal to 32 mg DCW). *G. cerinus* X512 has the highest potential to produce these two products.

2.11. Activity comparison using NAD or PMS/DCPIP as the electron acceptor for membrane and soluble cytoplasmic fractions

To determine whether the prepared membrane fractions of *G. oxydans* 621H have dehydrogenase activity on D-sorbitol and galactitol, when using NAD or PMS/DCPIP as the electron acceptor, 10 μL 10 mg/mL membrane proteins, 40 μL 50 $g{\cdot}L^{-1}$ D-sorbitol or galactitol (the final concentration of sugar alcohols was 20 $g{\cdot}L^{-1}$), 10 μL 5 mM PQQ, 10 μL 10 mM Ca^{2+}, 10 μL 1 mM DCPIP, 10 μL 6.7 mM PMS and 10 μL 40 mM sodium azide were mixed and reacted at 30 °C for 48 h. For comparison, NAD at a final concentration of 5 mM was used as an electron acceptor instead of PMS/DCPIP.

On the other hand, dehydrogenase activity of soluble cytoplasmic fractions on D-sorbitol and galactitol was also assayed using NAD or PMS/DCPIP as the electron acceptor. The 100 μL reaction mixture contained 20 μL 10 mM PBS buffer (pH 6.0), 10 μL 10 mg/mL soluble cytoplasmic proteins, 40 μL 50 $g{\cdot}L^{-1}$ D-sorbitol or galactitol (the final concentration of sugar alcohols was 20 $g{\cdot}L^{-1}$), 10 μL 10 mM Ca^{2+}, 10 μL 40 mM sodium azide, 10 μL 50 mM NAD, and reacted at 30 °C. As a comparison, PQQ, PMS, and DCPIP at a final concentration of 0.5 mM, 0.67 mM, and 0.1 mM were used as the electron acceptor instead of NAD.

When using PMS/DCPIP as the electron acceptor, the activity was determined by measuring the decrease in absorbance of DCPIP at 600 nm using PMS as a redox mediator as described by Hölscher et al. [16], or by measuring the increase in absorbance of NAD at 340 nm. Reactions were initiated by the addition of substrate, and absorbance values in the first 3 min were recorded. One unit of dehydrogenase activity was defined as the quantity of enzyme that reduces 1 μmol DCPIP or NAD min^{-1}, corresponding to the oxidation of 1 μmol substrate min^{-1}. The specific activity was calculated using a molar extinction coefficient for DCPIP of 17.2 mM^{-1} cm^{-1} at 600 nm and pH 6 [16], and for NAD of 6.22 mM^{-1} cm^{-1} at 340 nm and pH 6. The reaction products were quantified by HPLC.

2.12. Galactitol catalysis by pqqA knockout mutant of G. oxydans 621H

The *pqqA* knockout mutant of *G. oxydans* 621H was constructed according to the strategy of Hölscher and Görisch [17]. The *pqqA* knockout mutant was cultivated in a complex medium containing 20 $g{\cdot}L^{-1}$ D-glucose. When OD_{600} reaches 1.5, the cells were harvested, washed with PBS buffer (10 mM, pH 6.0), and disrupted to obtain crude extract. The membrane fraction was obtained by centrifugation (at 200,000 *g* for 60 min), washed with 10 mM PBS buffer (pH 6.0), centrifuged again (at 200,000 *g* for 60 min), and resuspended with the same buffer containing 1% Triton X-100 and 1 mM PMSF. The membrane protein concentration was determined and used to react with L-fucitol (20 g/L) and galactitol (20 g/L) at a final concentration of 1 $mg{\cdot}mL^{-1}$ membrane protein. For reconstitution of quinoprotein apoenzymes to the holoenzyme, membrane fractions of PQQ-deficient *G. oxydans* 621H were incubated with PQQ, Ca^{2+}, and PQQ plus Ca^{2+}, each at a final concentration of 0.5 mM and 1 mM, for 1 h at 4 °C, and then used as an enzyme to react with galactitol or L-fucitol. The reaction mixture (100 μL) contains 20 μL 10 mM PBS buffer (pH 6.0), 40 μL 50 $g{\cdot}L^{-1}$ galactitol or L-fucitol (the final concentration was 20 $g{\cdot}L^{-1}$), 10 μL 10 mg/mL apoenzyme or holo-enzyme, 10 μL 1 mM DCPIP, 10 μL 6.7 mM PMS and 10 μL 40 mM sodium azide, or in the presence of PQQ, Ca^{2+}, and PQQ plus Ca^{2+}, each at a final concentration of 0.5 mM and 1 mM, and reacted at 30 °C. The dehydrogenase activities were measured as described above.

2.13. HPLC analytical method

The polyol and ketone compounds were separated and quantified by HPLC coupled with a Shodex SP0810 ion exclusion column (300 mm × 8 mm), and a refractive index detector (Shodex RI101), using distilled water as the mobile phase at a flow rate of 1.0 $mL{\cdot}min^{-1}$ at 70 °C.

3. Results

3.1. Oxidation of galactitol by resting cells of Acetobacter and Gluconobacter strains

The acetic acid bacteria *Acetobacter* and *Gluconobacter* are well characterized by their ability to oxidize various sugars and sugar alcohols by cytoplasmic soluble or membrane-bound dehydrogenases using PQQ, FAD, or NAD(P) as cofactors [1–6]. It was reported that

Table 2
Galactitol conversion rate by various acetic acid bacteria.

