

Oral Session V

Oxidative Fermentation and Useful Product Formation, and Enzymology of Acetic Acid Bacteria

V-1: Analysis of structure and function of membrane-bound alcohol dehydrogenase from acetic acid bacteria

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Many Gram-negative aerobic bacteria can grow on alcohols and sugars as the sole carbon and energy sources. In the periplasm of acetic acid bacteria, quinoprotein alcohol dehydrogenases (ADH) containing pyrroloquinoline quinone (PQQ) instead of nicotinamide or flavin compounds as the prosthetic group catalyze the first step of acetic acid production, oxidation of ethanol to acetaldehyde. There are three types of quinoprotein ADHs. Type I ADH is a soluble, dimeric protein of identical subunits having a PQQ and a calcium ion in each active center, but no other redox cofactors. Type II ADH is a soluble, monomeric quinoxinoprotein, having a PQQ-containing catalytic domain and an additional cytochrome *c* domain with a covalently bound heme *c*. Type III ADH is a quinoxinoprotein–cytochrome complex with three nonidentical subunits that catalyzes the oxidation of ethanol and the subsequent reduction of ubiquinone, and attached on the cytoplasmic membrane of acetic acid bacteria.

Crystals of a heterotrimeric membrane-bound ADH have been obtained by hanging drop vapor diffusion at 277 K using Ammonium sulfate as precipitant^{1,2}.

We report here 3.0 Å crystal structure of the type III membrane-bound quinoxinoprotein ADH from *Gluconobacter suboxydans* refined to *R*-factor 29 %. Our structure reveals that the enzyme contains a large subunit A similar to the type II quinoprotein ADHs which have a eight-stranded β propeller domain and a cytochrome *c* domain, a membrane-bound subunit B which has a novel three-heme cytochrome *c* structure, and a small subunit C which has unknown function. The PQQ is located near the axis of the propeller domain about 14 Å from the heme in subunit A. The shortest distances between four hemes are about 9 Å, 4 Å, and 8 Å, respectively.

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V-2: Enzymatic Synthesis of L-Fucose by Using Dehydrogenase and Oxidase Obtained from Acetic Acid Bacteria.

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A new method for the synthesis of L-fucose from L-fucitol has been considered. Among known chemical production methods of L-fucose, D-galactose has been used as the start material [1, 2]. In these methods, D-galactose is converted to L-fucitol by two chemical steps and then L-fucitol is converted to L-fucose through intricate protection-removal steps. In order to simplify the latter steps for the conversion of L-fucitol to L-fucose, we aimed to establish a more effective method by enzymatic reactions. For this purpose, we designed a scheme consisting of the oxidation of L-fucitol to L-fuculose and the isomerization of L-fuculose to L-fucose.

On the basis of the above scheme, we tried to discover a new enzyme catalyzing the oxidation of L-fucitol to L-fuculose and considered to use the enzyme in combination with a known enzyme, L-fucose isomerase (FucI), which had been reported to catalyze the isomerization of L-fuculose to L-fucose with a good yield (L-fucose/L-fuculose = approx. 10). For screening the enzyme, we focused on acetic acid bacteria, which have been known to possess dehydrogenase enzymes reacting on various kinds of sugar alcohols. Through the screening, a strain *Gluconobacter oxydans* IFO3255 was selected as the most effective catalyst under an appropriate reaction condition using intact cells.

From this strain, a novel enzyme responsible for the conversion of L-fucitol to L-fuculose was identified and isolated to be electrophoretically homogeneous. The enzyme, L-fucitol dehydrogenase (FcDH), catalyzed the regio-selective oxidation of L-fucitol depending on the existence of cofactor NAD. The oxidative production of L-fuculose was observed by using cell-free extract of *G. oxydans*. In the reaction, the amount of L-fuculose produced was much more than that of NAD added in the reaction mixture without any sources for NAD generation. This observation was considered to be explained by NAD regeneration catalyzed by another enzyme. On further investigations, another novel enzyme, NADH oxidase (NOX), responsible for the oxidation of NADH to NAD was also identified and isolated. Using purified enzymes, enzymatic characteristics of both FcDH and NOX were determined. FcDH exhibited low substrate specificity and could catalyze the oxidation of various sugar alcohols, ex. D-arabitol, D-mannitol, D-sorbitol, xylitol etc. NOX required a flavin coenzyme such as riboflavin, FAD or FMN for its activation and did not recognize NADP as the substrate.

Aiming to establish the effective process, the gene encoding FcDH and NOX were identified and then co-expressed with a gene encoding FucI in an *E. coli* strain. The recombinant strain could convert L-fucitol to L-fucose with a good yield in intact cell reactions. The best result obtained so far was 78 g/L accumulation of L-fucose in 48 h with an 80% molar yield to L-fucitol.

