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MINI-REVIEW

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New developments in oxidative fermentation

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Abstract Oxidative fermentations have been well established for a long time, especially in vinegar and in L-sorbose production. Recently, information on the enzyme systems involved in these oxidative fermentations has accumulated and new developments are possible based on these findings. We have recently isolated several thermotolerant acetic acid bacteria, which also seem to be useful for new developments in oxidative fermentation. Two different types of membrane-bound enzymes, quinoproteins and flavoproteins, are involved in oxidative fermentation, and sometimes work with the same substrate but produce different oxidation products. Recently, there have been new developments in two different oxidative fermentations, D-gluconate and D-sorbitol oxidations. Flavoproteins, D-gluconate dehydrogenase, and D-sorbitol dehydrogenase were isolated almost 2 decades ago, while the enzyme involved in the same oxidation reaction for D-gluconate and D-sorbitol has been recently isolated and shown to be a quinoprotein. Thus, these flavoproteins and a quinoprotein have been re-assessed for the oxidation reaction. Flavoprotein D-gluconate dehydrogenase and D-sorbitol dehydrogenase were shown to produce 2-keto-D-gluconate and D-fructose, respectively, whereas the quinoprotein was shown to produce 5-keto-D-gluconate and L-sorbose from D-gluconate and D-sorbitol, respectively. In addition to the quinoproteins described above, a new quinoprotein for quinate oxidation has been recently isolated from *Gluconobacter* strains. The quinate dehydrogenase is also a membrane-bound quinoprotein that produces 3-dehydroquinate. This enzyme can be useful for the production of shikimate, which is a convenient salvage synthesis system for many antibiotics, herbicides, and aromatic amino acids synthesis. In order to reduce energy costs of oxidative fermentation in industry, several thermotolerant acetic acid bacteria that can grow up to 40°C have been isolated. Of such isolated strains, some thermotolerant *Acetobacter* species were found to be useful for vinegar fermentation at a high temperature such 38–40°C, where mesophilic strains showed no growth. They oxidized higher concentrations of ethanol up to 9% without any appreciable lag time, while alcohol oxidation with mesophilic strains was delayed or became almost impossible under such conditions. Several useful *Gluconobacter* species of thermotolerant acetic acid bacteria are also found, especially L-erythrulose-producing strains and cyclic alcohol-oxidizing strains. *Gluconobacter frateurii* CHM 43 is able to rapidly oxidize *meso*-erythritol at 37°C leading to the accumulation of L-erythrulose, which may replace dihydroxyacetone in cosmetics. *G. frateurii* CHM 9 is able to oxidize cyclic alcohols to their corresponding cyclic ketones or aliphatic ketones, which are known to be useful for preparing many different physiologically active compounds such as oxidized steroids or oxidized bicyclic ketones. The enzymes involved in these *meso*-erythritol and cyclic alcohol oxidations have been purified and shown to be a similar type of membrane-bound quinoproteins, consisting of a high molecular weight single peptide. This is completely different from another quinoprotein, alcohol dehydrogenase of acetic acid bacteria, which consists of three subunits including hemoproteins.

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Introduction

It has been established that acetic acid bacteria produce acetate from ethanol by two sequential oxidation reactions of membrane-bound quinohemoprotein alcohol dehydrogenase (ADH) and membrane-bound quinohemo-

protein aldehyde dehydrogenase (ALDH)(Matsushita et al. 1994). Such oxidation reactions are termed "oxidative fermentation," since they involve incomplete oxidation of a substrate accompanied by accumulation of large amounts of the corresponding oxidation product in the growth medium. In addition to the well-established oxidative fermentation for vinegar production (acetate fermentation) and L-sorbose fermentation, different oxidative fermentations have been proposed for industrial production of useful materials over the last 100 years. They were well developed for manufacturing useful oxidation products; however, little information on the enzymes concerned and the reaction mechanism of oxidative fermentation had been accumulated until numerous membrane-bound dehydrogenases were characterized and the structures and mechanism of oxidative fermentation elucidated (Matsushita et al. 1994). In early studies, there was a great deal of confusion over the enzyme localization for oxidative fermentation; namely, whether they are membrane-bound enzymes or cytosolic enzymes. Most came from insufficient cell fractionation. Moreover, localization of enzymes in the periplasmic space, which may appear in the soluble fraction on cell disruption, was not well characterized and there was confusion over whether they were localized in the cytoplasmic fraction. Even recently, there has still been misunderstanding, with the belief that oxidative fermentation is brought about by cytosolic NAD(P)-dependent dehydrogenases.

Extensive studies on membrane-bound dehydrogenases in acetic acid bacteria, all of which are important in oxidative fermentation, have allowed us to conclude that the functioning coenzyme in oxidative fermentation is either PQQ or FAD (Matsushita et al. 1994). Substrate oxidation by membrane-bound dehydrogenases is coupled with the respiratory chain of the organisms, and the electrons generated are transferred to the terminal oxidase yielding oxidation energy. Thus, they form a specific machinery in substrate oxidation as a membrane-bound dehydrogenase-dependent oxidase system (Matsushita et al. 1994; Matsushita et al. 2002a, b). On the other hand, the cytosolic NAD(P)-dependent dehydrogenases make no contribution to oxidative fermentation (Matsushita et al. 1994). Vinegar production, L-sorbose production, and D-gluconate production are catalyzed by PQQ-dependent dehydrogenases (quinoproteins), while 2-keto-D-gluconate (2KGA) production, 2,5-diketo-D-gluconate production, and 5-keto-D-fructose production involve flavoprotein dehydrogenases. Vinegar production and L-sorbose production are the most-typical examples of oxidative fermentation and can be regarded as the classic microbial bioconversion, because the individual enzymes from acetic acid bacteria catalyze an incomplete one-step oxidation reaction and equivalent amounts of oxidized substrates are accumulated in the culture media.