Strains	Conversion rate[a] of D-tagatose	conversion rate of L-xylo-3-hexulose	Ratio of L-xylo-3-hexulose to D-tagatose	Total conversion rate	Production rate[b] of L-xylo-3-hexulose
Gluconobacter cerinus X512	0.129	0.285	2.21	0.414	3.56
Gluconobacter oxydans CGMCC 1.110	0.039	0.142	3.64	0.181	1.78
Acetobacter pasteurianus DSM 3509	0.044	0.133	3.02	0.177	1.66
Gluconobacter frateurii DSM7146	0.078	0.089	1.14	0.167	1.11
Gluconobacter oxydans 621H	0.071	0.085	1.19	0.156	1.06
Gluconobacter japonicus CGMCC 1.49	0.042	0.104	2.48	0.146	1.3
Acetobacter aceti DSM2002	0.011	0.055	5	0.065	0.69

[a] Conversion rate: the amount of product to the amount of substrate galactitol (g/g).
[b] Production rate: the amount of product (g) produced by per g dry cell biomass per hour per liter medium (g/L·h·g dry biomass).

galactitol can be oxidized into D-tagatose by *G. oxydans* [18]. At the first purpose, we screened acetic acid bacteria with the best performance to synthesize D-tagatose from galactitol by the whole-cell transformation. However, we found that all acetic acid bacteria selected can produce another unknown product in addition to D-tagatose (Fig. 2). It attracts our interest to investigate their efficiency to produce this unknown product from galactitol. The highest total conversion rate of 0.414 was observed for *G. cerinus* X512, followed by *G. oxydans* CGMCC1.110 (0.181) and *A. pasteurianus* DSM 3509 (0.177). The most commonly used strain *G. oxydans* 621H showed a conversion rate of 0.156, while the lowest yield of 0.065 was recorded by *A. aceti* DSM2002 (Table 2). Among these acetic acid bacteria, *G. cerinus* X512 resting cells yielded the three-fold higher production rate of the unknown product than that to *G. oxydans* 621H, with 3.56 g/L·h·g dry biomass to 1.06 g/L·h·g dry biomass. HPLC profiles of galactitol biotransformation by various acetic acid bacteria further corroborated that *G. cerinus* X512 has the highest production yield (Fig. 2). The strain *G. cerinus* X512 was isolated from sludge in the acetic acid production plant and identified as *G. cerinus* by its 16S rDNA sequences compared with those of other known acetic acid bacteria. The isolated strain was deposited in China General Microbiological Culture Collection Center with an accession number CGMCC19520. Based on those rDNA sequences, the phylogenetic tree was constructed that showed a close relationship with other known *G. cerinus* (Fig. S3). Due to the higher conversion and production rate of the unknown product, *G. cerinus* X512 was used to prepare the unknown product from galactitol for further identification.

3.2. Biorefinery process to improve the purity of the unknown product

After biotransformation of *G. cerinus* X512 in complex medium, the purity of the unknown product was around 28.5%. The main byproduct, namely D-tagatose and residual substrate galactitol should be removed to recover the pure unknown product. It is challenging to separate the unknown product and D-tagatose due to their similar retention time. Therefore, biotransformation supernatant of *G. cerinus* X512 was used as a substrate for the different yeasts to eliminate these two undesired compounds (D-tagatose and galactitol). The yeast X535 strain was selected because of its ability to utilize galactitol and D-tagatose but not the unknown product. Then the yeast X535 was identified as *Candida* sp. by its 26S rDNA sequence and deposited in China General Microbiological Culture Collection Center with an accession number CGMCC3268. This yeast can utilize glycerol, erythritol, xylitol, ribitol, D-arabitol, galactitol, mannitol, sorbitol, xylose, L-arabinose, D-tagatose, as well as glucose. As depicted in Fig. 3, X535 yeast cells first consumed D-tagatose within 24 h followed by galactitol after 60 h of culture. At this time, the unknown product purity was recorded to be 95% from the HPLC chromatogram. Then it was further purified through ion-exchange chromatography and identified by the following methods.

3.3. Identification of the unknown product

The unknown compound might be the biochemical oxidation product by dehydrogenation of the hydroxyl group at C1, C2, C3, C4, C5, or C6 position. Provided that dehydrogenation was taken place at C1 or C6, the unknown compound is L-galactose or D-galactose. If dehydrogenation was taken place at C2 or C5, the unknown compound is ketose L-tagatose or D-tagatose. However, the retention times of L-galactose, D-galactose, L-tagatose, or D-tagatose were different from that of the unknown compound, indicating that dehydrogenation was not taken place at C1, C2, C5, or C6, but might be dehydrogenated at C3 or C4, yielding a 3-ketose or 4-ketose, respectively. No discoloration by the addition of bromine further indicates that the unknown compound was a ketose (inset in Fig. 4B). The purified unknown product (Fig. 4B) was reduced by $NaBH_4$. Due to no stereoselectivity of chemical hydrogenation, the carbonyl compound would be expected to yield galactitol and another isomeric alcohol at the oxidized C-OH position in the ratio of approximately 1:1. The $NaBH_4$ reduction products were galactitol and the isomeric alcohol of galactitol (Fig. 4C, iii), which were then isolated by HPLC (Fig. 4D). The HPLC isolated isomeric alcohol of galactitol was then identified by different methods. First, the HPLC retention time was found the same as that of authentic D-sorbitol and L-sorbitol (Fig. 4E). Then the isomeric alcohol of galactitol was further identified by optical rotation, ^1H NMR, and ^{13}C NMR. The specific optical rotation was -2.14^o, which is identical to authentic D-sorbitol, indicating its D-configuration. The main signals in the ^1H NMR and ^{13}C NMR of the isomeric alcohol of galactitol were identical with authentic D-sorbitol (Fig. S4). These results strongly suggested that the isomeric alcohol of galactitol, ultimately reconfirming as D-sorbitol. The reducing products were galactitol and D-sorbitol, which enabled us to deduce that the carbonyl compound oxidized from galactitol by acetic acid bacteria was L-xylo-3-hexulose. If the OH group at the C4 position of galactitol was oxidized, the reducing products by $NaBH_4$ should be galactitol and L-sorbitol, which specific optical rotation was $+2.14^o$.

To further confirm that the oxidation product of galactitol by *G. cerinus* X512 was authentic L-xylo-3-hexulose, the GC/MS spectra of both oxidation products of AnLadB enzyme and *G. cerinus* X512 were compared (Fig. S5). It showed that the BSTFA-derivatized galactitol oxidation products by *G. cerinus* X512 yielded the same characteristic mass spectrum ion fragments to the authentic L-xylo-3-hexulose produced by AnLadB. These combined results indicated that the unknown product produced by *G. cerinus* X512 from galactitol was L-xylo-3-hexulose, which is hard to be crystallized and keep syrup through concentration.

