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# Studies on the Oxidation of Ribitol and D-Arabitol by *Acetobacter aceti* IFO 3281

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**Abstract:** *Acetobacter aceti* IFO 3281, grown on 1% glycerol, was found to have the best conversion potential for ribitol and D-arabitol than other acetic acid bacteria and has broad substrate specificity on various *polyols*. Strain can convert upto 20% ribitol and 50% D-arabitol to L-ribulose and D-xylulose, respectively without any tendency of by-product formation or substrate/product consumption.

**Key words:** *Acetobacter aceti* IFO 3281, D-Arabitol, L-Ribulose, D-Xylulose

## Introduction

The biochemical capabilities of microorganisms are vast and a wide variety of new or unusual compounds can be produce by various microbial isolates. Using new techniques of biotechnology, scientists will continue to build on current research to improve existing products and processes and to generate many new ones by construction of microorganisms that might utilize a specific low cost substrate to make a desirable end product. Carbohydrate substrates are the classical example for traditionally applied biotransformation and next to significance of obtaining monosaccharide from the corresponding biopolymer, the microbiological transformation of monosaccharide has become an important biotechnological method. A genus of gram type-negative bacteria of the family Acetobacteraceae has been known to oxidize polyols with particular stearic configurations to ketoses. Among this group, *Acetobacter aceti,* is known to be a ketogenic microbes with glycerol or sorbitol substrate. The present study was undertaken to optimize the transformation of ribitol to L-ribulose and 0-arabitol to D-xylulose using the *Acetobacter aceti* strain IFO 3281 (Fig. 1).

## Materials and Methods

Chemicals Ribitol, D-arabitol, D-xylulose, D-mannitol, polypepton and yeast extract were obtained from Difco Laboratories (USA). Other carbohydrates and chemicals were purchased from Sigma Chemical Co. (USA) and were certified as reagent grade.

**Analytical method:** Accumulation of ketoses in the reaction mixture was detected by the method of Dische and Borenfreund (1) and high-performance liquid chromatography analysis (HPLC, Nihonbunko HPLC 880 PU liquid chromatography, Shimadzu RID-6A refractive index detector and Shimadzu C-R6A chromatopac using a Hitachi HPLC column GL-611. Separation was achieved at 60°C using 10 4M NaOH at a flow rate of 1.0 ml/min.

**Microorganisms and cultivation condition:** The strains collected from the Institute for Fermentation Osaka (IFO), Japan were grown and preserved in a medium containing 0.3% yeast extract, 0.5% polypepton and 1.0% glycerol for 48 h at 30°C with shaking at 160 rpm.

**Production of ketoses by various strain:** Investigation was done to know the production of L-ribulose and D-xyluloe using various acetic acid bacteria. The selected bacterial strains were as follows: *A. aceti* IFO 3281, *Gluconobacter frateuni* IFO 3254, *G. oxydans* IFO 3172, IFO 3290, IFO 3291, IFO 3130, IFO 3255, IFO 3256 and IFO 3289. After cultivation, cells (~0.05 g) were washed with 0.05 M Na-Phosphate buffer (pH 7.51 and used for the production of L-ribulose/D-xylulose from 0.5 g ribitol/D-arabitol in 5.0 ml reaction mixture at 30°C for 5 h with shaking. The production of L-ribulose/D-xylulose was determined by cysteine-carbazole reaction (1) and also by HPLC.

**Media design:** *A. aceti* IFO 3281 was grown on various carbohydrates in order to determine the most suitable growth carbon and inducer for transformation of ribitol and D-arabitol. The cells of *A. aceti* IFO 3281 were grown aerobically with continuous shaking for 48 h at 30°C in tubes containing medium of the following composition: $(NH_4)2SO_4$, 0.26%; $KH_2PO_4$, 0.24%; $K_2HPO_4$, 0.56%; $MgSO_4.7H_20$, 0.01%; yeast extract, 0.05% and sugar or polyol, 1%. Test for growth and pH in every case was determined where growth was measured at 600 nm absorbance. Various media were composed for the determination of suitable growth medium for optimal transformation of ribitol and D-arabitol by *A. aceti* IFO 3281. The compositions are as follows- (1) media A: yeast extract, 0.5%; polypepton, 0.5%; glycerol, 1.0% (2) media B: composition of media A with NaCI, 0.5% (3) media C: Trypto soy broth (TSB), 2.0%; glycerol, 1,0% (4) media D: composition of media A with $(NH_4)_2HPO_4$, 0.6%; $KH_2PO_4$, 0.3% (5) media E: corn steep liquor, 0.5%; polypepton, 0,5%; glycerol, 1.0% (6) media F: composition of media E with $INH_412HPO_4$, 0.6%; $KH_2PO_4$, 0.3%. In every case, the culture medium was inoculated with 5.0 ml of preculture grown in the same medium. After growth, the cells were harvested by centrifugation at 9,000 rpm for 10 min and washed with 0.05 M Na-Phosphate buffer (pH 7.0). After washing twice, the cells were checked for the transformation of ribitol/D-arabitol. The transformation was carried out in 0.05 M Na-Phosphate buffer (pH 7.0) with the reaction mixture of ribitol/D-arabitol, 1.0% and washed cell of strain IFO 3281 cultivated in different growth medium (Absorbance 20 at 600 nm) with shaking at 30^C for 48 h. The product accumulation in the reaction mixture was determined by cysteine-carbazole reaction (1) by increase in absorbance of ketoses at 540 nm in 20°C for 20 min.