We have added experimental evidence that the enzymes obeying the rule contain PQQ as the coenzyme without exception. However, a new D-sorbitol dehydrogenase with a covalently bound FAD as the coenzyme has been indicated in acetic acid bacteria, which does not obey the Bertrand-Hudson's rule and catalyzes oxidation of D-sorbitol to D-fructose. This new finding will likely lead to the development of a more-successful method for high D-fructose syrup production (Adachi et al. Japanese Patent Application 2001, 358707). Oxidative fermentation leading to the production of 5-keto-D-gluconate (5KGA) was discovered during observation of the reaction mechanism of polyalcohol dehydrogenase in acetic acid bacteria (Adachi et al. 2001). It is said that, in oxidative fermentation, enzymes in acyclic sugar alcohol oxidation of the erythro form and R-configuration exclusively obey the Bertrand-Hudson's rule (Hann et al. 1938; Kersters et al. 1965). The finding of a membrane-bound quinoprotein quinate dehydrogenase in acetic acid bacteria has led us to the shikimate pathway. 3-Dehydroquinate production by oxidative fermentation would enable us to produce shikimate on an industrial scale (Adachi et al. Japanese Patent Application 2001, 267257). This new finding has led to the rebirth of cinchona agriculture in tropical area, thus ensuring adequate supplies of quinate in the future.

Recent new discoveries in oxidative fermentation by acetic acid bacteria have led us to a new perspective on oxidative fermentation, which is attractive from both academic and practical aspects. The finding of thermotolerant acetic acid bacteria has allowed a reduction in energy costs, including heating-cooling power, as exemplified by vinegar fermentation at higher temperatures (Saeki et al. 1997b). L-Erythrulose production was also more effective with a thermotolerant strain (Moonmangmee et al. 2002). The finding of a membrane-bound cyclic ADH has opened up a new field of application of the enzyme to enantioselective oxidation of cyclic alcohols. The reaction products have many important uses (Moonmangmee et al. 2002). In this article, several recent developments in oxidative fermentations are described together with the enzymology and their possible applications.

D-Fructose production is catalyzed by an alternative D-sorbitol dehydrogenase

The D-Sorbitol dehydrogenase contributing to L-sorbose production was identified as a quinoprotein in which PQQ functions as the coenzyme (Adachi et al. 2001; Ameyama et al. 1985), while FAD-containing D-sorbitol dehydrogenase, an alternative D-sorbitol dehydrogenase, is important in D-fructose production (Adachi et al. Japanese Patent Application 2001, 358707). D-fructose production by oxidative fermentation must be superior to the well-established method employing D-glucose isomerase, since the oxidative fermentation catalyzes irreversible one-way oxidation and D-sorbitol is oxidized to D-fructose without any reaction equilibrium, unlike D-glucose isomerase. Some strains of the *Gluconobacter* genus, such as *G. suboxydans* IFO 3258, have a novel D-sorbitol oxidizing enzyme, which forms D-fructose as the oxidation product,

in addition to L-sorbose yielding quinoprotein D-sorbitol dehydrogenase. The majority of D-sorbitol dehydrogenase indicated so far in the *Gluconobacter* genus shows L-sorbose yielding, which is useful in the vitamin C industry. L-sorbose from D-sorbitol, D-fructose from D-mannitol, L-ribulose from ribitol, and D-xylulose from D-arabitol are examples of reactions that obey the Bertrand-Hudson's rule in sugar alcohol oxidation (Hann et al. 1938; Kersters et al. 1965). D-Gluconate conversion to 5KGA has been recently added to the reactions obeying the rule, although 2KGA production from D-gluconate does not follow the Bertrand-Hudson's rule. Likewise, if position 2 of D-sorbitol is oxidized preferentially by an enzyme, it must give D-fructose. Extensive screening of the enzyme yielding D-fructose from D-sorbitol was performed and a few strains were selected.

D-Fructose-producing SLDH purified from *G. suboxydans* IFO 3254 or IFO 3258 was homogenous, showing a molecular mass of 130 kilodaltons (kDa) and dissociating into three different subunits (61 kDa, 53 kDa, and 17 kDa) on SDS-PAGE. D-Fructose-producing SLDH showed a strict substrate specificity and only D-sorbitol was oxidized by the enzyme, the reaction mixture of which reacted with D-fructose dehydrogenase (Ameyama et al. 1981b), but not with NADPH-dependent L-sorbose reductase (Adachi et al. 1999c). The reaction product was highly reactive with NADP-dependent D-mannitol dehydrogenase (Adachi et al. 1999a) as well as NAD-dependent D-sorbitol dehydrogenase (Adachi et al.1999b). This evidence indicates that D-fructose-producing SLDH is different from L-sorbose-producing SLDH in D-sorbitol oxidation. The characteristics of two different SLDHs are summarized in Table 1. On the basis of these results, the oxidative fermentation of D-fructose would allow more-convenient D-fructose production. Unlike the currently used method of D-fructose production by D-glucose isomerase (originally referred to as pentose isomerase), there is no reaction equilibrium in D-sorbitol oxidation by acetic acid bacteria. In the D-glucose isomerase reaction, D-fructose production stops when the product concentration has increased to 42%–50%, due to the reaction equilibrium.

5KGA production by polyol dehydrogenase of acetic acid bacteria

5KGA is known to be formed only by acetic acid bacteria when grown on a medium containing D-glucose and/or D-gluconate. *Gluconobacter* strains produce two keto-D-gluconates, 5KGA and 2KGA, from D-glucose via D-gluconate. Several attempts to produce 5KGA have been made with *Gluconobacter* strains, as 5KGA was reported to be useful for L-ascorbate production by Gray's method (Gray 1945a, b). However, there is little biochemical information about the enzyme involved in 5KGA formation. The localization, catalytic properties, and nature of the prosthetic group of the enzyme remain to be clarified. In our recent studies on membrane-bound polyalcohol dehydrogenase (tentatively designated as D-arabitol dehydrogenase, ARDH) from *G. suboxydans* IFO 3257, purified ARDH was shown to be a versatile enzyme in oxidation of various sugar alcohols to their corresponding oxidation products, such as glycerol to dihydroxyacetone, ribitol to L-ribulose, D-arabitol to D-xylulose, D-sorbitol to L-sorbose, and D-mannitol to D-fructose (Adachi et al. 2001). Substrate oxidation proceeds according to the Bertrand-Hudson's rule (Hann et al. 1938; Kersters et al. 1965). ARDH was similar to quinoprotein glycerol dehydrogenase (Ameyama et al. 1985) in substrate specificity as well as molecular properties. At the same time, the coenzyme functioning in L-sorbose fermentation has been confirmed as PQQ.

