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Note

Production of Aldonic Acids from Monosaccharides by Washed Cells of *Burkholderia cepacia* and their Calcium Binding Capability

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Abstract: Microbial oxidation of monosaccharides and calcium binding capability of the resulting aldonic acids were investigated. When washed cells of a strain of *Burkholderia cepacia* were shaken with 15% (w/v) D-galactose, D-mannose, D-xylose and L-arabinose in the presence of $CaCO_3$, corresponding aldonates were produced with yields of 99.4, 93.6, 94.5 and 93.0%, respectively, after 2 d for D-galactose and 8 d for the others. Free aldonic acids of high purity were obtained from the reaction supernatants by the treatment with activated carbon followed by cation exchange resin. The aldonic acids showed lower sequestering capacities compared with EDTA and citrate, suggesting weak binding with a calcium ion. Such weak calcium binding and high aqueous solubility of the aldonates suggested their possible application not to builders of detergents, but to functional saccharides that promote intestinal absorption of minerals.

Key words: aldonic acid, *Burkholderia cepacia,* microbial conversion, oxidation, calcium binding

Aldonic acids are hydroxyl monocarboxylic acids derived from aldoses by oxidation of the aldehyde group at the C1 position. Chemically, for example, D-gluconic acid (GlcA) can be produced by mild oxidation of D-glucose (Glc) with bromine water. GlcA is produced biologically and is applied to a food additive for adjusting pH, acidifying, seasoning, *etc.* Biological oxidation of reducing saccharides, however, has not received much attention probably because of the lack of appropriate biocatalysts. We have been studying the bioconversion of oligosaccharides, especially the microbial conversion of lactose (Lac) to lactobionic acid (LacA),[1–4] which has several practical, potential uses[5–7] such as a component of a water-soluble antibiotic, erythromycin lactobionate, and as a functional saccharide that promotes intestinal absorption of minerals.[5] We screened several microbes including a *Burkholderia (B.) cepacia* strain as potent LacA producers.[1,2] An enzyme involved in the Lac oxidation was found in the membrane fraction of the disrupted cells of *B. cepacia,* which required O_2 for the reaction, indicating a kind of oxidase was responsible for the conversion.[1] The productivity of LacA was improved by the selection of a mutant strain (No. 24) that acquired a tolerance to high Lac concentrations.[2,3] Then the resting cells of the mutant were subjected to repeated batch reaction,[4] which not only saved the valuable catalyst but also facilitated the subsequent refining process of the product. The present report describes an efficient conversion of a few monosaccharides to corresponding aldonic acids with washed cells of the mutant bacterium. We also evaluated the calcium binding capability of the resulting aldonic acids, which had not been determined so far.

The mutant bacterium[2,3] was cultured in a medium (100 mL) containing 10% (w/v) Lac, 3.0% (w/v) corn steep liquor, 0.02% (w/v) yeast extract, and 1.5% (w/v) $CaCO_3$ (pH 7). Cultivation was carried out in 500-mL flasks at 30°C with shaking (120 oscillation/min).[4] After the removal of $CaCO_3$ by gentle centrifugation at 160 × *g* for 5 min, the cells were harvested by centrifugation at 7000 × *g* for 30 min. Then the cells were washed twice with 0.85% (w/v) NaCl solution and suspended in the saline solution. The cells in the suspensions were tentatively determined by the absorbance at 660 nm (A_{660}). The activity of the washed cells was assayed by the oxidation of Glc to GlcA as described previously[4]. One unit was defined as the amount of cells that generated 1 μmol of GlcA per min. A maximum specific activity of 0.75 U per A_{660} (15 U/mL of the culture fluid) was obtained after cultivation for 9 days.

The specificity of the washed cells on various substrates was investigated by measuring O_2-uptake rates with an O_2 probe (Yellow Spring Instrument Co., OH, USA). When the decreasing velocity of the dissolved O_2 in the oxidation of Glc was taken as 100%, Lac, D-galactose (Gal), D-mannose (Man), D-xylose (Xyl), L-arabinose (Ara) and D-ribose were oxidized with relative velocities of 93, 90, 80, 50, 46 and 15%, respectively. The cells exhibited no activity on D-fructose, L-sorbose, D-arabinose, D-erythrose and DL-glycerlaldehyde. In this study, therefore, aldonic acids were prepared from the following preferable monosaccharides: Gal, Man, Xyl and Ara. Figure 1 shows time courses for the conversion with the washed cells. The reaction progressed efficiently in the presence of a half-mole equivalent of $CaCO_3$ of the substrates under shaking conditions, where the aldonic acids were spontaneously neutralized by $CaCO_3$ to maintain pH of the mixture at 5–7. The oxidation of Gal to D-galactonic acid (GalA) was the most efficient and a yield of 99.4% was attained after 2 d. Such high efficiency was almost comparable with that of Lac, where a stoichiometric conversion occurred within approximately 24 h under the

