

## **Poster Session V**

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

## **PV-1: Development of thermotolerant sorbose fermentation and Physiological analysis of the thermotolerant *Gluconobacter* species**

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*Gluconobacter* is one genus of acetic acid bacteria, and well known to have a strong ability to oxidize a broad range of sugars and sugar alcohols. These oxidations called as oxidative fermentation are uniquely carried out by membrane-bound enzymes located on the outer surface of the cytoplasmic membrane, and the oxidation products are accumulated in the culture medium. These features of the microorganism lead to the applications in industry for fermentation of valuable products such as L-sorbose, dihydroxyacetone, D-gluconate, and keto-D-gluconates. But, these fermentations by *Gluconobacter* are normally done below 30°C, the optimum growth temperature. So, large amount of energy for cooling is needed in the industrial fermentation processes. Thus the development of thermotolerant acetic acid bacteria would release us from the strict temperature control.

Many thermotolerant *Gluconobacter* species were isolated from Thailand. They can grow and perform oxidative fermentation at 37°C, in which mesophilic strains can not grow<sup>1)</sup>. Among them, CHM43 (identified as *Gluconobacter frateurii*) was selected as the best strain to produce L-sorbose at higher temperature. It has been reported that *Gluconobacter* has two types of membrane-bound D-sorbitol-oxidizing enzymes, one is a glycerol dehydrogenase having pyrroloquinoline quinone (PQQ), and another is a sorbitol dehydrogenase containing FAD and heme *c* as the prosthetic groups<sup>2,3)</sup>. These dehydrogenases function by linking to terminal ubiquinol oxidases via ubiquinone in the respiratory chain to produce D-sorbose. To obtain more thermostable strain, CHM43 strain was grown successively at 38.5°C, which is the upper limited temperature for the growth. After several repeating of shaking cultures, the strain was adapted to grow well at 38.5°C and CHM43ad was isolated.

To understand the thermotolerant mechanism of CHM43ad strain, D-sorbitol-oxidizing respiratory chain, H<sub>2</sub>O<sub>2</sub> sensitivity, and generation of reactive oxygen species (ROS) in the cells were compared with CHM43 original strain or IFO3264<sup>T</sup> strain (mesophilic type strain of *Gluconobacter frateurii*). Although catalase and superoxide dismutase activities were not much changed, ROS level of CHM43ad strain at higher temperature (37, 38.5°C) was decreased compared with CHM43 original strain and IFO3264<sup>T</sup> strain. Thus it seems that CHM43ad strain generates much less ROS that it can grow better at higher temperature.

This study was supported by Program for Promotion of Basic Research Activities for Innovative Biosciences.

1. D. Moonmangmee, et al., *Biosci. Biotechnol. Biochem.*, **64**, 2306-2315 (2000)
2. T. Sugisawa, et al., *Biosci. Biotechnol. Biochem.*, **66**, 57-64 (2002)
3. E. Shinagawa et al., *Agric. Biol. Chem.*, **46** 135-141 (1982)

## **PV-2: *Gluconobacter frateurii* NBRC3271 possesses two types of FAD-gluconate dehydrogenases for 2-keto-D-gluconic acid production**

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2-Keto-D-gluconic acid (2KGA) in *Gluconobacter* has been known to be a product from D-gluconic acid oxidation, catalyzed by an FAD containing D-gluconate dehydrogenase (FAD-GADH). This enzyme has three subunits encoded by *gndSLC* genes which have an operon-like structure. By previous studies, disruption of *gndL* which encodes the dehydrogenase subunit of this enzyme could not abolish 2KGA production in *Gluconobacter frateurii* NBRC3271. In this study, we identified *gndFGH* genes that encode a similar, but different type of FAD-GADH. We named the *gndSLC* gene and *gndFGH* gene products as FAD-GADH-1 and FAD-GADH-2, respectively. Formation of 2KGA was observed in each GADH-1 or GADH-2 disrupted strain but completely disappeared in the mutant strain defected in both GADH-1 and GADH-2. GADH-2 was found in several *Gluconobacter* strains, whereas, until now, GADH-1 was found only in *G. frateurii* NBRC3271 and plays a main role in 2KGA production in this strain. It is supposed that GADH-2 may be found as a general enzyme in *Gluconobacter* strains, whereas GADH-1 is a special FAD-GADH that would confer high productivity of 2KGA and thus be found only in some dominant 2KGA-producing strains.