D-Gluconate was found to be well oxidized by ARDH, giving 5KGA as the sole oxidation product (Adachi et al. 2001). D-Gluconate is a substrate that obeys the Bertrand-Hudson's rule. When an ARDH-deleted mutant was derived by inserting a suicide plasmid into *ardh,* no 5KGA production was observed, although the mutant still produced 2KGA (Matsushita et al. 2003). These results suggest that D-gluconate is oxidized to 5KGA by ARDH and there is no specific 5KGA-producing D-gluconate dehydrogenase. When D-gluconate dehydrogenase encoding 2KGA is deleted, the *Gluconobacter* strain must produce 5KGA exclusively. It is noteworthy that ARDH catalyzes D-gluconate oxidation to 5KGA, whereas 2KGA is produced by a membrane-bound flavoprotein D-gluconate dehydrogenase. To confirm these results, ARDH was purified to a homogenous state (Adachi et al. 2001).

Table 1 Characterization of membrane-bound D-sorbitol dehydrogenase from *Gluconobacter suboxydans*

	FAD-SLDH	PQQ-SLDH
Molecular mass	130 kDa	91 kDa
Subunit	3 (61 kDa, 53 kDa, 17 kDa)	2 (77 kDa, 14 kDa)
Prosthetic group	Covalently bound FAD	PQQ
Substrate specificity	D-sorbitol	Various sugar alcohols
Oxidation product of D-sorbitol	D-fructose	L-sorbose
Specific activity (U/mg)	430	38
Optimum pH	pH 4.5	pH 5.0
Stable pH	pH 5.0	pH 4.5
Absorption peak	4 (280, 417, 522, 551 nm)	1 (280 nm)
Sedimentation coefficient	5.9 s	3.6 s
K_m for D-sorbitol	30 mM	40 mM
EDTA treatment	Not inhibited	Inhibited

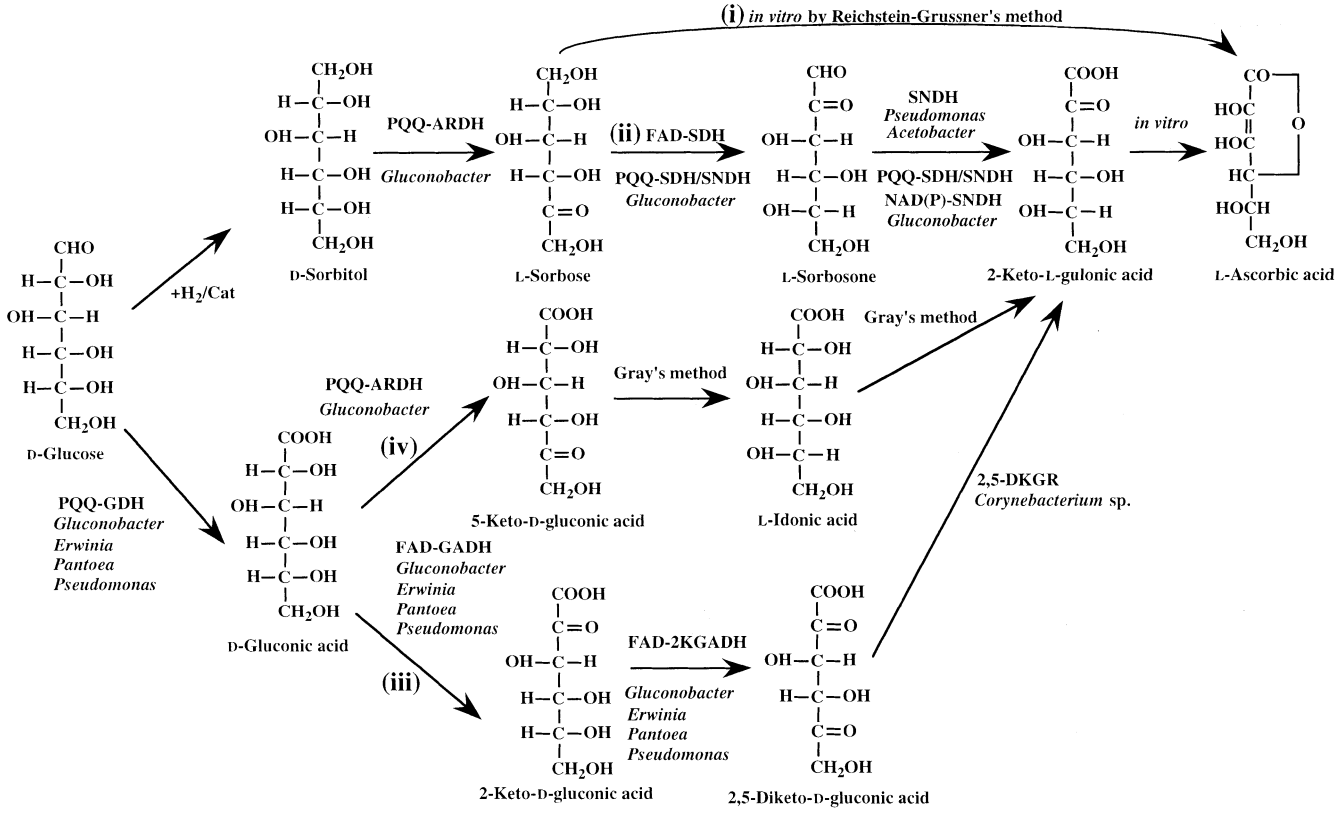

Fig. 1 Different routes for vitamin C production. (i) The classic method developed by Reichstein in 1938 to which one microbial oxidation step giving L-sorbose from D-sorbitol was introduced when L-sorbose fermentation became available (Anderson et al. 1985). (ii) Method involving sorbose/sorbosone dehydrogenase (Sugisawa and Hoshino 2002; Sugisawa et al. 1990, 1991). (iii) D-Gluconate route via 2-keto-D-gluconate (2KGA) to 2,5-diketo-D-gluconate (Sonoyama et al. 1982). (iv) D-Gluconate route via 5KGA to 2-keto-L-gulonate proposed by Gray as described in this article

ARDH is dissociated into two different subunits upon SDS-PAGE with molecular masses of 82 kDa (subunit I) and 14 kDa (subunit II), forming a heterodimeric structure. ARDH has been shown to be a typical quinoprotein. PQQ liberated from SDS-treated ARDH was detected by high-performance liquid chromatography (Adachi et al. 2001). Coenzyme PQQ and ubiquinone 10 were detected in a purified ARDH when enzyme solubilization was performed with dodecylmaltoside instead of Triton X-100. More preliminary results showed that the membrane fraction lost the ARDH activity upon EDTA treatment and the enzyme activity was restored to the original level by exogenous addition of PQQ and Ca^{2+}. However, it has been found that D-arabitol oxidation is always parallel to D-gluconate oxidation.