3.4. D-tagatose and L-xylo-3-hexulose can be produced by membrane-bound dehydrogenase(s) and the supernatants fractions

To determine whether the galactitol oxidation was mainly taken place in the membrane or supernatant, the disrupted crude cell extract

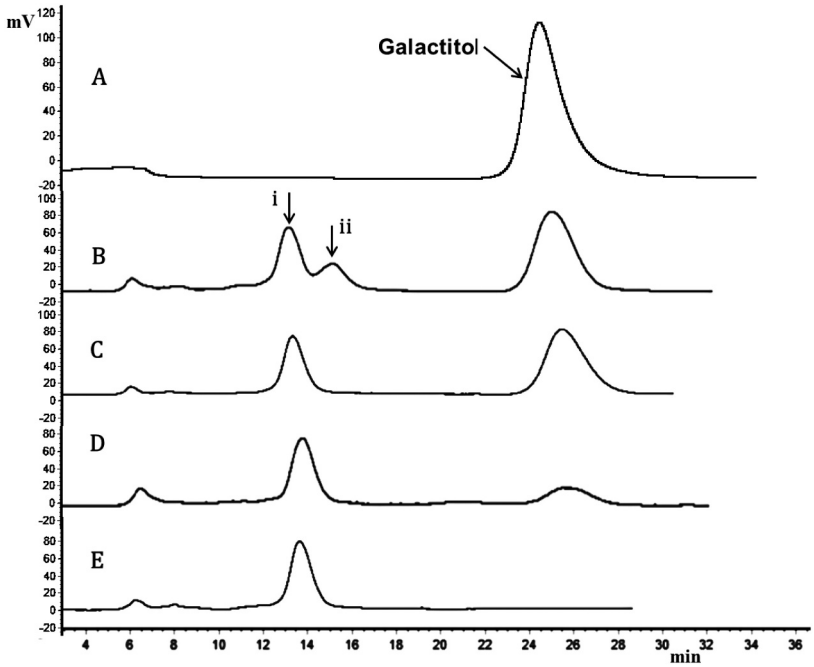

Fig. 3. The purity changes of unknown product during biodegradation by yeast *Candida* sp.
A, 20 g/L galactitol at the beginning of biotransformation; B, 60 h biotransformation by *G. cerinus* X512 with cell concentration at 1.5 g/L DCW, unknown product (i) and D-tagatose (ii) were produced; C, D-tagatose was first utilized by the yeast *Candida* sp.; D, after D-tagatose was exhausted, galactitol was then consumed; E, after D-tagatose and galactitol were exhausted, the main unknown product reached up to 95% purity.

was ultra-centrifuged (at 200,000 *g* for 1 h), and the supernatants and membrane fractions were used as crude enzymes to catalyze galactitol oxidation. With 20 g/L galactitol, more L-xylo-3-hexulose and D-tagatose were produced by membrane fractions than that of the supernatant fractions. The total production rate of L-xylo-3-hexulose and D-tagatose was 0.52 g/L·h·g for membrane fractions protein, and 0.21 g/L·h·g for supernatant fractions protein, 2.5-fold higher than that of supernatant fractions protein.

Comparing to the reaction mixture without supplement of PQQ and Ca^{2+} (Fig. 5A), the membrane reaction mixture produced more L-xylo-3-hexulose and D-tagatose (inset in Fig. 5B) by addition of PQQ and Ca^{2+} to a final concentration of 0.5 mM and 1 mM (Fig. 5B). The total production rate of L-xylo-3-hexulose and D-tagatose increased to 0.94 g/L·h·g from 0.52 g/L·h·g, about 2-fold higher than that of without the addition of PQQ and Ca^{2+}. When using supernatant fraction as an enzyme source, the amount of L-xylo-3-hexulose and D-tagatose were decreased after treatment with 10 mM EDTA (Fig. 5C,D). The total production rate of L-xylo-3-hexulose and D-tagatose reduced to 0.08 g/L·h·g from 0.21 g/L·h·g.

These results indicated that galactitol could be oxidized to L-xylo-3-hexulose and D-tagatose by both membrane and supernatant fractions, PQQ and EDTA have a significant effect on L-xylo-3-hexulose and D-tagatose production. So, we further determined the effects of EDTA and PQQ on membrane galactitol dehydrogenase activity.

The galactitol dehydrogenase activity of native membrane fractions was around 0.026 U/mg protein, while the activity was increased to 0.057 U/mg protein by the addition of PQQ at the final concentration 0.5 mM to replenish the PQQ loss during the membrane preparation by ultra-centrifugation at 200,000 *g*. Addition of 1 mM Ca^{2+} plus 0.5 mM PQQ only resulted in a slight increase than that of 0.5 mM PQQ addition. Whereas the membrane-bound galactitol dehydrogenase activity was reduced by 93% after treatment with 10 mM EDTA (Fig. 6, pink