[1] Kristen, H., Vogel, G., Wrubel, F., Mahrwald, R., and Schick, H., *J. Carbohydr. Chem.*, **7**, 277-281 (1988)

[2] Sarbajna, S., Das S. K., and Roy, N., *Carbohydr. Res.*, **270**, 93-96 (1995)

V-3: Cytoplasmic NAD(H)- and NADP(H)-dependent Enzymes Involved in Glycerol Metabolism in *Gluconobacter* Strains

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It is well known that, in oxidative fermentation, microbial growth is improved by the addition of glycerol. In a wild strain, glycerol was converted rapidly to dihydroxyacetone (DHA) quantitatively in the early growth phase by the action of quinoprotein glycerol dehydrogenase (GLDH), and then DHA was incorporated into the cells in the late growth phase.

Two DHA reductases (DHARs), NADH-dependent one (NADH-DHAR) (EC 1.1.1.6) and NADPH-dependent one (NADPH-DHAR) (EC 1.1.1.156), were detected in the same cytoplasm of *Gluconobacter suboxydans* IFO 3255. The former appeared to be inducible and labile in nature while the latter was constitutive and stable. The two DHARs were separated each other and finally purified to crystalline enzymes. This might be the first report dealing with NADPH-DHAR that has been crystallized. The two DHARs, having almost same molecular weight of 120k and consisted of four identical subunits, were specific only to DHA reduction to glycerol and thus contributed to cytoplasmic DHA metabolism, resulting in an improved biomass yield with the addition of glycerol.

In addition to the two different DHARs as above, at least four different NADH and NADPH dehydrogenases were identified in the same cytoplasmic fraction of *G. suboxydans* IFO 3255. NADPH dehydrogenase (NADPH-dh) appeared to be the same as the old yellow enzyme (EC 1.6.99.1) and to have FMN as the coenzyme. A novel NADH dehydrogenase (NADH-dh), distinct from NADPH-dh, was isolated and shown to have FAD non-covalently as the coenzyme. Molecular oxygen was available as an electron acceptor in the NADH-dh and also NADPH-dh, which is different from those flavoproteins classified in EC 1.1.99.2 and EC 1.6.99.3, most of which are unable to use oxygen as the electron acceptor. The most significant properties of NADH-dh and NADPH-dh were indicated by their catalytic properties: NADH-dh was concluded to be DHA reductase and NADPH-dh as glyceraldehyde reductase. Enzymatic properties of other two yellow enzymes will also be reported together.

Taking consideration above all, in the cytoplasm of *Gluconobacter* strains, enzymatic machinery for glycerol metabolism was well equipped and DHA incorporated into the cytoplasm would be processed immediately without any metabolic retardation caused by unusual accumulation of DHA and glyceraldehyde in cytoplasm.

V-4: Exploring the reductive potential of *Gluconobacter oxydans* based on the genome sequence

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Many useful organic compounds, such as pharmaceuticals and food additives, with symmetric carbons and enantiomeric forms exist. It is common to have only one biologically active enantiomer, while the other not showing activity and sometimes having a harmful effect. In such cases, chemically synthesized racemic mixtures cannot be used. This is especially true for pharmaceuticals. Therefore, the racemic mixture must be made optically pure by conventional organic synthesis. *Gluconobacter oxydans* was used in new strategies for regio- and stereoselective biotransformations. It is known that *G. oxydans* contains over 70 genes that encode putative oxidoreductases with unknown enzymatic activities¹. Additionally, the organism was found to be able to reduce a wide variety of α -ketoaldehydes, α,β -diketones, vinyl ketones, sugars, and aldehydes². Eight proteins responsible for these reductions were identified by heterologous expression of the corresponding genes in *E. coli*, followed by analysis of their substrate spectra. The products of these biotransformations often produced chiral centers and may find industrial uses (e.g. pharmaceuticals, food additives or fragrance industries). These enzymatic reactions often require expensive cofactors (e.g. NADPH). However, increased yields and substantially decreased production cost can be achieved if cofactor can be regenerated³. Therefore, we have employed a coupled enzyme biotransformation system that allows the efficient regeneration of cofactor and increased product production.

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2. Schwieger, P. et al., *Appl. Microbiol. Biotechnol.*, in press (2008)

3. Schweiger, P. et al., *J. Mol. Microbiol. Biotechnol.* **13:147-155 (2007)**

V-5: Enzymatic method for the production of xylitol from D-arabitol by *Gluconobacter oxydans*

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Xylitol is a naturally occurring pentahydroxy sugar alcohol and has numerous applications in the fields of food and pharmaceuticals. Xylitol is produced commercially by chemical reduction of D-xylose derived from hemicellulose-xylan hydrolysates of substrates such as birchwood. Here we report a novel method for the production of xylitol from D-glucose. Since D-arabitol can be produced efficiently from D-glucose by fermentation with osmophilic yeasts, we developed a microbial conversion of xylitol from D-arabitol.