Exclusive 5KGA production would be possible by a mutant in which D-gluconate dehydrogenase yielding 2KGA is deleted. As new information on 5KGA production accumulates, 5KGA production should become practical, which will allow us to utilize the new route for vitamin C production proposed by Gray (Gray 1945a, b). According to the proposal, half of the 5KGA is converted to L-idonate and then to 2-keto-L-gulonate by another oxidative fermentation before finally being converted to L-ascorbate. The other half of 5KGA is reversed to D-gluconate, which is again oxidized to 5KGA. There are at least four routes in vitamin C production (Fig.1): (i) the classic method developed by Reichstein in 1938 to which one microbial oxidation step giving L-sorbose from D-sorbitol was introduced when L-sorbose fermentation became available (Anderson et al. 1985), (ii) a method involving sorbose/sorbosone dehydrogenase (Sugisawa and Hoshino 2002; Sugisawa et al. 1990, 1991), (iii) a D-gluconate route via 2KGA to 2,5-diketo-D-gluconate (Sonoyama et al. 1982), and (iv) a D-gluconate route via 5KGA to 2-keto-L-gulonate proposed by Gray and described in this article. When the stability of the intermediates is compared among the vitamin C processes, 5KGA is more stable than 2,5-diketo-D-gluconate and L-sorbosone.

Production of 3-dehydroquinate by oxidative fermentation and subsequent bioconversion to shikimate by the same acetic acid bacteria

Quinate utilization is an important index in the systematic evaluation of *Pseudomonas* and many other aerobic bacteria. 3-Dehydroquinate dehydratase (synonymous to

3-dehydroquinase, EC 4.2.1.10) was isolated from a cell extract of *Escherichia coli* (Mitsuhashi and Davis 1954a). Quinate 3-dehydrogenase [synonymous to NAD(P)-dependent quinate dehydrogenase, EC 1.1.1.24] and shikimate 3-dehydrogenase [synonymous to 3-dehydroshikimate reductase, EC 1.1.1.25] were reported in *Aerobacter aerogenes* (Mitsuhashi and Davis 1954b) and 3-dehydroshikimate reductase in *E. coli* (Yaniv and Gilvarg 1955). These enzymes were also indicated in plants in a series of aromatic biosyntheses. The first report of a quinate-oxidizing enzyme in acetic acid bacteria was by Whiting and Coggins in 1967. They observed quinate oxidation to 3-dehydroquinate by NAD(P)-independent quinate dehydrogenase (QDH) (EC 1.1.99.25) and shikimate oxidation to 3-dehydroshikimate by an enzyme associated with a particulate enzyme in the cytoplasmic membrane. van Kleef and Duine (1988) indicated the occurrence of QDH in the periplasm of *Acinetobacter calcoaceticus* LMD 79.41 and suggested that QDH is a quinoprotein containing PQQ as the coenzyme. Elsemore and Ornston (1994) conducted genetic analysis of protocatechuate catabolism and claimed that the consecutive action of three genes, *quiA* encoding quinate dehydrogenase, *quiB* for 3-dehydroquinate dehydratase, and *quiC* for 3-dehydroshikimate dehydratase, is required in the catabolism of quinate to protocatechuate (Elsemore and Ornston 1995). Aromatic compounds administered to microorganisms such as *Acinetobacter, Pseudomonas, Aerobacter*, or *Escherichia* are degraded and it is difficult to collect the metabolic intermediates. To produce metabolic intermediates, acetic acid bacteria would be more useful for oxidative fermentation.

Several bacterial strains carrying QDH were identified through screening acetic acid bacteria and other bacteria. A strong enzyme activity was found in the membrane fraction of *G. melanogenus* IFO 3294, *G. oxydans* IFO 3292, and *G. oxydans* IFO 3244. In the cytosolic soluble fraction, no QDH activity was observed, indicating the metabolic importance of QDH functioning in the membrane (Matsushita et al. 1994). Stoichiometric oxidation of quinate to 3-dehydroquinate was observed with the *Gluconobacter* strains and 3-dehydroquinate was accumulated in the culture medium. This was different from the pseudomonads and *Acinetobacter* strains in which a complete degradation of quinate was observed. Oxidation of quinate by the resting and immobilized cells of *Gluconobacter* strains successfully produced 3-dehydroquinate. Production of shikimate from 3-dehydroquinate via 3-dehydroshikimate was also conducted by NADP-dependent shikimate dehydrogenase from the same organism, which was readily crystallized (Adachi et al. unpublished data) in dried cells of *G. oxydans* IFO 3244 (Adachi et al. Japanese Patent Application 2001, 267257). The reaction can be driven forward to yield shikimate by a coupling reaction with NADP-dependent D-glucose dehydrogenase (EC 1.1.1.119), which functions as an NADPH generator (Adachi et al. 1980). The three steps shown by the large arrow in Fig. 2 have

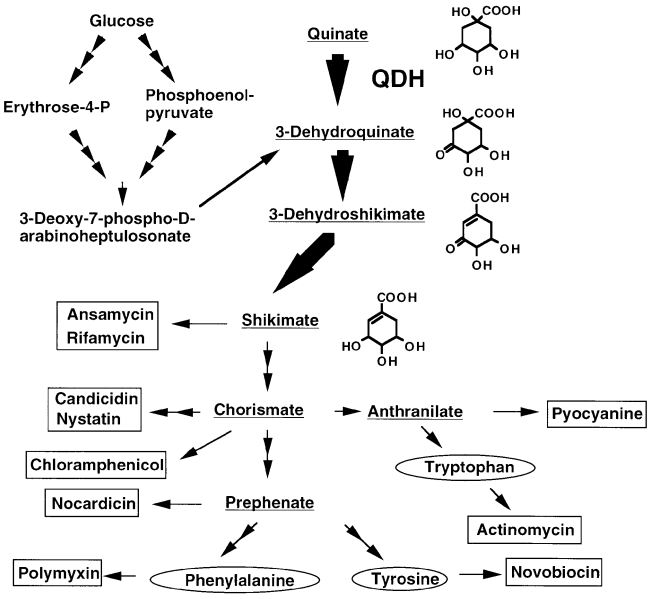

Fig. 2 Entrance of quinate into the shikimate pathway led by quinoprotein quinate dehydrogenase (QDH)

been confirmed with acetic acid bacteria and shikimate production led by the oxidation of quinate by QDH seeming promising.