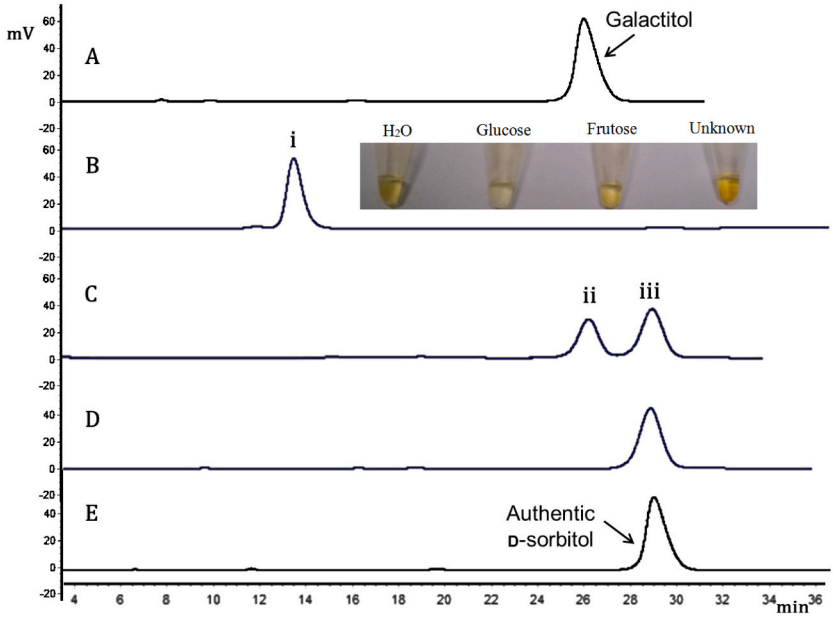

Fig. 4. Identification of the galactitol transformation products by chemical reduction with $NaBH_4$.
A, HPLC profile of authentic galactitol, retention time 25.99 min; B, HPLC profile of the purified unknown product (i). C, chemically reduced product by $NaBH_4$, resulting in two polyols, one is galactitol (ii), and the other polyol (iii) with the same retention time to D-sorbitol (29.1 min). D, isolated polyol (iii) with the same retention time to D-sorbitol. E, the authentic D-sorbitol. By addition of bromine into the unknown product, no discoloration was observed as the same to that of fructose (was still bromine colour), whereas discoloration was observed for glucose due to oxidation of bromine by aldehyde group in glucose, indicating that the unknown compound was a ketose (inset in Figure).

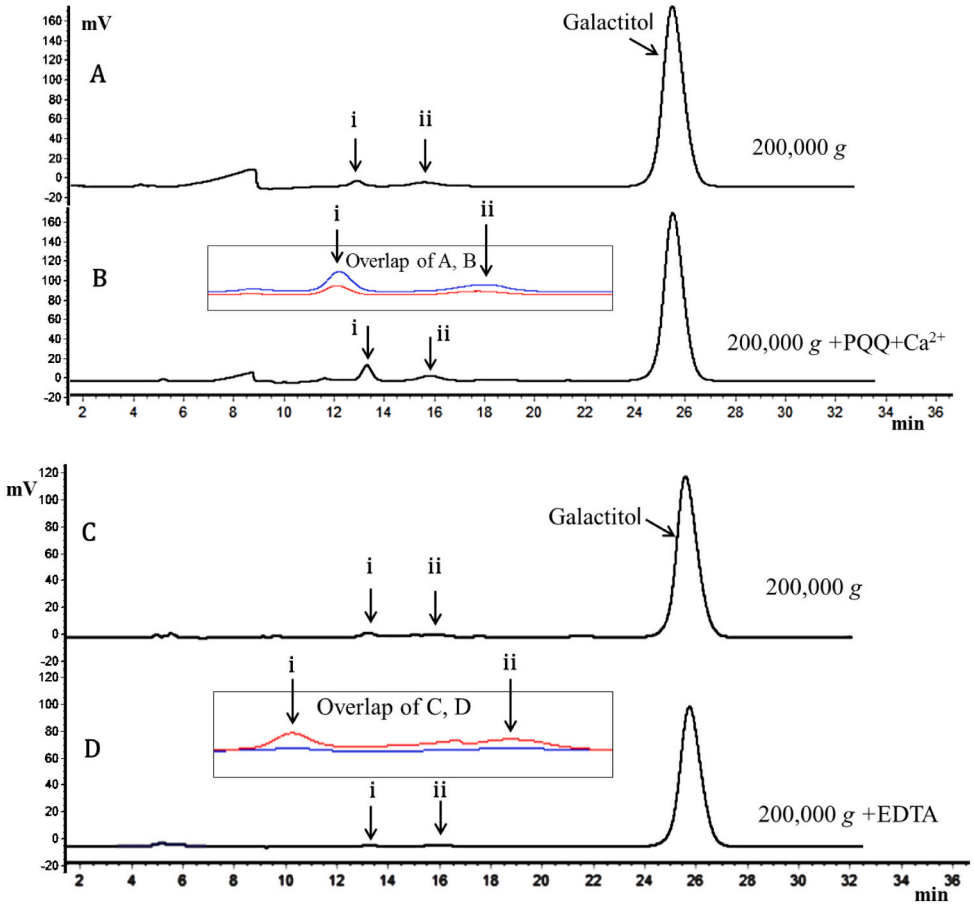

Fig. 5. Effects of PQQ and EDTA on products formation.

For A and B, membrane-bound fractions used were obtained by centrifugation 200,000 *g*, then were used as catalyst to react on galactitol. A, the reaction product with membrane fraction; B, the reaction product with membrane fraction by the addition of PQQ and Ca^{2+} at a final concentration of 0.5 mM and 1 mM respectively; The inset is the zoom and overlap of A and B. Red line, A. Blue line, B. For A, the reaction mixture (100 μL) contains 50 μL 10 mM potassium phosphate buffer (pH 6.0), 40 μL 50 $g{\cdot}L^{-1}$ galactitol (the final concentration was 20 $g{\cdot}L^{-1}$), 10 μL 10 mg/mL membrane proteins, reacted at 30 °C for 48 h. For B, the reaction mixture (100 μL) contains 30 μL 10 mM potassium phosphate buffer (pH 6.0), 40 μL 50 $g{\cdot}L^{-1}$ galactitol (the final concentration was 20 $g{\cdot}L^{-1}$), 10 μL 10 mg/mL membrane proteins prepared at 200000 g, 10 μL 5 mM PQQ and 10 μL 10 mM Ca^{2+}, reacted at 30 °C for 48 h. Then the reaction products were analyzed by HPLC.

For C and D, *G. oxysans* 621H cells crude extract was centrifuged at 200,000 *g*, and supernatants were used as a crude enzyme to catalyze 20 g/L galactitol at 30 °C for 48 h. C, HPLC analysis of reaction product with supernatant; D, HPLC analysis of reaction product with supernatant by the addition of 10 mM EDTA. The inset is the overlap and zoom of C and D. Red line, C profile. Blue, D profile. Addition of 10 mM EDTA decreased product formation, indicating the enzyme in supernatant oxidizing galactitol to L-xylo-3-hexulose and D-tagatose might from the soluble membrane fraction.

column), which converts quinoprotein galactitol dehydrogenase into apoenzyme form. When PQQ, Ca^{2+}, or both were added, their activities were significantly increased from 0.002 U/mg to 0.037, 0.045, and 0.064 U/mg (Fig. 6). These results revealed the PQQ -dependent nature of membrane-bound galactitol dehydrogenase.