**Optimization of pH, cell and substrate concentration:** Effects of pH on L-ribulose production were studied using the

following buffers (0.05 M); citrate buffer (pH 3.0-5.0), maleate buffer (pH 5.0-7.01, Na-phosphate buffer (pH 6.0-8,01, Tris-HCl (pH 7.0-9.0) and glycine-NaOH buffer (pH 9.0-11.0). For the optimization of suitable cell concentration of *A. aceti* 1F0 3281, reaction was carried out in L tube in the reaction mixture where the absorbance of cells was adjusted to 5, 10 and 20 at 600 nm on L-ribulose/D-xylulose production from 2.0% ribitol/D-arabitol. After determining the best cell concentration for conversion of ribitol/D-arabitol to Lribulose/D-xylulose, conversion rates from different concentrations of ribitol/D-arabitol were determined. The concentration of the substrate was adjusted to 5.0, 10.0, 15.0 and 20.0% in the final reaction mixture for transformation of ribitol and for D-arabitol transformation, the substrate was adjusted to 10, 20, 30, 40 and 50% in the final reaction mixture. Transformation conditions, determination of substrate oxidation and product accumulation in the reaction mixture were done using the same method described earlier.

**Product recovery and determination of the product:** After the transformation, the cells were removed by centrifugation and the supernatant fluid was treated with activated charcoal, filtered after centrifugation to remove the charcoal, then treated with a microacilyzer (Asahi Kasei, Model G-1) for deionization. The microacilyzer treated sample was again deionized with a mixture of diaion (SK1B, H+) and amberlite (IRA-411, $Co_3^{2-}$) ion-exchange resins. The deionized content was evaporated and concentrated under vacuum at 40°C. After concentration, the content was identified by HPLC as described above.

## Results

**Suitable medium:** The transformation and growth was found higher in media-A (Fig. 2A) followed by media-B and media-C. Among the various carbohydrates, *A. aceti* IFO 3281 showed good growth only in a medium containing 1.0% glycerol, followed by those grown on D-mannitol, erythritol, D-mannose and could not grow on any other carbohydrates (data not shown). So, glycerol was selected as the carbohydrate for the culture and the concentration needed to obtain optimum transformation was also investigated. Following the determination of a suitable carbohydrate, glycerol for growth, cells of strain IFO 3281 were cultivated on various concentration of glycerol in order to find out suitable concentration of glycerol for transformation. The cells were cultivated in a tube of 5.0 ml medium (described above) containing 0.5 to 3.0% glycerol with continuous shaking at 30°C for 48 h. Transformation was carried out in a reaction mixture containing 1.0% ribitol and the cells concentration of strain IFO 3281 was adjusted at 20 in 600 nm absorbance (final reaction mixture) in 0.05 M Na-Phosphate buffer (pH 7.0) at 30°C with shaking and L-ribulose was measured by cysteine-carbazole method (Chiang *et al.*, 1981). After 3 h reaction, cells grown on 1.0% and 2.0% glycerol was found to achieve almost same effect on the transformation of 1.0% ribitol followed by 0.5% glycerol (Fig. 2B). Therefore the strain was cultivated at 30°C in a medium containing 0.3% yeast extract, 0.5% polypepton and 1.0% glycerol.