This work was supported by Program for Promotion of Basic Research Activities for Innovative Biosciences.

### **PV-3: Role of CIO on the respiratory chain and ketogluconate fermentation**

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*Gluconobacter* oxidizes a broad range of organic compounds and accumulates the corresponding products into the culture medium. These reactions are called oxidative fermentation which is carried out by many different primary dehydrogenases localized on the outer surface of cytoplasmic membrane of the organisms. Such the oxidative fermentation is important for industrial production of L-sorbose, ketogluconates or dihydroxyacetone etc, of which the ketogluconate production has been shown to occur by the two different enzymes, PQQ-dependent glycerol dehydrogenase (GLDH) and FAD-dependent gluconate dehydrogenase (GADH). GADH selectively oxidizes gluconate to 2-ketogluconate (2KGA), while GLDH oxidizes a broad range of sugar alcohols including gluconate, of which the reaction product was shown to be 5-ketogluconate (5KGA). These membrane-bound enzymes work with terminal oxidases, in the respiratory chain, which are involved in the generation of an electrochemical proton gradient. The respiratory chain of *Gluconobacter suboxydans* IFO 12528 has been shown to be terminated in two different ways, cyanide-sensitive cytochrome *bo*<sub>3</sub> oxidase and cyanide-insensitive bypass oxidase. Recently, in *Gluconobacter* genome, *cydAB* gene encoding cytochrome *bd*, has been shown to be present and further to have much higher homology to *Pseudomonas cioAB*. Thus, it was expected that the CioAB would correspond to the bypass oxidase of *Gluconobacter*. To elucidate the role of CioAB on the oxidative fermentation, ketogluconate fermentation and the respiratory chain were examined.

We identified by gene disruption and over-expression that CioAB actually works as the cyanide-insensitive oxidase (CIO) in the respiratory chain in *G. suboxydans* IFO 12528. CIO exhibited the apparent Km value of 40  $\mu$ M for Q<sub>1</sub>H<sub>2</sub> and IC<sub>50</sub> value of 13 mM for KCN, but no clear spectroscopic features even in the membranes of CIO-overproducing strain. When ketogluconate fermentation was done in *cioAB* disruptant and overproducing strains, 5KGA production was increased in the disruptant but decreased in the overproducer, while 2KGA production was vice versa. In the resting cell reaction, 5KGA production was largely inhibited in the presence of NaN<sub>3</sub> while 2KGA production was vice versa again. Thus, the role of CIO on ketogluconate fermentation is discussed together with the results obtained from GLDH or GADH disruptants.

## PV-4: Formaldehyde-Oxidizing Enzyme and Formaldehyde Elimination by *Acetobacter* sp. SKU 14

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Formaldehyde is a toxic compound that is ubiquitous in our environment and one of the most common indoor air pollutants originating from various synthetic materials. Formaldehyde oxidation is a central and critical step in methylotrophic bacteria because formaldehyde is a key intermediate in the metabolism of C<sub>1</sub>-compound such as methanol and methylamine. Some organisms are able to oxidize formaldehyde to formate and formate to carbon dioxide. Although *Acetobacter* sp. SKU 14 is not methylotrophic, this strain was found to oxidize formaldehyde and also formate with membrane fraction. The enzymes involved in oxidation of these compounds are NAD(P)-independent formaldehyde and formate dehydrogenase.