Shikimate is remote from D-glucose in the metabolic map and many steps are required to reach shikimate. D-Erythrose-4-phosphate from the pentose phosphate pathway and phosphoenolpyruvate from the glycolytic pathway must come together to yield 3-deoxy-7-phospho-D-arabinoheptulosonate before entering the shikimate pathway. Therefore, it is quite difficult to control the shikimate pathway using molecular engineering. However, quinate can be prepared by extracting cinchona bark or cinchona leaves, since quinine hydrochloride was extracted for antimalarial drugs more than 50 years ago. Cinchona agriculture in South Asian countries was stopped after synthetic antimalarial agents were developed. A rebirth of cinchona agriculture may be possible if the oxidative fermentation of quinate to shikimate has a practical value in salvage synthesis of numerous antibiotics, herbicides, and aromatic amino acids derived from the shikimate pathway.

For QDH purification, *Gluconobacter* strains were not suitable due to the existence of large amounts of quinohemoprotein ADH in the same membrane fraction, which was co-solubilized with QDH making it difficult to isolate pure QDH. Purification of QDH was performed with *Acinetobacter* sp. SA1 instead, which contains no ADH. QDH was readily solubilized from the membrane fraction by an alkylglucoside such as β-octylglucoside in the presence of 0.1 M KCl. A simple purification procedure involving butyl-Toyopearl column chromatography gave a highly purified QDH that was monodispersed with a molecular mass of 85 kDa and showed one major band by SDS-PAGE. When QDH purification was

performed in the absence of PQQ with *A. calcoaceticus* AC3, which is not possible in PQQ synthesis, purified apo-QDH appeared to be a dimer (170 kDa), which was converted to the monomer (85 kDa) on addition of PQQ (Tanasupawat et al. 2000).

Thermotolerant acetic acid bacteria and vinegar fermentation

Despite the long history of vinegar production, biochemical and molecular studies on ADH and ALDH were only started in the early 1980s after PQQ, a novel quinone coenzyme, or a related quinone cofactor, was indicated in the function of the enzymes, in addition to the cytochrome components (Adachi et al. 1978a, b). ADH and ALDH have been extensively investigated and are well characterized (Matsushita and Adachi 1993a, b; Matsushita et al. 1992, 1994). The two enzymes are bound to the outer surface of the cytoplasmic membrane and catalyze oxidation reactions in the periplasmic space. Understanding of the role of the heme *c* moieties in the intramolecular electron transport during alcohol oxidation has progressed greatly. On the basis of recent results, it is suggested that the heme *c* in subunit I of ADH and two of the three heme *c* moieties in subunit II of ADH are involved in the intramolecular electron transport of ADH to ubiquinone, from which the electron is mediated in vivo to the terminal oxidase (Matsushita et al. 1996, 2002a).

The recent hot summer has raised indoor temperatures beyond 30°C, even at night time, in many countries. This is a serious problem for vinegar fermentation and other fermentation industries, since they have to pay increased energy costs to keep the fermentation at the optimum. Domestic vinegar production by acetic acid bacteria is usually carried out at 30°C and strict, temperature control is necessary irrespective of whether the culture is static or submerged. A temperature increase of 2–3°C causes a serious deterioration in both the fermentation rate and fermentation efficiency. In submerged cultures, a large amount of heat is generated during fermentation and thus cooling costs become rather expensive. Therefore, if favorable strains of acetic acid bacteria that can work optimally at 37–40°C were available, the cooling expenses would be reduced greatly. However, there have been few reports of vinegar fermentation by thermotolerant acetic acid bacteria. Isolation, identification, and characterization of thermotolerant acetic acid bacteria were investigated to develop new microbial resources for oxidative fermentation (Saeki et al. 1997b). The course of vinegar fermentation at higher temperatures with the isolated strains was compared with mesophilic strains. There are several advantages of vinegar fermentation cultures at higher temperatures with thermotolerant acetic acid bacteria.

Thermotolerant acetic acid bacteria that can grow at 37–40°C were collected and divided into several groups according to their taxonomic and physiological properties, such as rapid ethanol oxidation, rapid acetate oxidation, cellulose biopolymer formation, growth at 40°C, growth in 3% acetate, growth in 8% ethanol, and formation of thermotolerant ADHs and ALDHs. Thus, the screening conditions were adjusted in order to isolate *Acetobacter* strains preferentially over *Gluconobacter* strains. The majority of the acetic acid bacteria isolated were classified as *Acetobacter rancens* subsp. *pasteurianus*, *A. lovaniensis* subsp. *lovaniensis*, *A. aceti* subsp. *liquefaciens*, and *A. xylinum* subsp. *xylinum*. They produced acetate even at high temperatures of up to 40°C. When acetate was initially added at 4%, they still oxidized ethanol to accumulate acetate, while 2% of the initial acetate was the upper limit for mesophilic strains at higher temperatures.[1] They oxidized higher concentrations of ethanol up to 9% without any appreciable lag time, while alcohol oxidation with mesophilic strains was delayed or became almost impossible under such conditions. Fermentation efficiency of vinegar production with the thermotolerant strains at 38–40°C was almost the same as that of mesophilic strains at 30°C. However, the thermotolerant strains worked rapidly, with a higher fermentation rate at higher temperatures, at which the mesophilic strains were unable to work. Vinegar fermentation at higher temperatures was successful in submerged culture as well as static culture. The fermentation rate and efficiency of continuous vinegar fermentation at higher temperatures by the thermotolerant strains in a jar fermentor were also more than 2 to 3 times higher than those of mesophilic strains at 30°C. Thus, thermotolerant acetic acid bacteria are useful for vinegar fermentation at higher temperatures, allowing a possible reduction in cooling expenses and other costs (Saeki et al. 1997b). Vigorous growth in the presence of high acetate and high ethanol concentrations was the outstanding characteristic of the thermotolerant isolates. About 50% of the isolates grew in seed culture medium containing 3% acetate, at which only three strains obtained from the Institute for Fermentation, Osaka (IFO), *A. rancens* IFO 3298, *A. aceti* IFO 3299, and *G. sphaericus* IFO 12467, showed growth. This is consistent with the fact that most IFO strains are useful for vinegar fermentation at 30°C without any noticeable acetate oxidation.