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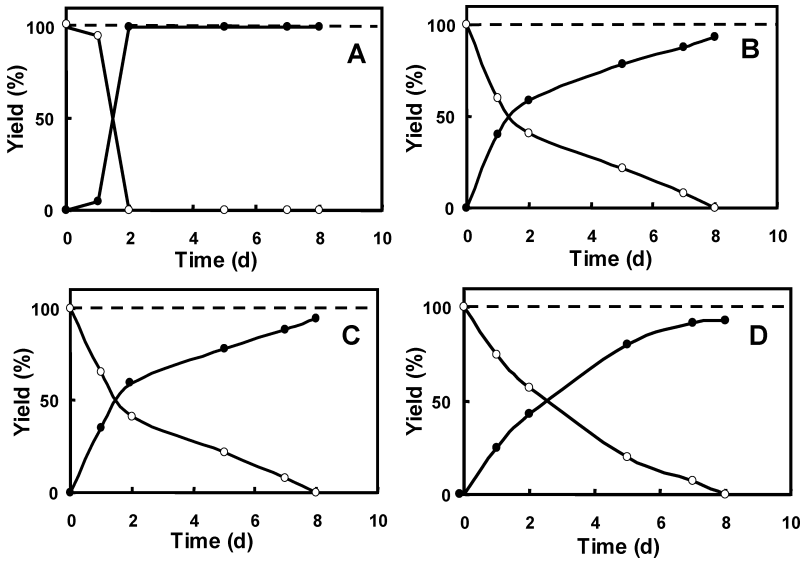

Fig. 1. Conversion of monosaccharides by the washed cells of *B. cepacia* No. 24.

Conversion was performed in 500-mL culturing flasks containing mixtures (100 mL) of monosaccharides (15 g), the washed cells (2.2 g-wet weight, 2.0 U/mL), and $CaCO_3$ (4.2 g, half-mole equivalent of the saccharides) at 40°C on a reciprocal shaker (110 oscillation/min). The substrate solutions and solid $CaCO_3$ were sterilized separately prior to the reaction. The substrates and the products were measured by HPLC under the following conditions: pump, LC-10A (Shimadzu Co., Kyoto); column, Asahipak NH2P-50 (Shodex Co., Tokyo); solvent, 60% (v/v) CH_3CN in 40 mM Na_2HPO_4-20 mM citric acid buffer, (pH 5.0); flow rate, 0.8 mL/min; column temperature, 40°C; detector, Shimadzu RID-10A refractometer. A, Gal to GalA; B, Man to ManA; C, Xyl to XylA; D, Ara to AraA; open circle, substrate; closed circle, product.

same conditions.[4] Man, Xyl, and Ara were oxidized to D-mannonic acid (ManA), D-xylonic acid (XylA) and L-arabinonic acid (AraA), respectively, at somewhat slower rates: The substrates had almost disappeared after 8 d and respective final yields were 93.6, 94.5 and 93.0%. Such high yields were attributable not only to the high oxidation activity of the cells, but also to deficiency in assimilating both the substrates and the products.

After the conversion, the cells were removed by centrifugation at 7000 × *g* for 30 min. Each supernatant solution was passed through a column (2.5 × 12 cm) of activated carbon. The passed solutions contained neither starting substrates nor other saccharides, which was confirmed by HPLC, and gave odorless white powders after lyophylization. Calcium aldonates were then changed to free acids by passing through a column (2.5 × 6 cm) of a cation exchange resin (Diaion PK-216, H^+ form, Mitsubishi Chemical Co., Tokyo). The overall yields of GalA, ManA, XylA, and AraA were 86.1, 70.7, 92.7 and 89.4%, respectively. Their structures were confirmed by ^{13}C-NMR (75 MHz, D_2O, JEOL AL-300 FT-NMR spectrometer, Tokyo). The signals were reasonably assigned as follows (δ ppm): (1) GalA; 182.5 (C1), 74.6 (C2), 74.1 (C3), 72.7 (C4), 72.4 (C5), 65.9 (C6). (2) ManA; 181.7 (C1), 76.9 (C2), 73.3 (C3), 73.6 (C4), 73.2 (C5), 65.6 (C6). (3) XylA; 181.3 (C1), 75.7 (C2), 75.3 (C3), 74.8 (C4), 65.1 (C5). (4) AraA; 182.2 (C1), 74.8 (C2), 74.6 (C3), 73.8 (C 4), 65.7 (C5). A high purity of each product was also suggested by the simple spectra profiles.