Microorganisms capable of producing xylitol from D-arabitol were screened for. Three acetic acid bacteria were found to produce xylitol from D-arabitol with resting cell reaction¹. Among them, *Gluconobacter oxydans* ATCC 621 produced 29.2 g/l xylitol from 52.4 g/l D-arabitol after incubation for 27 h. The production of xylitol was markedly enhanced by addition of 5% (v/v) ethanol and 5 g/l D-glucose to the reaction mixture. Under these conditions, 51.4 g/l xylitol was obtained from 52.4 g/l D-arabitol, a molar yield of 98%, after incubation for 27 h. This conversion consisted of two successive reactions, oxidation of D-arabitol to D-xylulose by membrane-bound D-arabitol dehydrogenase, and reduction of D-xylulose to xylitol by soluble NADH-dependent xylitol dehydrogenase (XDH). The mechanism of the effect of ethanol on the xylitol production was investigated by use of disruptants of membrane-bound alcohol dehydrogenase genes, and it was suggested that NADH was generated in the presence of ethanol via the NAD-dependent soluble alcohol dehydrogenase.

Since the reduction of D-xylulose to xylitol by XDH was a rate-limiting step, we constructed recombinant *G. oxydans* strains harboring *xdh*-expression vectors. We constructed a host-vector system of *G. oxydans* ATCC 621, and also cloned the *xdh* gene². Expression plasmids for the *xdh* gene were constructed and used to produce recombinant strains of *G. oxydans* that possessed up to 11-fold greater XDH activity than the wild-type strain. The strain harboring the *xdh* expression vectors produced 57 g/l xylitol from 225 g/l D-arabitol, whereas the control strain produced 27 g/l xylitol. These results demonstrated that enhancement of XDH activity in *G. oxydans* improved xylitol productivity. But it also suggested that the enhancement of NADH supply was required to increase an accumulation of xylitol.

The NADH-regeneration mechanism of *G. oxydans* was unclear due to the lack of key enzymes in glycolysis. So next, we investigated the NADH-regeneration pathway of *G. oxydans*. Using an in vitro xylitol production assay, we identified two soluble proteins that can increase xylitol production from D-arabitol, one was transaldolase / glucose-6-phosphate isomerase bifunctional enzyme and the other was ribulokinase³. We also revealed that both the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase can reduce NAD and NADP⁴. These results suggested that NADH can be regenerated through the pentose phosphate pathway in *G. oxydans*, and gave information as a guide for further molecular breeding of acetic acid bacteria, important industrial microorganisms.

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V-6: Oxidation of Lactose to Lactobionic Acid by Acetic Acid Bacteria

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When lactose is acted on by an oxidases and dehydrogenases, the product is lactobionic acid (4-O- β -D-galactopyranosyl-D-gluconic acid; LacA), an aldonic oligosaccharide in which galactose is linked to gluconic acid via an ether-like linkage. Presently, LacA and its salts are used mainly as highly priced specialty products. Lactobionate is, for example, added to solutions used for stabilizing organs prior to transplantation. LacA is used to enhance the solubility of macrolide antibiotics such as erythromycin. Further, calcium lactobionate is used in human and veterinary medicine to stabilize supersaturated solutions of calcium gluconate, a traditional calcium supplement.

The occurrence of LacA in foods and beverages has not been previously demonstrated. We demonstrated that LacA was found in a Caucasian fermented milk product, known in Japan as "Caspian Sea Yogurt." The presence of LacA in fermented milk was indicated by both chromatographic analyses using high-performance anion-exchange chromatography with pulsed amperometric detection and mass spectrometric analysis. A substantial amount of LacA was found in the upper layer of the yogurt, especially in the top 1 cm. Forty five milligrams of LacA per 100 g of the upper yogurt layer was accumulated in 100g of the upper layer yogurt after 4 days of fermentation. And the 100 g of the yogurt contained 2.1 mg of LacA on average. This implies that the annual LacA intake of individuals consuming 100 g of the yogurt daily would range from 0.5 to 1.0 g.

A lactose-oxidizing bacterium was isolated from the fermented milk and was identified as *Acetobacter orientalis*. Therefore, we investigated the oxidization of lactose to LacA in acetic acid bacteria. Almost all the acetic acid bacteria tested oxidized lactose to LacA, with *Gluconobacter cerinus* UTBC-427 showing the highest activity.