**L-RibulosefD-xylulose production by various bacterial strains:** Figure 3 shows that among the strain tested, the highest activity for ketose production was found in *G. frateurii* IFO 3254, followed by *A. aceti* IFO 3281. Other strains like *G. oxydans* IFO 3172, IFO 3290, IFO 3291 and IFO 3130 have also some activity. Therefore HPLC analysis was done in order to know whether these strains produce any by-products. From the HPLC analysis (data not shown), it was found that all strains except *A. aceti* IFO 3281 concomitantly produced by-product in addition to L-ribulose/D-xylulose in the reaction mixture. Therefore, *A. aceti* IFO 3281 was used as the most suitable and potent L-ribulose/D-xylulose producer throughout the experiments.

**Ketose production from various polyols:** The substrate specificity of the strain IFO 3281 for ketose production from various polyols: galactitol, L-arabitol, xylitol, allitol, D-mannitol, ribitol, D-sorbitol, erythritol and D-arabitol was studied. The reaction for ketoses production from 5.0% various polyols was carried out at 30'C in L tube with shaking for 12 h where the cells concentration of strain IFO 3281 was adjusted at 20 in 600 nm absorbance (final reaction mixture) and accumulation of ketoses was determined by cysteine-carbazole method (1). The strain showed broad substrate specificity. Among the polyols checked D-arabitol appeared to have the maximum affinity towards this strain followed by erythritol, D-sorbitol and ribitol (Fig. 4).

**Effect of pH, cell and substrate concentration:** The reaction for L-ribulose production was inhibited in citrate buffer of pH 5.0 and in Tris-HCl buffer of all pHs (data not shown). L-Ribulose production was the highest when the reaction mixture was carried out at an initial pH of 7.0 using the 0.05 M Na-phosphate buffer. Figure 5 shows that the reaction speed increased with increasing cell concentration. The conversion rates were almost similar in case of the cell absorbance of 10 and 20 at 600 nm. The effects of substrate concentration are shown in Fig. 6. The initial concentration of ribitol in the reaction mixture was increased from 5.0% to 20.0%. The time course gradually prolonged with increasing substrate concentration and 20.0% substrate was converted to Lribulose after 72 h of the reaction. Figure 6 shows that *A. aced* 1F0 3281 can convert upto 50% D-arabitol to D-xylulose after about 80 h which means that it is a potent microbes for transformation but the conversion was also prolonged with the increase of substrate concentration.

**Production and identification of L-ribulose and D-xylulose:** The complete conversion of ribitol took about 48 h (Fig. 7A) and the conversion rate was about 98%, which indicated that significant conversion could be achieved, Fig. 7B shows the HPLC chromatograms of D-xylulose production at different time. It was found from our analysis that about 100% conversion of D-arabitol to D-xylulose was achieved by this washed cell and no other peak was found indicating no by product was formed during the reaction. The HPLC results were compared with those of authentic L-ribulose and Dxylulose. The HPLC retention time of the products corresponded to that of authentic one. Considering the above result, the product formed from ribitol and D-arabitol was identified as L-ribulose and D-xylulose, respectively.

## Discussion

Pentitol catabolism is an interesting phenomena in microbial metabolism which can serve as an excellent model system for studying the acquisition of new metabolic capabilities of microbes. The bacterial strain, *A. aceti* IFO 3281 used in this study has considerable potential for L-ribulose and D-xylulose

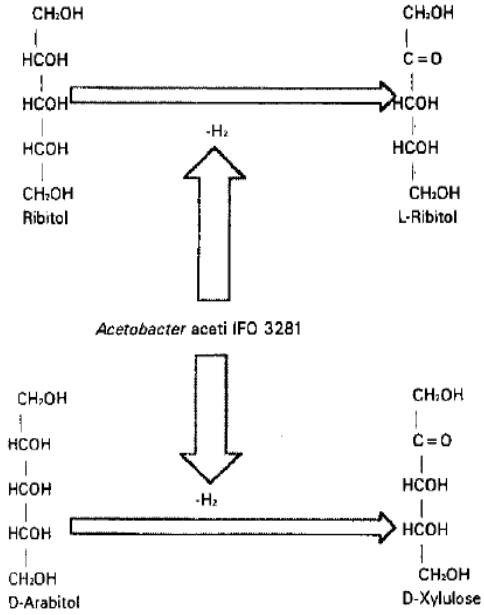


Fig. 1: Schematic diagram for the production of L-ribulose and D-xylulose by *Acetobacter aceti* IFO 3281

