Faculty of Biosciences
Department of Biological and Environmental Sciences
Division of Biochemistry
University of Helsinki, Helsinki, Finland

Characterisation, cloning and production of industrially interesting enzymes: gluconolactone oxidase of *Penicillium cyaneo-fulvum* and gluconate 5-dehydrogenase of *Gluconobacter suboxydans*

Tuomas Salusjärvi

Danisco Innovation, Kantvik, Finland

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Reviewed by: D.Tech., Docent, Kristiina Kruus
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Ph.D. Jari Vehmaanperä
Roal Oy

Opponent: Research Prof. Merja Penttilä
VTT Biotechnology

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Abstract

The work covered in this thesis is focused on the development of technology for bioconversion of glucose into D-erythorbic acid (D-EA) and 5-ketogluconic acid (5-KGA). The task was to show on proof-of-concept level the functionality of the enzymatic conversion or one-step bioconversion of glucose to these acids. The feasibility of both studies to be further developed for production processes was also evaluated.

The glucose–D-EA bioconversion study was based on the use of a cloned gene encoding a D-EA forming soluble flavoprotein, D-gluconolactone oxidase (GLO). GLO was purified from *Penicillium cyaneo-fulvum* and partially sequenced. The peptide sequences obtained were used to isolate a cDNA clone encoding the enzyme. The cloned gene (GenBank accession no. AY576053) is homologous to the other known eukaryotic lactone oxidases and also to some putative prokaryotic lactone oxidases. Analysis of the deduced protein sequence of GLO indicated the presence of a typical secretion signal sequence at the N-terminus of the enzyme. No other targeting/anchoring signals were found, suggesting that GLO is the first known lactone oxidase that is secreted rather than targeted to the membranes of the endoplasmic reticulum or mitochondria. Experimental evidence supports this analysis, as near complete secretion of GLO was observed in two different yeast expression systems. Highest expression levels of GLO were obtained using *Pichia pastoris* as an expression host. Recombinant GLO was characterised and the suitability of purified GLO for the production of D-EA was studied. Immobilised GLO was found to be rapidly inactivated during D-EA production. The feasibility of *in vivo* glucose–D-EA conversion using a *P. pastoris* strain co-expressing the genes of GLO and glucose oxidase (GOD, E.C. 1.1.3.4) of *A. niger* was demonstrated.

The glucose–5-KGA bioconversion study followed a similar strategy to that used in the D-EA production research. The rationale was based on the use of a cloned gene encoding a membrane-bound pyrroloquinoline quinone (PQQ)–dependent gluconate 5-dehydrogenase (GA 5-DH). GA 5-DH was purified to homogeneity from the only source of this enzyme known in literature, *Gluconobacter suboxydans*, and partially sequenced. Using the amino acid sequence information, the GA 5-DH gene was cloned from a genomic library of *G. suboxydans*. The cloned gene was sequenced (GenBank accession no. AJ577472) and found to be an operon of two adjacent genes encoding two subunits of GA 5-DH. It turned out that GA 5-DH is a rather close homologue of a sorbitol dehydrogenase from another *G. suboxydans* strain. It was also found that GA 5-DH has significant polyol dehydrogenase activity. The *G. suboxydans* GA 5-DH gene was poorly expressed in *E. coli*. Under optimised conditions maximum expression levels of GA 5-DH did not exceed the levels found in wild-type *G. suboxydans*. Attempts to increase expression levels resulted in repression of growth and extensive cell lysis. However, the expression levels were sufficient to demonstrate the possibility of bioconversion of glucose and gluconate into 5-KGA using recombinant strains of *E. coli*.

An uncharacterised homologue of GA 5-DH was identified in *Xanthomonas campestris* using *in silico* screening. This enzyme encoded by chromosomal locus NP_636946 was found by a sequencing project of *X. campestris* and named as a hypothetical glucose dehydrogenase. The gene encoding this uncharacterised enzyme was cloned, expressed in *E. coli* and found to encode a gluconate/polyol dehydrogenase without glucose dehydrogenase activity. Moreover, the *X. campestris* GA 5-DH gene was expressed in *E. coli* at nearly 30 times higher levels than the *G. suboxydans* GA 5-DH gene. Good expressability

of the *X. campestris* GA-5DH gene makes it a valuable tool not only for 5-KGA production in the tartaric acid (TA) bioprocess, but possibly also for other bioprocesses (e.g. oxidation of sorbitol into L-sorbose).

In addition to glucose–5-KGA bioconversion, a preliminary study of the feasibility of enzymatic conversion of 5-KGA into TA was carried out. Here, the efficacy of the first step of a prospective two-step conversion route including a transketolase and a dehydrogenase was confirmed. It was found that transketolase convert 5-KGA into TA semialdehyde. A candidate for the second step was suggested to be succinic dehydrogenase, but this hypothesis was not tested.

The analysis of the two subprojects indicated that bioconversion of glucose to TA using *X. campestris* GA 5-DH should be prioritised first and the process development efforts in future should be focused on development of more efficient GA 5-DH production strains by screening a more suitable production host and by protein engineering.

Preface

This work was carried out in the Danisco Innovation in Kantvik in the group of Gene Technology.

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Above all, I want to show my gratefulness and appreciation to my family. My wife Laura has patiently been there for me and given her support during our now over 12 years together. Thank you for having our sweet children Akseli and Olavi with me! You all have taught me what really is important in life. I want also thank the rest of our family, my parents Markku and Marja and parents-in-law Ilmari and Ulla, my brothers Joonas, Aleksis, and Johannes, Laura's siblings Klaus and Anna, and all our friends for their support, example and tolerance during my studies and life.