In the culture medium, 4% ethanol and 1% acetate were initially added, thus allowing the final acetate concentration to be about 5%. Two strains of *A. rancens,* SKU 1108 and SKU 1112, grew at 40°C and normal accumulation of acetate was observed. Vinegar fermentation with *A. rancens* SKU 1108 was carried out in the main culture medium at 37°C in a jar fermentor. The course of fermentation and efficiency of vinegar production by the thermotolerant strains were compared with those of *A. rancens* IFO 3298 and *A. aceti* IFO 3283, both

[1] To characterize the thermotolerant properties of acetic acid bacteria described here, the term mesophilic is tentatively used for strains that cannot grow at 37–40°C, although all strains dealt with in this paper are typical mesophiles. Growth temperature of the thermotolerant strains was adapted to a higher range by several degrees.

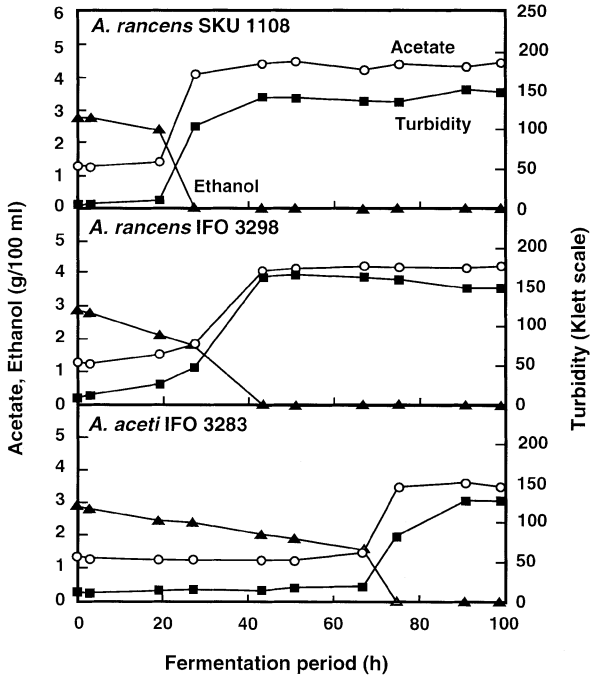

Fig. 3 Comparison of fermentation efficiency of a thermotolerant strain with mesophilic strains at 37°C. Vinegar fermentation with *Acetobacter rancens* SKU 1108 was compared with two well-known mesophilic vinegar producers, *A. rancens* IFO 3289 and *A. aceti* IFO 3283. The culture medium contained 0.2% yeast extract, 0.2% polypeptone, 1% acetate (v/v), and 4% ethanol (v/v). Vinegar fermentation was carried out at 37°C for the period indicated in a jar fermentor with an agitation rate of 1,000 rpm and aeration was controlled to 0.2 vvm

well-known mesophilic vinegar producers (Fig. 3). Ethanol was rapidly consumed by *A. rancens* SKU 1108 and stoichiometric amounts of acetate accumulated in the culture medium. A delayed ethanol oxidation was observed with *A. rancens* IFO 3289 and more than 10 h were further required for the acetate concentration to reach the maximal level. In the case of *A. aceti* IFO 3283, the bacterial growth at 37°C appeared to be almost impossible and ethanol oxidation was greatly delayed. It is generally agreed that acetic acid bacteria show more thermostability when grown under static cultures than submerged cultures. Marked acetate tolerance as well as high ethanol tolerance was observed with thermotolerant acetic acid bacteria (Saeki et al. 1997b). The appearance, taste, and fragrance of vinegar produced by thermotolerant acetic acid bacteria seemed similar to those produced by mesophilic strains, although many other points remain to be checked. It has been reported that there were no significant differences in taste and fragrance when vinegar prepared at 30°C and 37°C was subjected to sensory tests as well as chemical analyses (Masai 1979).

Through the studies on thermotolerant acetic acid bacteria, the enzymatic mechanism of acetate oxidation has been investigated (Saeki et al. 1997a; Saeki et al. 1999). Several strains of acetic acid bacteria belonging to the genus *Acetobacter* show a strong acetate oxidation, which is known as an unfavorable phenomenon in vinegar manufacturing. *A. rancens* SKU 1111, a typical acetate oxidant, grew rapidly on consumption of acetate giving the second growth accompanying a typical biphasic growth curve, when compared with that of *A. aceti* IFO 3284, a silent strain for acetate oxidation. Increasing the concentrations of glycerol in the culture medium caused an elevated biomass yield forming the second stationary phase and acetate was rapidly consumed as glycerol concentrations in the culture medium increased. The enzyme activities of acetyl-CoA synthetase (EC 6.2.1.1) and phosphotransacetylase (EC 2.3.1.8) during acetate oxidation became predominant. Among the enzymes concerned with glycerol catabolism, the enzyme activity of phosphoenolpyruvate carboxylase (EC 4.1.1.31) was significantly stimulated by a trace amount of acetyl-CoA. Phosphoenolpyruvate carboxylase purified from *A. rancens* SKU 1111 showed a typical sigmoidal saturation curve with acetyl-CoA with Michaelis-Menten kinetics. It was suggested that acetyl-CoA formation occurs first by acetyl-CoA synthetase and/or phosphotransacetylase and phosphoenolpyruvate carboxylase is activated thereafter by acetyl-CoA. These data correlate well with findings over the last century. To avoid acetate oxidation in vinegar fermentation, it is better to terminate the fermentation by separating the cell biomass from the vinegar mash when the accumulation of acetate reaches the required level; this has been considered the most-effective way to avoid acetate oxidation. Vinegar fermentation allowing acetate accumulation to be more than 4% has become an alternative means of overcoming acetate oxidation. In commercial vinegar production, the final acetate concentration is usually controlled at 4%–5%. Selected acetic acid bacteria that show the least acetate oxidation are currently used, although a prolonged incubation beyond ethanol exhaustion sometimes causes acetate oxidation. Although it is difficult to compare the data for thermotolerant strains directly with mesophilic strains, the usefulness of the thermotolerant strains for vinegar fermentation as well as other oxidative fermentations is emphasized here and below.