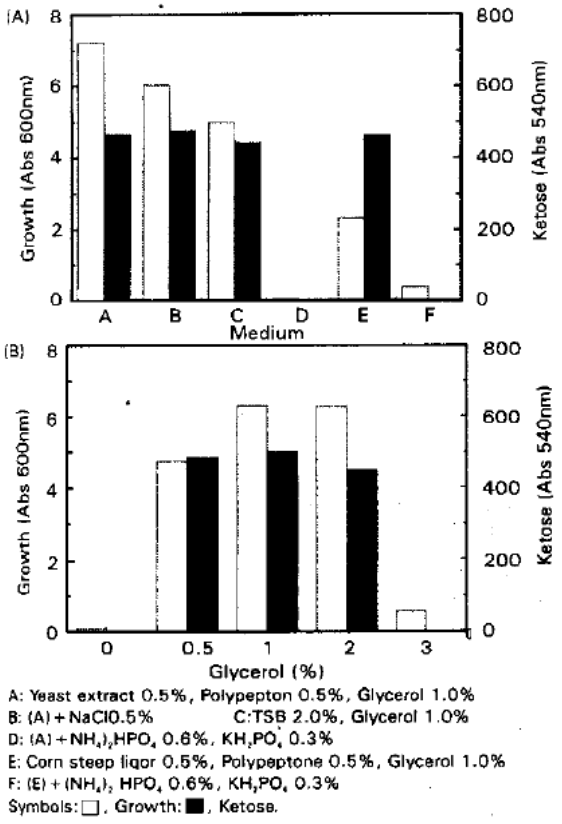


Fig. 2: Transformation of ribitol to L-ribulose by the cells grown on various medium (A) and effect of glycerol concentration on the growth and transformation potential of *Acetobacter aceti* IFO 3281

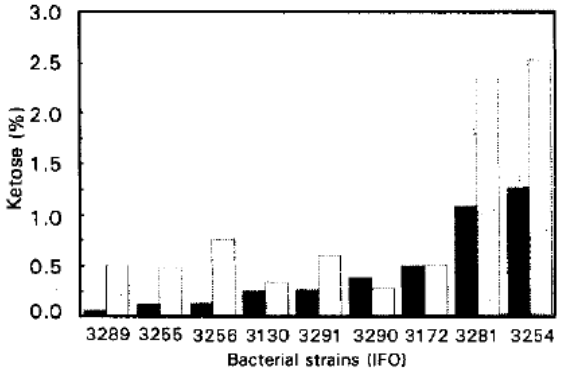


Fig. 3: Production of L-ribulose and D-xylulose by various acetic acid bacteria
Symbols: ■, L-ribulose: □, D-xylulose.

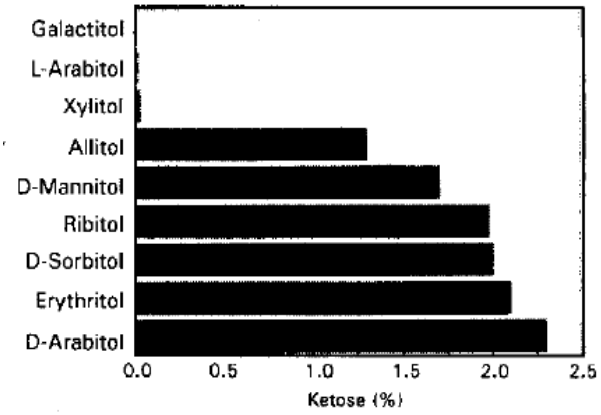


Fig. 4: ketose production from various polyois by *Acetobacter aceti* IFO 3281

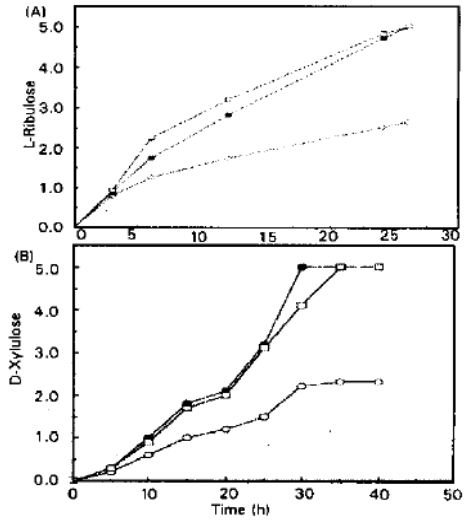


Fig. 5: Effect of cell concentration of Acetobacter aceti IFO 3281 for conversion of ribitol to L-ribulose (A) and D-xylulose (B)
Symbole: ○,5: ●,10: □,20.