Formaldehyde-oxidizing activity decreased when *Acetobacter* sp. SKU 14 was cultivated with EDTA. The cultivation with molybdenum increased formate-oxidizing activity, especially formate dehydrogenase activity. NAD(P)-independent formaldehyde dehydrogenase was purified from membrane fraction of glycerol-grown cells<sup>1</sup>. The enzyme was solubilized with 1% Tween 20 at pH 2.85 and purified to homogeneity through two column chromatographies on DEAE-Sephadex A-50 and Q-Sepharose in the presence of 0.1% Tween 20 and 0.1% Triton X-100. The solubilized enzyme was very stable during two column chromatographies, bringing about a high overall yield of 89%. The enzyme purified together with a cytochrome *c* showed a single protein band on native-PAGE and was dissociated into three different subunits upon SDS-PAGE with molecular masses of 78 kDa, 55 kDa, and 18 kDa. The two subunits of 78 and 55 kDa contained cytochrome *c* because of positive heme staining. The purified enzyme was finally characterized as a quinoprotein alcohol dehydrogenase (QADH). The substrate specificity of the enzyme was found to be broad toward aldehydes and alcohols, and alcohols, especially *n*-butanol, *n*-propanol, and ethanol, were oxidized more rapidly than formaldehyde.

Formaldehyde elimination in solution was tried using the membrane fraction and the resting cells of *Acetobacter* sp. SKU 14<sup>2</sup>. Formaldehyde was oxidized to carbon dioxide *via* formate successively with formaldehyde dehydrogenase and formate dehydrogenases found in the organism. Since NAD(P)-independent formaldehyde dehydrogenase was found to be QADH, formaldehyde elimination was also examined with *Gluconobacter suboxydans* IFO 12528, known as high production of QADH. The resting cells of *G. suboxydans* IFO 12528 also degraded formaldehyde. However, different from *Acetobacter* sp. SKU 14, formate production was found with *G. suboxydans* IFO 12528 due to the absence of formate dehydrogenase. The resting cells of *Acetobacter* sp. SKU 14 were effective at higher temperature than *G. suboxydans* IFO 12528 because the former strain is thermotolerant. Thus *Acetobacter* sp. SKU 14 is a useful strain for formaldehyde elimination and applicable for food processing because acetic acid bacteria are nonpathogenic and edible microorganisms.

1. Shinagawa, E. et al., *Biosci. Biotechnol. Biochem.*, **70**, 850-857 (2006)

2. Shinagawa, E. et al., *J. Biosci. Bioeng.*, **105**, 292-295 (2008)

## **PV-5: Membrane-bound D-Glucono- $\delta$ -lactonase in Acetic Acid Bacteria**

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Many different lactonases hydrolyzing lactones such as D-glucono- $\delta$ -lactone, 6-phospho-D-glucono- $\delta$ -lactone, xylonolactone, L-rhamnono- $\gamma$ -lactone, aldonate lactones, and aromatic lactones, are known as an important enzyme in cytoplasmic metabolism of eukaryotes to prokaryotes. When D-glucose or D-glucose-6-phosphate is oxidized in cytoplasm by NAD(P)-dependent D-glucose dehydrogenase or NADP-dependent D-glucose-6-phosphate dehydrogenase, corresponding lactone is formed before further hydrolyzed to D-gluconic acid or 6-phospho-D-gluconic acid by the action of cytoplasmic lactonases (sGLase).