L-Erythrulose production by thermotolerant acetic acid bacteria

No information has accumulated on oxidative fermentation of C4 sugar alcohols, although there are some earlier reports of *meso*-erythritol oxidation by aerobic bacteria. Since L-erythrulose is not readily available from commercial sources, it is important to investigate the fermentation profile of L-erythrulose production, to identify the enzyme responsible for *meso*-erythritol oxidation, and to purify and characterize the responsible enzyme. All oxidative fermentation by acetic acid bacteria is only catalyzed by the membrane-bound dehydrogenases localized on the outer surface of the cytoplasmic membrane (Matsushita et al. 1994). With respect to cytosolic NAD(P)-dependent *meso*-erythritol dehydroge-

nases, only one enzyme has been reported as L-erythrulose reductase (EC 1.1.1.162) from beef liver and L-erythrulose reduction to *meso*-erythritol is more predominant than *meso*-erythritol oxidation (Uehara et al. 1974). It has also been reported that NADPH is more reactive than NADH in L-erythrulose reduction with the mammalian enzyme. However, there is no contribution of NAD(P)-dependent dehydrogenases in the cytosolic fraction in oxidative fermentation. After extensive screening, a thermotolerant *G. frateurii* CHM 43 was selected as the best strain for L-erythrulose production (Moonmangmee et al. 2002).

This strain accumulated the most L-erythrulose after 24–36 h of cultivation when grown at 37°C, although the total cell mass was less than observed at 30°C. It appeared that the optimal production of L-erythrulose at higher temperatures was better than at lower temperatures. The oxidation product from *meso*-erythritol formed by the action of *Gluconobacter* is based on the fact that this substrate has an erythro form of two secondary hydroxyl groups adjacent to the primary alcohol moiety, according to the Bertrand-Hudson's rule (Hann et al. 1938; Kersters et al. 1965). Growth of *G. frateurii* CHM 43 at 30°C was faster than that at 37°C, but it was interesting that production of L-erythrulose at 37°C was higher than at 30°C (Fig. 4). The strain produced L-erythrulose to 90% after 24 h at 30°C. However, it was decreased to 34% after 48 h incubation, while it was maintained at almost 100% when grown at 37°C. These data suggested that at 30°C the microorganism used both *meso*-erythritol and L-erythrulose as growth substrates. However, at higher temperatures the organism might have increased oxidization of *meso*-erythritol to L-erythrulose in order to generate energy for survival at higher temperatures. Similar control of oxidative fermentation to produce a higher yield of oxidation product by stressing the bacterial growth at higher temperatures was successful for L-sorbose and D-fructose production (Moonmangmee et al. 2000). The industrial advantages of L-erythrulose production at higher temperatures would be the higher conversion rate, shorter incubation time, and reduced cost for cooling expenses.

The *meso*-erythritol dehydrogenase activity was remarkably decreased after EDTA treatment, while exogenous addition of PQQ or PQQ and $CaCl_2$ reactivated the enzyme activity to the original level (Moonmangmee et al. 2002). These results suggested that membrane-bound *meso*-erythritol dehydrogenase has PQQ as the prosthetic group. Therefore, it can be classified as a member of the quinoprotein dehydrogenases like methanol dehydrogenase (EC 1.1.99.8) in methylotrophs (Anthony 1982), alcohol dehydrogenase (EC 1.1.99.8) (Adachi et al. 1978a, b) and glycerol dehydrogenase (EC 1.1.99.) in acetic acid bacteria (Ameyama et al. 1985), D-glucose dehydrogenase (EC 1.1.99.17) from acetic acid bacteria (Ameyama et al. 1981a), pseudomonads (Matsushita and Ameyama 1982), *Acinetobacter calcoaceticus* (Duine et al. 1979), and *E. coli* (Ameyama et al. 1986). However, membrane-bound *meso*-erythritol dehydrogenase described here is composed of two different subunits and distinct from the membrane-bound dehydrogenases, including alcohol dehydrogenases (EC 1.1.99.8) (Adachi et al. 1978a, b), D-sorbitol dehydrogenase (Shinagawa et al. 1982), D-fructose dehydrogenase (Ameyama et al. 1981b), D-mannitol dehydrogenase (Oikawa et al. 1997), and D-gluconate dehydrogenase (Matsushita et al. 1979), most of which are composed of three different subunits and cytochrome *c,* while *meso*-erythritol dehydrogenase has no cytochrome *c* component.

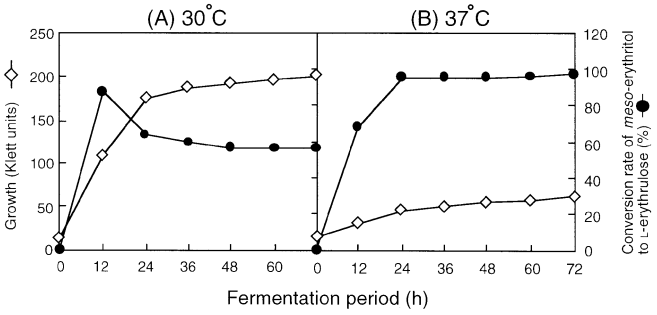

Fig. 4 Time course of L-erythrulose production by growing cells of *Gluconobacter frateurii* CHM 43 at 30°C (**A**) and 37°C (**B**)