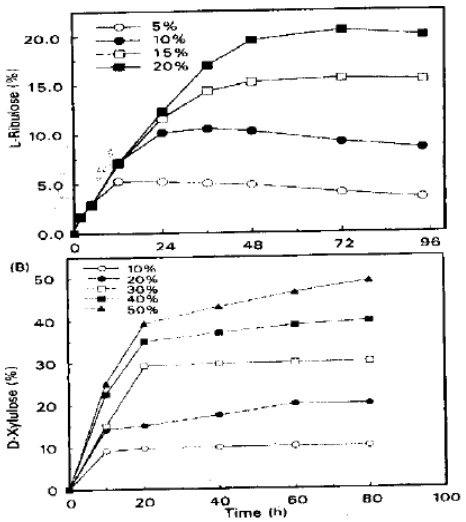


Fig. 6: Donversion of ribitol to L-ribulose (A) to D-xylulose (B) at different substrate concentration by *Acetobacter aceti* IFO 3281

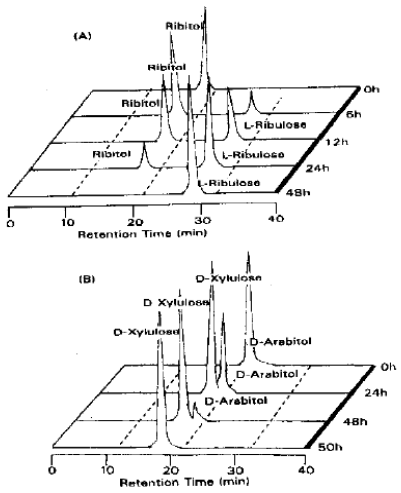


Fig. 7: HPLC Chromatograms of the conversion of ribitol to L-ribulose (A) and 0-arabitol to D-xylulose (B)

production from ribitol and D-arabitol, respectively. *A. aceti* IFO 3281 attacks carbon 4 of ribitol to produce L-ribulose same as that of *A. suboxydans* (Compello, 1973). The oxidation of ribitol follows the rule in that a secondary hydroxyl group of a *cis* pair adjacent to a terminal alcohol group is oxidized (Dische and Borenfreund, 1951). In one report, L-ribulose was prepared from xylitol by refluxiing with dry pyridine from the corresponding aldopentose (Mortlock *et al*., 1965) where the oxidation of ribitol to L-ribulose was followed by a long lag period to attain complete oxidation; resulting in the formation of by-products. Microbiological and enzymatic production of D-xylulose from D-arabitol have been reported previously (Compello, 1973; Moses and Ferrier, 1962; Olsson *et al*., 1994). D-Xylulose can also be prepared from D-xylose (Schwartz *et al*., 1994; Wood *et al*., 1961). In contrast, the results described here demonstrate that *A. aceti* IFO 3281 cultivated for 48 h at 30°C in a medium containing 0.3% yeast extract, 0.5% polypepton and 1.0% glycerol potentially transformed ribitol to L-ribulose as well as D-arabitol to D-xylulose at high substrate concentration without any by-product formation and did not show any tendency of product and substrate consumption. The strain WO 3281 can produce various ketoses from many polyols and showed highest conversion in Na-phosphate buffer (pH 7.0). Tris (hydroxymethyl) amino methane (Tris) which has been widely used as a buffer for the enzyme reaction system, inhibited the preparation of L-ribulose in a non-competitive manner. Cells grown on 1.0% glycerol showed very high transformation activity, which is indeed advantageous since this organism does not require any expensive growth carbon for obtaining better transformation. Ribitol and D-arabitol are naturally occurring polyols, which is commercially available and considerably cheaper. In the past few years, the use of L-carbohydrates and their derived nucleosides in medicinal applications have greatly increased. In particular, several modified nucleosides derived from L-sugars have been shown to be pdtent antiviral agents and may find use in antisense therapy. It was also found that L-monosaccharides has antineoplastic characteristics. Researchers found that L-isomeric form of monosaccharides exhibited cytostatic and cytotoxic properties with regards to neoplastic cells that can be used for cancer therapy (Eicher, H. I., USA patent no. 941222, 1997). The success of these synthesis are due to on two factors: (a) all reactions are selective only for product formation and (b) most importantly the final product (L-ribulose and D-xylulose) are very easily obtained from the reaction mixture with no effect on the desired product and without producing by-products where almost all the substrates are converted to products.

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