Acetic acid bacteria and other aerobic bacteria participated in oxidative fermentation are accommodated by a highly developed periplasmic oxidase system on the outer surface of the cytoplasmic membrane. Quinoprotein D-glucose dehydrogenase (QGDH), one of the representative membrane-bound dehydrogenases in oxidative fermentation, rapidly oxidizes D-glucose and D-gluconate is accumulated in culture medium. In this process, D-glucono- $\delta$ -lactone is the direct oxidation product of D-glucose, which is in turn converted to D-gluconate enzymatically by D-glucono- $\delta$ -lactonase or spontaneously. In oxidative fermentation, besides D-aldonate production, 2-keto-D-gluconate, 5-keto-D-gluconate, and 2,5-diketo-D-gluconate are formed from D-glucose. Other kinds of aldonates such as galactonate and xylonate are also oxidation products of respective aldoses catalyzed by QGDH or other kinds of oxidases of membrane-bound. Half a century ago (1958), King and Cheldelin predicted the existence of D-glucono- $\delta$ -lactonase in the membrane fraction of acetic acid bacteria. In 1962, accumulation of lactones in culture medium of acetic acid bacteria and other aerobic organisms were indicated. In 1988, Buchert and Viikari confirmed existence of membrane-bound D-glucono- $\delta$ -lactonase (mGLase) in the membrane fraction of *G. oxydans* ATCC 621, in the study on D-xylose metabolism. However, purification and characterization of mGLase remained to be investigated.

Many times of trial and error have finally led us to stable solubilization of mGLase from the membrane fraction of *G. oxydans* IFO 3244 and the enzyme was purified to an apparent homogeneity. Hydrophobic nature of mGLase was striking and mGLase was inactivated soon, unless the hydrophobic region was protected after solubilization. Unlike sGLases of which optimum pH show at pH 7-7.5, mGLase showed the optimum pH at 5.5. This report may be the first one dealing with mGLase, unlike many numbers of sGLase of eukaryotes to prokaryotes.

## **PV-6: Molecular cloning of D-fructose dehydrogenase from *Gluconobacter frateurii* and its biotechnological applications**

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Bioelectrocatalysis is concerned with the use of biological materials as catalysts for electrochemical processes. Of particular value in this context are those enzymes that catalyze oxidations and reductions. These often have or require cofactors, other proteins or coenzymes, involved in the electron transfer steps. Direct electron transfer (DET)-based catalytic phenomena have been reported for limited species of enzymes<sup>1</sup>. Acetic acid bacteria produce a various kinds of redox enzymes, and some of them can directly transfer electrons to electrodes.

D-Fructose dehydrogenase from *Gluconobacter frateurii* (FDH) is a membrane-bound flavohemoprotein and works as a good catalyst in DET-type bioelectrocatalytic oxidation of D-fructose<sup>2</sup>. This enzyme shows high substrate specificity to D-fructose and is expected to be utilized in the field of food analysis and clinical use. In this study, we have cloned the gene cluster encoding three subunits of FDH. In addition, the  $\alpha$  subunit of FDH, catalytic subunit containing the flavin, was expressed in *E. coli*, and its properties were investigated.

We have cloned a 3.9-kb nucleotide gene encoding FDH, about 1200-amino acid long 128 kDa. A protein similarity search on data base by the BLAST program revealed that FDH is similar to some redox enzymes which can directly transfer electrons to electrodes, including gluconate dehydrogenase from *Gluconobacter* sp. (30% identical).

The 1.7-kb gene fragment consisting of the structural gene of *fdhL*, encoding the  $\alpha$  subunit, was ligated into pET26b(+) cloning vector to produce pET26b(+)-*fdhL*, and the *E. coli* strain Rosetta(DE3) cells containing pET26b(+)-*fdhL* were grown in liquid LB medium supplemented with 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside and 0.5 mg mL<sup>-1</sup> rivoflavin. The  $\alpha$  subunit monomer was capable of the oxidation of D-fructose. We will discuss the electrochemical properties of the DET-type enzymes from *Gluconobacter* sp.