3.5. Activity determination using NAD or PMS/DCPIP as the electron acceptor for membrane and soluble cytoplasmic fractions

The activities of the soluble cytoplasmic and the membrane fraction prepared at 200,000 g with PMS/DCPIP and NAD as electron acceptors were determined using galactitol and D-sorbitol as substrates, respectively, and the results are shown in Fig. 7. When using galactitol as

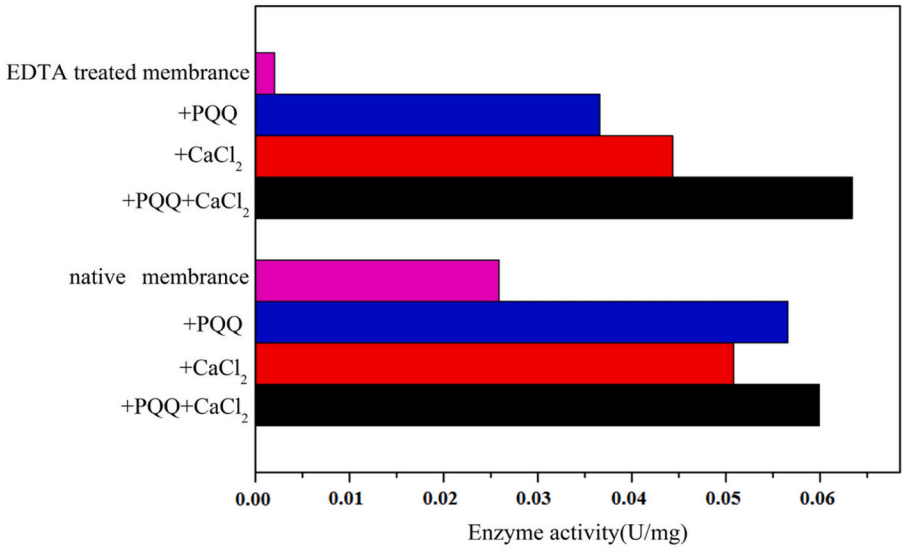

Fig. 6. Effects of EDTA and PQQ on membrane-bound galactitol dehydrogenase activities.

When EDTA was added to the reaction mixture, the dehydrogenase activity using galactitol as substrate was decreased, while the activity can be restored when adding PQQ or/Ca^{2+}. The results showed the membrane-bound galactitol dehydrogenase activity was inhibited when treated with EDTA and was PQQ dependent.

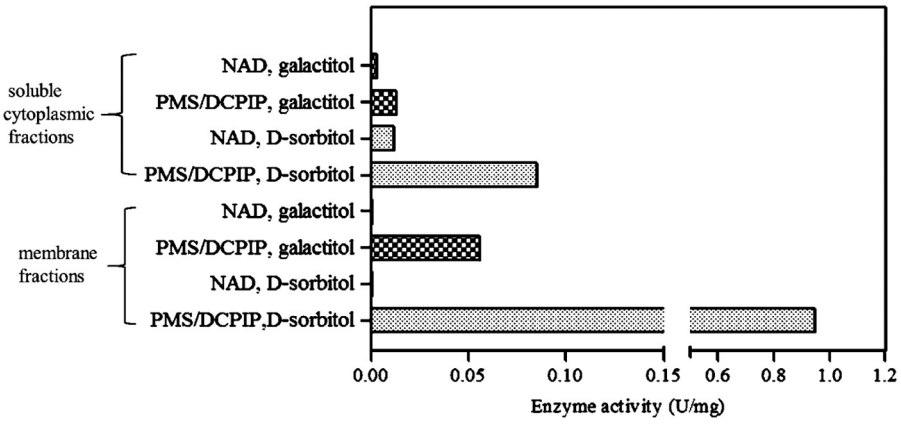

Fig. 7. Effects of different electron acceptors on the activities of soluble cytoplasmic and membrane-bound fractions.
When using NAD as an electron acceptor, the membrane fractions prepared at 200,000 g only have background activity whatever using galactitol or D-sorbitol as substrates. Whereas using PMS/DCPIP as the electron acceptor, the membrane fractions have strong activities using galactitol or D-sorbitol as substrates. For soluble fractions, the activities can be detected using NAD or PMS/DCPIP as the electron acceptor and galactitol or D-sorbitol as substrates. The results indicated that the main dehydrogenases in the membrane fractions utilized PMS/DCPIP as electron acceptor and were PQQ-dependent.

substrate and PMS/DCPIP as an electron acceptor, the detected activity was 0.056 U/mg for membrane fraction proteins. On the other hand, the activity was only 0.001 U/mg of membrane fraction proteins using NAD as an electron acceptor, almost the same as the background activity without the addition of NAD. This indicates that the membrane enzyme activity can only use PMS/DCPIP as the electron acceptor rather than NAD.

By using a soluble fraction of cytoplasm as an enzyme source in the presence of PMS/DCPIP as the electron acceptor and galactitol as substrate, the activity decreased to 0.013 U/mg for soluble fraction protein, only one-fourth of the activity of membrane fraction proteins. When using NAD as an electron acceptor and galactitol as substrate, the galactitol dehydrogenase activity can be detected (0.003 U/mg), although only one-fourth of activity to that of using PMS/DCPIP as an electron acceptor. The results indicate that the soluble fraction contains the NAD-dependent galactitol dehydrogenase, which might oxidize galactitol to D-tagatose, which was similar to that of early reported by Rollini and Manzoni [19].