Aldonic acids usually form mineral salts of high aqueous solubility due to the hydrophilic glycosyl moiety with a carboxyl group. Since nothing was known of the calcium binding capability of these uncommon aldonic acids, we measured "sequestering capacity", the capability to prevent the binding between calcium ion and sodium dodecyl sulfate (SDS), which is generally used to evaluate performance as a builder, an agent that increases the cleaning power of detergents.[8,9] In this study, the improved method of Hart[8] was used for the measurement: Mixtures containing 0.5% (w/v) aldonic acids (20 mL) and 10% (w/v) SDS (0.5 mL) were placed in glass beakers and titrated with 1.0% (w/v) calcium acetate solution. The developed turbidity of calcium salt of SDS that obscured a figure on the opposite side of the beaker was taken as the end point of the titration, when the figure was observed horizontally through the solution. Sodium citrate and EDTA (disodium salt) in addition to GlcA and LacA were used as reference compounds. As shown in Table 1, insoluble calcium salt of SDS became apparent with the additions of relatively small amounts of calcium acetate to the solutions of the aldonic acids. The results suggested ManA, GalA, AraA and ManA as well as LacA and GlcA had lower calcium binding capability compared with EDTA and citrate, and thus the chelating capability seemed insufficient for builders. The other property of a builder termed as "calcium stability" was also measured:[8] As a result, the solutions of the free aldonic acids (0.5%,

Table 1. Sequestering capacities of several aldonic acids and relating compounds.

Compound	Sequestering capacity (mg/g)*
LacA	15
ManA	21
AraA	27
GalA	29
XylA	45
GlcA	55
Citrate	85
EDTA	375

*mg of $CaCO_3$ per g of testing compound. The method is described in the text.

w/v, 20 mL) did not form any precipitates after mixing with calcium acetate solution (5.0%, w/v, 25 mL). The results were expressed as the calcium stability of more than 6300 ppm as calcium carbonate according to the definition.[8] The values corresponded to a maximum level of calcium stability, which suggested the aldonic acids should not form troublesome precipitations of calcium salts as far as they were used as builders.

From physiological or nutritional viewpoints, strong chelating of calcium ion is not necessarily preferable. Some natural chelating agents such as phytic acid and oxalic acid are known as antinutritional factors to disturb absorption of dietary minerals by forming insoluble complexes.[10,11] As described here, therefore, weak binding with calcium ion and high aqueous solubility of the aldonates suggested their possible application not to builder of detergents, but more favorably to functional saccharides that promote intestinal absorption of minerals.

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Burkholderia cepacia 洗浄菌体による単糖からアルドン酸の生産とそのカルシウム結合力

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数種類の単糖を微生物酸化してアルドン酸を生成させ，それらのカルシウム結合力を調べた．*Burkholderia cepacia* 洗浄菌体を $CaCO_3$ の存在下で 15%(w/v)の D-ガラクトース，D-マンノース，D-キシロース，L-アラビノースと振とうした結果，99.4，93.6，94.5，93% の収率でそれぞれのアルドン酸が得られた．変換には，D-ガラクトースで 2 日，他では 8 日を要した．反応液上清を活性炭および陽イオン交換樹脂で処理すると高純度の遊離アルドン酸が得られた．アルドン酸類のカルシウム封鎖能は EDTA やクエン酸よりも低く，カルシウム結合力が弱いことが示唆された．アルドン酸類はカルシウムとの結合が弱く，高水溶性の塩を形成することから，ミネラル塩を捕捉して洗剤の働きを助けるビルダーなどとしてではなく，ミネラル吸収を促進する機能性糖質としての利用が有望と考えられた．