1. Ikeda, T., et al., in *Frontiers in Biosensorics I*, 243-266 (1997)
2. Kamitaka Y., et al., *Chem. Lett.*, **36**, 218-219 (2007)

## **PV-7: Characterization of levansucrase and levan produced by *Acetobacter nitrogenifigens***

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Recently described *Acetobacter nitrogenifigens* strain RG1 isolated from Kombucha tea, a nitrogen fixing bacteria from Acetobacteraceae family, has been found to secrete out this interesting enzyme Levansucrase (E.C. number 2.4.1.10) is sucrose-2,6- $\beta$ -D-fructan 6- $\beta$ -D-fructosyltransferase. Levansucrase catalyzes the transfer of the fructosyl residue, from sucrose to a variety of acceptors including water (sucrose hydrolysis), glucose (exchange), sucrose (oligofructoside synthesis) and fructan (polymerase reaction). Levansucrase represents almost 10% of the total extracellular protein which has an average molecular weight of around 50KDa. It has dual characteristic of hydrolysis of sucrose and synthesis of levan on 5% sucrose agar plate. Levan has a degree of polymerization more than 15 as inferred from TLC plate. Optimization for condition of hydrolytic and levan synthase activity at a temperature of 30°C and pH 5.0 within 5 days of bacterial growth. Effect of different metal ions on the activities have been studied that exhibited a diverse effect on hydrolytic and levan synthase activity indicating probable two different sites for each function. Studies on levansucrase from *Acetobacter nitrogenifigens* is the first in the *Acetobacter* genus.

## PV-8: Synthesis of Useful Carboxylic Acids from Aromatic Aldehydes and Prochiral Diol Using Oxidation System of *Acetobacter* Strains

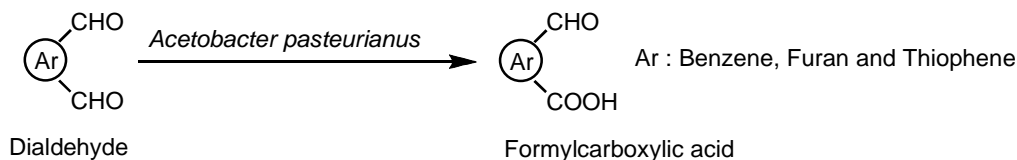
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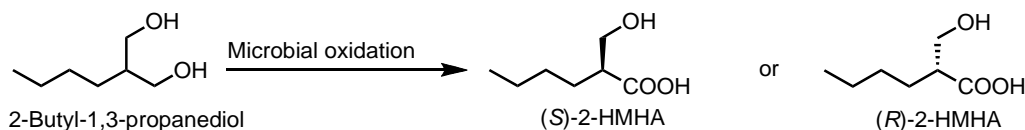
Acetic acid bacteria have extremely high oxidation activity for ethanol, which have been used for vinegar production as an industrial application. Furthermore, various studies on oxidation of various alcohols and aldehydes by acetic acid bacteria have been developed for making full use of the characteristics of chemo-, regio- or stereo-selectivity for hydroxyl group and/or formyl group.

Recently, we found that *Acetobacter rancens* NBRC 3297 showed very high oxidation activity for furfural and vanillin. Under optimized conditions, *A. rancens* NBRC 3297 produced 981 mM (110 g l<sup>-1</sup>) 2-furoic acid and 233 mM (39.2 g l<sup>-1</sup>) vanillic acid by a sequential addition of the corresponding aldehyde, respectively<sup>1</sup>. In *A. pasteurianus* NBRC13753, there had high oxidation activity for one formyl group of aromatic dialdehydes (Fig 1). Dialdehydes such as isophthalaldehyde, 2,5-furandicarbaldehyde and 2,5-thiophenedicarbaldehyde were selectively oxidized to give the corresponding formylcarboxylic acid with 86-91% molar conversion yield<sup>1</sup>.



**Fig.1 Oxidation of aromatic dialdehyde by *Acetobacter pasturianus***

There are optically active hydroxycarboxylic acids as promising chiral building block for the synthesis of pharmaceuticals and agrichemicals. We examined the synthesis of optically active 2-hydroxymethylhexanoic acid (2-HMHA) which can be applied to antibacterial agent for methicillin-resistant *Staphylococcus aureus* (MRSA). We have focused on the asymmetric oxidation of 2-butyl-1,3-propanediol and have surveyed 2-HMHA-producing strains with high stereoselectivity (Fig. 2). Among strains tested, *A. pasteurianus* IAM 12073 showed the highest oxidation activity for 2-butyl-1,3-propanediol and afforded 12 g l<sup>-1</sup> (*S*)-2-HMHA with 89% enantiomeric excess (e.e.) and 83% molar conversion<sup>2</sup>. However, there was not found high *R*-2-HMHA-producing activity in our stocked acetic acid bacteria. On the other hand, *Pseudomonas putida* NBRC 3738 produced (*R*)-2-HMHA (94% e.e.)<sup>2</sup>.