We examined different LacA bioconversion conditions, aiming to develop a microbial production system for LacA. Microbial conversion of lactose to LacA was carried out using resting *G. cerinus* UTBC-427 cells. To obtain resting *G. cerinus* cells for bioconversion, the strain was cultivated at 25°C for 24 h in a medium (pH 7.0) consisting of 0.5% (w/v) glucose, yeast extract, polypepton, and 0.1% magnesium sulfate. The effects of reaction conditions on lactose oxidizing activity were studied to develop an efficient conversion system. The oxidizing activity of resting cells was most active at pH 6.0 and 50°C. The cells converted 30% (w/v) lactose and 3% CaCO₃ to calcium lactobionate in 80% yield within 48 h at 40°C.

We also tested if a diet supplemented with LacA enhanced calcium absorption in growing rats. Male SD rats were fed a control diet or diets containing LacA for 5 weeks. Our data indicated that calcium absorption in rats fed a diet supplemented with LacA (LacA/Ca w/w ratio >0.5) was higher than that in rats fed a control diet, suggesting that a diet supplemented with LacA produced by acetic acid bacteria can potentially increase calcium absorption.

V-7: Current and future industrial L-ascorbic acid production

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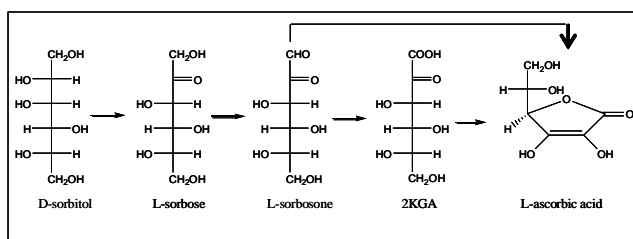
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L-ascorbic acid (vitamin C) was isolated for the first time from various natural sources in 1928 by Szent-Györgyi, who soon after identified the compound as the long-sought antisorbutic factor. Today, L-ascorbic acid is widely used in the feed, food and pharmaceutical industry as preservative and nutritional supplement making use of its anti-oxidative properties. The current L-ascorbic acid world market reaches approximately 100'000 metric tons.

The Reichstein-Grüssner process designed 1933 and improved ever since provided industrial L-ascorbic acid for decades via 2-keto-L-gulonic acid (2KGA) as a key intermediate. Starting from D-glucose the process consists of a series of high yield steps: (i) catalytic reduction at C1 affording D-sorbitol, (ii) *G. oxydans* catalysed bio-oxidation at C5 affording L-sorbose, (iii) chemical oxidation at C6 affording L-sorbosone and 2KGA, (iv) cyclisation of 2KGA affording L-ascorbic acid. The chirality at C2 and C3 of glucose is preserved providing the L-threo-configuration at C4 and C5 of the product.

The chemical oxidation steps at C1 of L-sorbose (i.e. C6 of the starting D-glucose) can be replaced by bio-oxidation with *Ketogulonicigenium sp.* as biocatalyst. For efficient oxidation co-cultivation with a helper strain, e.g. *Bacillus megaterium*, is mandatory. Using this technology, Chinese producers have emerged during the 1990ies and became the dominant producers in today's L-ascorbic acid market.

The cyclization of 2KGA through 1,4 lactonization followed by tautomerization, is achieved in an acid or base catalysed reaction, the latter via the 2KGA methyl ester. Several attempts geared to the natural L-ascorbic acid pathway in plants involving L-galactose to produce the vitamin directly have been met with difficulties. Recently, two dehydrogenases were discovered in *Ketogulonicigenium sp.* and *Gluconobacter sp.* that oxidise L-sorbosone at C1 affording L-ascorbic acid instead of 2KGA. Purification and characterization of these enzymes showed that both of them have PQQ as a redox cofactor, but are otherwise distinct. The enzyme from *Ketogulonicigenium sp.* designated SNDH has similarity to soluble PQQ-dependent glucose dehydrogenases, whereas the one from *Gluconobacter* designated SNDHai resembles membrane-bound PQQ-dependent glucose dehydrogenases. It was suggested that SNDH oxidizes the 1,4 furanose isomere of L-sorbosone to ascorbic acid, whereas 2KGA is derived from the oxidation of one of the L-sorbosone pyranose isomers. The discovery of SNDH and SNDHai opens the possibility for a direct route to L-ascorbic acid via L-sorbosone.



Current industrial L-ascorbic acid synthesis from D-sorbitol via 2KGA: In the Reichstein-Grüssner process L-sorbose 2,6 pyranosid is chemically oxidized at C1 after protection of the OH-groups at C2, C3 and C4, C6 with acetone. Fermentative 2KGA is obtained by microbial oxidation of L-sorbose at C1, no protection of the other OH-groups required.

Possible future direct process: Similar to fermentative 2KGA, but direct conversion of L-sorbosone to L-ascorbic acid with microorganisms expressing SNDH or SNDHai.