The membrane fractions in acetic acid bacteria, such as *G. suboxydans* contain PQQ-dependent dehydrogenases, which can efficiently oxidize D-sorbitol to L-sorbose [20]. The soluble cytosolic fraction contains the NAD-dependent dehydrogenases, which can oxidize D-sorbitol to D-fructose [21]. For comparison, D-sorbitol was used as a substrate instead of galactitol to assay the dehydrogenase activity, using PMS/DCPIP or NAD as an electron acceptor, membrane fractions, and soluble cytosolic fractions as crude enzymes. In the presence of soluble fraction as enzyme source and PMS/DCPIP and NAD as an electron acceptor, the sorbitol dehydrogenase activity was 0.085 and 0.012 U/mg, respectively, indicating that the soluble fraction of enzyme contains both PQQ-dependent and NAD-dependent sorbitol dehydrogenase, mainly PQQ-dependent one. Using the membrane fractions as an enzyme source, and PMS/DCPIP as an electron acceptor, the activity was 0.95 U/mg, 15-fold higher than the activity using galactitol as a substrate. Only background activity was determined using NAD as an electron acceptor, indicating that the PQQ-dependent D-sorbitol dehydrogenase was the main dehydrogenase in the membrane fractions.

3.6. pqqA gene deletion resulting in no galactitol oxidation

The *pqqA* gene was deleted by homologous replacement to determine the PQQ-dependent activity of membrane-bound galactitol dehydrogenase. The mutant was stretched five times on mannitol solid medium to dilute residual PQQ originated from the wild type cells before electroporation [17]. The obtained *pqqA* mutant was designated as GOX-621H-CHR1. Hölscher and Görisch reported that *pqqA* mutant could not grow on a complex medium containing D-sorbitol, D-mannitol, glycerol, and glucose, but able to slowly grow on D-gluconate [17]. However, in contrast to the result obtained by Hölscher and Görisch [17], the *pqqA* mutant GOX-621H-CHR1 can grow on complex medium containing glucose but with some slower growth rate (0.05 h^{-1} to 0.22 h^{-1} of wild type). On the complex medium plate containing glucose and $CaCO_3$, the *pqqA* mutant cannot oxidize glucose to gluconate, hence unable to dissolve $CaCO_3$ to form a transparent circle, but the wild type can form a transparent circle (Fig. S6). *G. oxydans* has two pathways to acquire energy from glucose, one is to incompletely oxidize glucose to gluconate by a PQQ-dependent glucose dehydrogenase [17,22], the other is to form acetate from glucose via glycolysis in which pyruvate was converted to acetaldehyde by pyruvate decarboxylase (PDC) and then oxidized to acetate by acetaldehyde dehydrogenase (AcDH) with the generation of a molecular NADPH [23], or to form keto-gluconate from glucose by cytoplasmic soluble $NADP^+$-dependent glucose dehydrogenase [24]. Though the PQQ-dependent glucose dehydrogenase gene was deleted, *G. oxydans* could retain growth on glucose [22].

The *pqqA* mutant was grown on a complex medium containing 20 g/L D-glucose, and the membrane fraction was prepared by ultracentrifugation at 200,000 *g* for 60 min. The isolated membrane fraction and supernatant were applied to react with galactitol and 6-deoxy-L-galactitol (L-fucitol). In *pqqA* mutant membrane or supernatant, both the activity of galactitol or L-fucitol dehydrogenase was absent, and no L-xylo-3-hexulose and D-tagatose or L-fuco-4-ketose were detected by HPLC. When *pqqA* mutant membrane fraction was incubated with 0.5 mM PQQ and/or 1 mM Ca^{2+} to reconstitute quinoprotein apoenzyme to holoenzymes, galactitol dehydrogenase activity can be restored to about the half of those of wild type membrane (Fig. 8) and produce L-xylo-3-hexulose and D-tagatose or L-fuco-4-ketose (Fig. 9).

4. Discussion

In recent years, rare sugars received great interest in nutrition, fine chemicals, and medicine. For instance, D-mannose has recently been found to be an excellent rare sugar that impairs several tumors growth and enhances cell death in response to chemotherapy [25]. D-allose induces programmed cell death in prostate cancer and human retinal progenitor *cells* (hRPC) by modulating the intrinsic apoptotic pathway [26]. D-tagatose, another important rare sugar, plays a significant role in healthy foods, management of type 2 diabetes, and the ingredient in drug manufacture [27]. With the original purpose of producing D-tagatose from galactitol according to the strategy of Manzoni [18] and Rollini [19], we found unexpectedly that L-xylo-3-hexulose, a 3-ketose, was produced as well as 5-ketose D-tagatose from galactitol by acetic acid bacteria (Fig. 1, Fig. 2). Recently, 3-ketose sugars have garnered attention owing to their potential value as building blocks in the synthesis of branched-chain sugars, amino sugars, and sugar nucleotides [28–30].

It was found that the acetic acid bacterium such as *Gluconobacter* sp. and *Acetobacter* sp. possess the ability to incompletely oxidize various sugar alcohols regiospecificity at the C-2 position to the corresponding

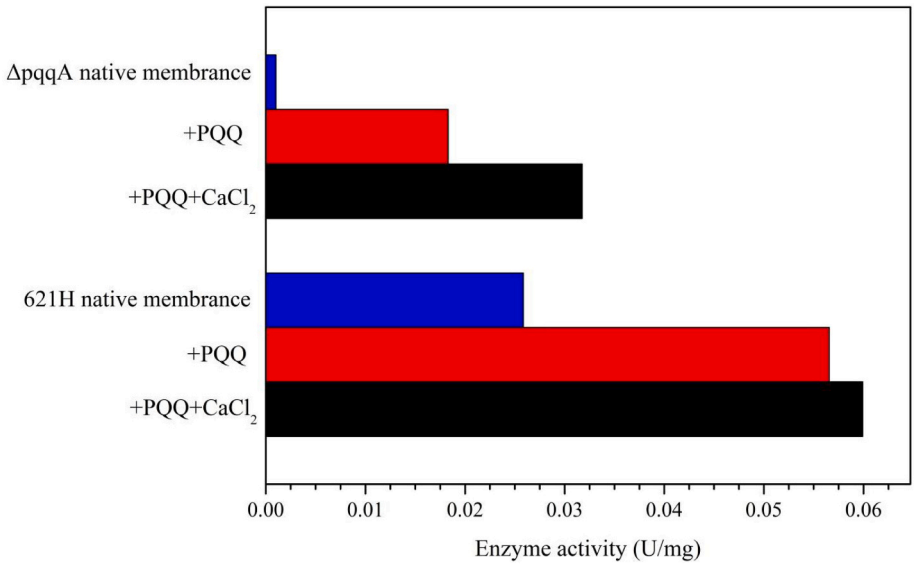

Fig. 8. Comparison of membrane-bound activities of galactitol dehydrogenase prepared from *pqqA* gene disruption mutant and wild type of *G. oxydans* 621H. When *pqqA* gene was disrupted, the membrane-bound galactitol dehydrogenase activity reduced to the background level, and can be restored at some extent by addition of PQQ with or without Ca^{2+}.