**Fig. 2 Microbial asymmetric oxidation of 2-butyl-1,3-propanediol**

1. Mitsukura, K. et al., *Biotechnol. Lett.*, **26**, 1643-1648 (2004)
2. Mitsukura, K. et al., *Appl. Microbiol. Biotechnol.*, **76**, 61-65 (2007)

## **PV-9: Production of Glyceric Acid from Glycerol by Acetic Acid Bacteria**

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Glycerol, a nontoxic, edible, biodegradable compound mostly derived from natural sources such as vegetable oils and animal fats, is produced as a by-product in the growing oleochemical industry that produces soaps, fatty acids, waxes, and surfactants. Glycerol is also a by-product from biodiesel production, which has increased dramatically during the last 10 years, resulting a large in excess of glycerol, especially in Europe. Hence, the price of raw glycerol dropped significantly from about 650–700 €/ton in 1996 to about 250–300 €/ton in 2004, and further decreased in 2005 (roughly to 150–200 €/ton). This indicates that glycerol is an attractive feedstock for producing useful chemicals. Fermentation or bioconversion processes using glycerol as a substrate have been increasingly explored in the last decade. However, no highly productive, promising chemicals for industrial use have been found, except for 1,3-propanediol and dihydroxyacetone (DHA).

One promising glycerol derivative, glyceric acid (GA), is obtained by metal-catalytic oxidation of the primary hydroxyl groups of glycerol. To date, the market for GA is limited owing to the high cost of chemical synthesis. However, GA has the potential to be a building block for several chemical compounds used in the pharmaceutical and cosmetics industry. Chemical synthesis of GA from glycerol yields a racemic mixture of DL-glyceric acid, whereas D-glyceric acid (D-GA) is specifically formed as a product of fructose breakdown. However, little is known about the production of D-GA from glycerol via bioprocesses.

Acetic acid bacteria are obligate aerobes that show efficient oxidation of a wide range of substrates, such as alcohols, sugars, sugar acids, and sugar alcohols. For such oxidative reactions, these bacteria have numerous membrane-bound dehydrogenases and oxidoreductases, and therefore, energy-consuming transport of substrates into the cell and products out of the cell is not required. Among acetic acid bacteria, *Gluconobacter oxydans* has been used for the industrial production of DHA, which is used as a tanning agent in the cosmetics industry. To produce DHA, the bacteria oxidize a secondary hydroxyl group of glycerol, and at the same time produce a small amount of glycerate as a byproduct.

We searched for a producer of D-GA among acetic acid bacteria and investigated the productivity of D-GA by the selected strain. We selected *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* species as screening sources of acetic acid bacteria. Most of the tested acetic acid bacteria could produce 1.6 to 17.9 g/l GA from 10% (v/v) glycerol when intact cells were used as the enzyme source. *Acetobacter tropicalis* NBRC16470 and *Gluconobacter* sp. NBRC3259 were the best GA producers among respective genera tested and were therefore further investigated. The enantiomeric composition of the produced GA was suggested to be D-GA. The effects of initial glycerol concentration, nitrogen sources, and initial pH of the medium on GA production were investigated. Under the optimized conditions, *A. tropicalis* NBRC16470 and *Gluconobacter* sp. NBRC3259 produced 20.0 g/l and 24.6 g/l D-GA, respectively, from c.a. 200 g/l glycerol within 5 days of incubation. This study was supported by Industrial Technology Research Grant Program in 2008 from New Energy and Industrial Technology Development Organization (NEDO) of Japan.