Production of cyclic ketones by oxidative fermentation

Different investigations on microbial and enzymatic oxidation of cyclic alcohols have been performed with various bacteria. Most are related to NAD-dependent cyclohexanol dehydrogenase (EC 1.1.1.245) from pseudomonads and *Acinetobacter* sp. (Dangel et al. 1988, 1989; Donoghue and Trudgill 1975; Tanaka et al. 1977). Cyclohexanol oxidation was also studied via cyclohexane metabolism (Trower et al. 1985). Genetic analysis of a gene cluster for cyclohexanol oxidation was studied with *Acinetobacter* sp. (Cheng et al. 2000). Reduction of cyclohexanone to cyclohexanol by NAD-dependent ADH by coupling with hydrogenase was proposed to produce cyclohexanol (Adlercreutz et al. 1998). This is similar to the case of cyclic ketone or cyclic aldehyde reductases. In earlier studies, NADPH-dependent acetone reductase yielding isopropanol was reported (Hoshino 1960a, b). In mammalian systems, a similar ketone reductase was reported (Wermuth 1981). Numerous aldo-keto reductases catalyzing the asymmetric reduction of carbonyl compounds have been proved to be useful for the synthesis of optically active compounds (Kataoka et al. 1999; Kita et al. 1999; Wada et al. 1999; Yamada et al. 1990). Microbial cyclopentanol oxidation was also studied with *Pseudomonas* sp., and NAD-dependent cyclopentanol dehydrogenase (EC 1.1.1.163) was indicated as the responsible enzyme at the first step of the oxidative degradation of cyclic alcohol (Davey and Trudgill 1977; Griffin and Trudgill 1972). Cyclohexanol oxidation was also observed by a secondary alcohol oxidase from

Pseudomonas sp., in which a hydrogen peroxide-producing flavin-dependent enzyme is functional (Morita et al. 1979; Suzuki 1976, 1978). Enzymes catalyzing the oxidation of sterols are also known (Shikita and Talalay 1979; Uwajima et al. 1978). NAD-dependent quinate dehydrogenase is also known as a cyclic alcohol metabolizing enzyme (Mitsuhashi and Davis 1954b).

However, since there are no reports about membrane-bound cyclic alcohol dehydrogenase (MCAD), there is no information on its function, localization, catalytic properties, physicochemical properties, and physiological functions. Therefore, it is worth investigating the the enzyme extensively and comparing it with cytosolic NAD(P)-dependent enzymes. As an initial approach, purification and characterization of MCAD are reported in this paper. A quinoprotein catalyzing the oxidation of cyclic alcohols was found in the membrane fraction of acetic acid bacteria after extensive screening (Moonmangmee et al. 2001). MCAD was solubilized and purified to a homogenous state from *G. frateurii* CHM 9. The enzyme preferentially oxidized various cyclic alcohols and aliphatic secondary alcohols to their corresponding cyclic ketones and aliphatic ketones, respectively. When it was compared with the cytosolic NAD-dependent cyclic alcohol dehydrogenase (CCAD) crystallized from the same strain, the reaction rate in cyclic alcohol oxidation by MCAD was 100 times higher than CCAD. CCAD was unfavorable for cyclic alcohol oxidation, but favorable for the reduction of cyclic ketones to cyclic alcohols. As shown in Fig. 5, various cyclic alcohols were well oxidized by MCAD, producing the corresponding cyclic ketones.

MCAD was found specifically in the membrane fraction of acetic acid bacteria and PQQ was functional as the primary coenzyme. Unlike already known cytosolic NAD(P)H-dependent alcohol-aldehyde or alcohol-ketone oxidoreductases, MCAD only catalyzed the oxidation reaction of cyclic alcohols irreversibly to their corresponding ketones. MCAD was solubilized and purified from the membrane fraction of acetic acid bacteria to high homogeneity. The purified MCAD had a molecular mass of 83 kDa and one smaller subunit of about 10 kDa by SDS-PAGE (Adachi et al. unpublished data). Investigation of substrate specificity showed that MCAD was an enzyme oxidizing a wide variety of cyclic alcohols. Some minor enzyme activity was found with aliphatic secondary alcohols and sugar alcohols, but not primary alcohols, distinguishing MCAD from quinohemoprotein ADH (Adachi et al. 1978a, b). Moreover, MCAD is completely different from quinohemoprotein ADH in subunit composition. CCAD in the same organism was crystallized and the catalytic and physicochemical properties were characterized. Judging from the catalytic properties of CCAD, it was apparent that CCAD was distinct from MCAD in many respects and seemed to make no contribution to cyclic alcohol oxidation (Moonmangmee et al. 2001).

Since cyclic alcohols are sometimes toxic to living cells, it is advantageous to catalyze cyclic alcohol oxidation by oxidative fermentation, since oxidative fermentation is carried out in the periplasmic space (Matsushita et al. 1994). Thus, purified MCAD was the first indication of an enzyme catalyzing cyclic alcohol oxidation in the bacterial membrane. MCAD is capable of catalyzing enantioselective oxidation of cyclic alcohols, bicyclic alcohols, and aliphatic secondary alcohols yielding their corresponding cyclic ketones, bicyclic ketones, and aliphatic ketones, respectively. Some of such oxidized products are identical to the naturally occurring products and thus they are important as starting materials for various physiologically active substances. In acetic acid bacteria, MCAD may contribute significantly to the production of useful cyclic ketones from sterols, bicyclic alcohols, most of which are valuable as the starting materials for many kinds of physiologically active compounds with pheromone activity (Emura and Kanisawa 2002; Kajiwara et al. 1997; Lebreton et al. 1996).

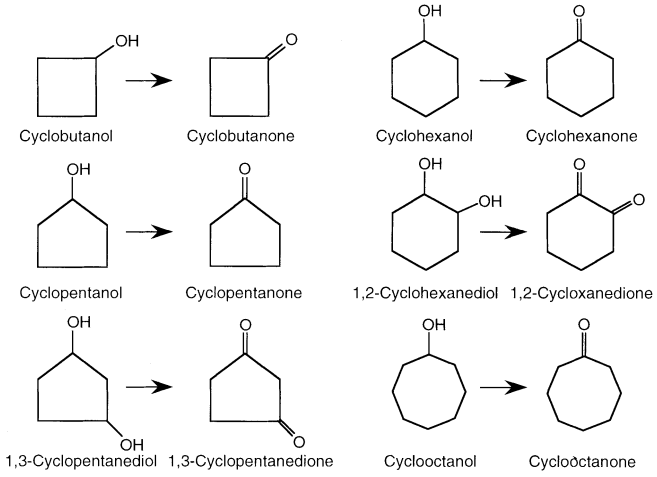

Fig. 5 Oxidation of various cyclic alcohols by membrane-bound cyclic alcohol dehydrogenase of acetic acid bacteria

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