2-ketose (for example, D-mannitol to D-fructose, D-sorbitol to L-sorbose, D-arabitol to D-xylulose, etc.) by using membrane-bound dehydrogenases in the presence of PQQ as a cofactor. These reactions obey the well-known Bertrand-Hudson's rule [10,31,32], in which polyols exhibiting D-*erythro* configuration are oxidized at the secondary alcohol group generating 2-ketose products within the pH 5–6.5 (Fig. S1a). No hydroxyl group at the C3 position of polyols (for example, hexitol, pentitol) being regio-specifically oxidized by acetic acid bacteria was observed so far in the literature. However, we found that galactitol can be spontaneously oxidized at the C5 and C3 position hydroxyl group to 5-ketose D-tagtose and 3-ketose L-xylo-3-hexulose by PQQ-dependent membrane-bound dehydrogenase in acetic acid bacteria, and do not follow the Bertrand-Hudson's rule.

Manzoni [18] and Rollini [19] reported D-tagatose production from galactitol by three *Acetobacter* sp. and five *Gluconobacter* sp. including *G. oxydans* NCIB 621 and DSM 2343, using either resting cells or growing cells, and achieved the highest D-tagatose titer of 4.4 g/L from 22.5 g/L galactitol using DSM 2343. Notably, no possible by-products such as galactose or fructose were detected in any of the samples using the Merck Polyspher OA-KC column at room temperature. However, 3-ketose L-xylo-3-hexulose along with D-tagatose was detected in all the tested samples, including the sample of DSM 2343 by HPLC using a shodex column SP0810 eluted at 1 mL/min with distilled water at 70 °C (Fig. 2). The reason why Manzoni [18] and Rollini [19] only detected

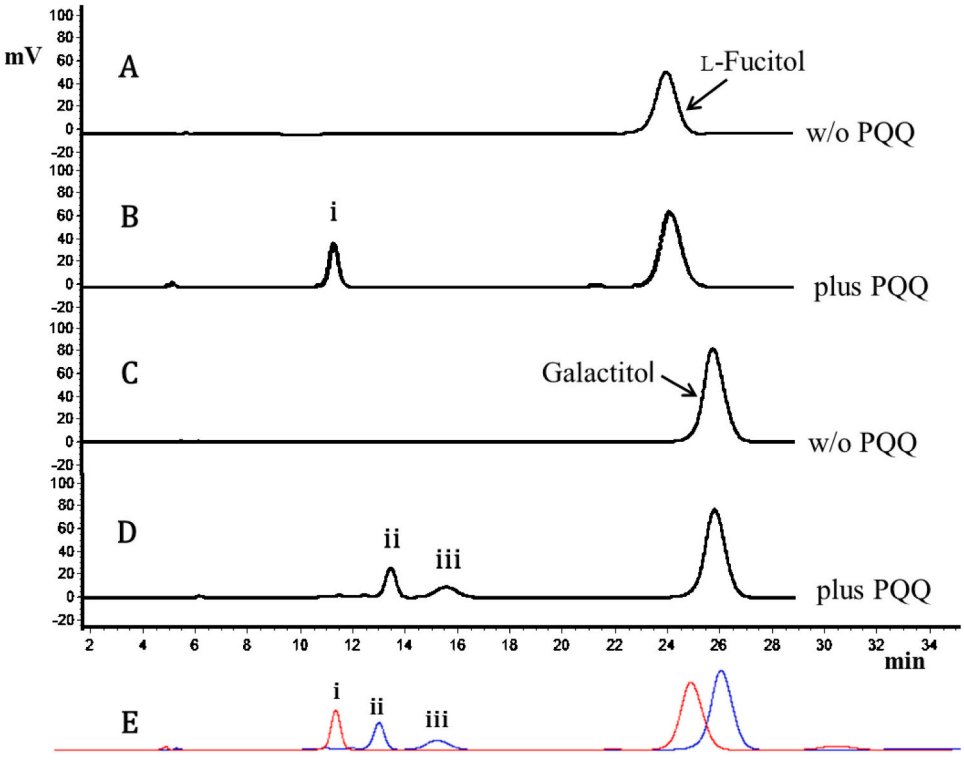

Fig. 9. PQQ effect on reaction products with membrane fraction of *pqqA* knockout mutant. Membrane fraction of mutant *G. oxydans* 621H with *pqqA* deficiency was obtained by centrifugation at 200,000 *g* for 60 min, and washed with 10 mM potassium phosphate sodium buffer (pH 6.0) containing 1% Triton X-100, centrifugation again at 200,000 *g* for 60 min to obtain membrane fraction, and resuspended with the same buffer and was used to react on L-fucitol and galactitol. A, *pqqA* knockout mutant membrane fraction act on L-fucitol without the addition of PQQ, no product L-fuco-4-ketose was produced; B, *pqqA* knockout mutant membrane fraction act on L-fucitol addition of PQQ and Ca^{2+}, product L-fuco-4-ketose was produced (peak i); C, *pqqA* knockout mutant membrane fraction act on galactitol without the addition of PQQ, no products L-xylo-3-hexulose and D-tagatose were produced; D, *pqqA* knockout mutant membrane fraction act on galactitol addition of PQQ and Ca^{2+}, products L-xylo-3-hexulose and D-tagatose were produced (peak ii and iii); E, overlap of B and D.

For A and C, the reaction mixture (100 μL) contains 50 μL 10 mM potassium phosphate buffer (pH 6.0), 40 μL 50 $g{\cdot}L^{-1}$ L-fucitol (A) or galactitol (C) (the final concentration was 20 $g{\cdot}L^{-1}$), 10 μL 10 mg/mL membrane proteins prepared at 200,000 g, reacted at 30 °C for 48 h. For B and D, the reaction mixture (100 μL) contains 30 μL 10 mM potassium phosphate buffer (pH 6.0), 40 μL 50 $g{\cdot}L^{-1}$ galactitol (the final concentration was 20 $g{\cdot}L^{-1}$), 10 μL 10 mg/mL membrane proteins, 10 μL 5 mM PQQ, 10 μL 10 mM Ca^{2+}, reacted at 30 °C for 48 h.

the 5-ketose D-tagatose but not the 3-ketose is that the two compounds D-tagatose and 3-ketose L-xylo-3-hexulose were co-eluted in the column they used, but can be efficiently separated by the column we used.

There are two sets of dehydrogenases in acetic acid bacteria such as *G. oxydans*. One set is cytosolic NAD(P)-dependent dehydrogenases, which oxidize sugar alcohols to biosynthetic pathways intermediates; the other is PQQ-dependent membrane-bound dehydrogenases that incompletely oxidize sugar alcohols to provide energy for growth [33]. For example, *G. oxydans* ATCC 621H contains a membrane-bound and a cytoplasmic NAD-dependent inositol dehydrogenase [16]. *Gluconobacter frateurii* CHM 43 possesses a PQQ-dependent membrane-bound *meso*-erythritol dehydrogenase as well as a NAD-dependent counterpart [34]. So, it is crucial to determine the oxidation of galactitol at C-5 and C-3 position to D-tagatose and L-xylo-3-hexulose are catalyzed by PQQ-dependent membrane-bound dehydrogenases and/or soluble NAD(P)-dependent dehydrogenases in the cytosol of acetic acid bacteria. As described above, the oxidation reaction of polyols (C3-C6) catalyzed by PQQ-dependent membrane-bound dehydrogenases obeys the well-known Bertrand-Hudson's rule. According to this rule, D-arabitol, D-mannitol, D-sorbitol, D-ribitol, and *Meso*-erythritol (D-*erythro* configuration) were oxidized by the polyol dehydrogenase, while L-arabitol, xylitol , D-threitol, L-threitol, and galactitol were not used as the substrates. In this study, we found that galactitol can be simultaneously oxidized into D-tagatose and L-xylo-3-hexulose by PQQ-dependent membrane-bound dehydrogenases, but not soluble NAD(P)-dependent cytosolic dehydrogenases. The addition of EDTA into membrane fraction results in decreased galactitol dehydrogenase activity (Fig. 7) and reduced D-tagatose and L-xylo-3-hexulose synthesis. Incorporation of PQQ and Ca^{2+} into *pqqA* knockout *G. oxydans* membrane fraction partly restores enzyme activity and enhances galactitol oxidation to produce both D-tagatose and L-xylo-3-hexulose (Fig. 8, Fig. 9).

These results indicated that the oxidation of galactitol into D-tagatose and L-xylo-3-hexulose was catalyzed by PQQ-dependent membrane-bound dehydrogenase(s). However, galactitol does not possess the D-*erythro* configuration and not obey Bertrand-Hudson's rule; thus, the galactitol oxidation by PQQ-dependent membrane-bound dehydrogenase of acetic acid bacteria such as *G. oxydans* and *A. suboxydans* may represent an exception to Bertrand Hudson's rule.

Many PQQ-dependent membrane-bound dehydrogenases have been recognized in *Gluconobacter* strains. Peters and coworkers constructed a set of mutants in which all putative membrane-bound dehydrogenases genes were consecutively deleted and investigated the substrate oxidation spectra of mutants. The resulting multi-deletion strains (BP.9 and BP.10) did not oxidize tested polyols, including glycerol, D-arabitol, D-sorbitol, D-mannitol, *meso*-erythritol, D-threitol, L-threitol, and ribitol [22]. However, they did not use galactitol as a substrate, so it was still challenging to scrutinize which membrane-bound dehydrogenase oxidizes galactitol to D-tagatose and L-xylo-3-hexulose. We deduced that the enzyme oxidizing galactitol to D-tagatose and L-xylo-3-hexulose was mainly PQQ-dependent membrane-bound in *Gluconobacter* bacteria (Figs. 5–9), and the enzyme identification is being undertaken in our group.

In addition to acetic acid bacteria, some filamentous fungi such as *Hypocrea jecorina* and *Aspergillus niger* can also utilize galactitol. The enzyme has been identified to be L-arabinitol 4-dehydrogenase (LAD), which catalyzes the transformation of L-arabinitol into L-xylulose, D-sorbitol to D-fructose, D-allitol to D-psicose, D-gulitol to D-sorbose, L-mannitol to L-fructose, all at the C-2 position, with NAD as a cofactor. However, it exclusively oxidizes galactitol at the C-3 position yielding L-xylo-3-hexulose [15,35], with the same spectra mass fractions to the oxidation product of galactitol by PQQ-dependent purified membrane of *G. oxydans*. Galactitol oxidation to L-xylo-3-hexulose (C-3 position) by acetic acid bacteria and fungi may represent two entirely different mechanisms, one is membrane-bound that uses PQQ as a cofactor, while the other is cytoplasmic and utilizes NAD as a cofactor.

5. Conclusion

Taken together, galactitol is a unique polyol, which not only can be oxidized at C5 to give D-tagatose but also at C3 position to yield another rare ketose L-xylo-3-hexulose by *G. oxydans* PQQ-dependent membrane-bound dehydrogenase that does not conform to Bertrand-Hudson's rule. Whereas other polyols can only be oxidized at the secondary alcohol group adjacent to the primary terminal alcohol by *G. xoydans* PQQ-dependent membrane-bound dehydrogenase and obey the Bertrand-Hudson's rule. Identification of the PQQ-dependent membrane-bound dehydrogenase and elucidation of the mechanism will better contribute to understand why galactitol can be simultaneously oxidized at C5 and C3 position hydroxyl group, which is being under-investigated in our laboratory.

Author contributions

HC conceived the conceptualization, funding acquisition, writing the original draft. YX, PC, JL performed the experiments. MB review and edit this manuscript.

Declaration of Competing Interest

The authors declare that they have no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2020.129740.

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