List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I Salusjärvi, T., Kalkkinen, N., and A. N. Miasnikov. (2004). Cloning and Characterization of Gluconolactone Oxidase of *Penicillium cyaneo-fulvum* ATCC10431 and Evaluation of Its Use for Production of D-Erythorbic Acid in Recombinant *Pichia pastoris*. *Appl. Environ. Microbiol*. 70, 5503–5510.

II Salusjärvi, T., Povelainen, M., Hvorslev N., Eneyskaya, E., V., Kulminskaya, A., A., Shabalin, K., A., Neustrov, K., N., Kalkkinen, N., and A. N. Miasnikov. (2004). Cloning of a Gluconate/Polyol Dehydrogenase Gene from *Gluconobacter suboxydans* IFO 12528, Characterisation of the Enzyme and Its Use for the Production of 5-ketogluconate on a Recombinant *Escherichia coli* Strain. *Appl. Microbiol. Biotechnol.* 65, 306–314.

III Salusjärvi, T., Hvorslev N., and A. N. Miasnikov. (2005). Characterisation of a Secondary Alcohol Dehydrogenase from *Xanthomonas campestris* DSM 3586. *Appl. Microbiol. Biotechnol.* 66, 664–667.

Additional previously unpublished data are also presented.

Contents

List of abbreviations

2-KGA	2-keto-D-gluconic acid
5-KGA	5-keto-D-gluconic acid
DCIP	dichlorophenolindophenol
DH	dehydrogenase
D-EA	D-erythorbic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FAD	flavineadenine dinucleotide
GA 5-DH	pyrroloquinoline quinone-dependent membrane bound gluconate 5-dehydrogenase
GLO	D-gluconolactone oxidase
GOD	glucose oxidase
HPLC	highperformance liquid chromatography
IPTG	isopropyl α-thiogalactoside
L-AA	L-ascorbic acid
NADH	β-nicotinamide adenine dinucleotide
NADPH	β-nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulphonylfluoride
PQQ	pyrroloquinoline quinone
SDS	sodium dodecyl sulphate
TA	L-(+)-tartaric acid
TLC	thin layer chromatography

1 Introduction

Despite the association with modern food production, food additives have been used for centuries. For example, salt has been used for thousands of years to preserve meat and fish, herbs and spices added to improve the flavour of foods, fruit and berries preserved with sugar, and vegetables canned with vinegar. Additives have been and continue to be used as ingredients to preserve, flavour, blend, thicken, and colour food. They play an important role in reducing serious nutritional deficiencies and promoting food safety by helping to ensure the availability of nutritional, appetizing, and safe foods.

A food additive, in its broadest sense, is any substance added to food. This includes any substance used in the production, processing, treatment, packaging, or storage of food. Additives perform a variety of useful functions in food that are often taken for granted, like safeness and appealiness. Food is subjected to many different environmental conditions such as temperature changes, oxidation, and exposure to microbes, which can change its original composition.

The modern food industry uses a broad range of ingredients in the preparation of different foods, specifically there are five main uses for food additives. First is to maintain product consistency. Certain ingredients such as emulsifiers, stabilizers and thickeners help to ensure consistent food texture and characteristics. Emulsifiers are added to keep emulsified products stable, reduce stickiness, control crystallization, keep ingredients dispersed (such as spices within a salad dressing) and to help products dissolve more easily (such as powdered coffee creamer). Many food products, especially reduced fat or low calorie foods, are only in existence due to emulsifiers. Emulsifiers work because their chemical structure attracts fats on one end and water on the other letting the two substances combine easily. Second is to improve or maintain nutritional value or promote health. Nutrients can either be lacking in a food or be lost during processing. Grains, flours, milk, margarine, and other foods are enriched or fortified by adding vitamins A and D, ascorbic acid, iron, calcium, etc. Specific health promoting ingredients such as xylitol, pre- and probiotics and plant stanol esters have been under increased worldwide interest lately. Third is to maintain palatability and appealiness of food. Food naturally loses flavour and freshness due to aging or exposure to air or microbes. Over time, fats and oils that come in contact with oxygen can become rancid developing unpleasant off-flavours and odours. Antioxidants are added to delay or prevent rancidity. Preservatives and antioxidants, such as ascorbic acid, erythorbic acid, and sodium nitrite help to reduce product spoilage and even toxicity while maintaining the taste. Foods to which antioxidants are added include fats and oils, cereals, meat and high-fat foods such as doughnuts and chips to extend their shelf life. Fourth use is to provide leavening of the dough or to control acidity or alkalinity of food. Leavening agents (baking soda or powder) help cakes, biscuits, etc. to rise during baking. Food acidulates, such as tartaric acid and citric acid help to modify the acidity of food. They are also used for proper flavour, taste, and colour of food, as preservatives, gelling and coagulation agent, and to prevent oxidation of fats and oils. A lemon-lime beverage would not have a characteristic bitterness without an acidulant ingredient. Fifth is to enhance flavour or impart the desired colour in food. Many spices and flavours, both natural and synthetic, like vanillin and ribonucleoside phosphates, enhance the taste of food. Colours enhance the appearance of food to meet consumer expectations.

White biotechnology is a field within modern biotechnology that uses living cells like moulds, yeasts or bacteria, as well as enzymes for the production of e.g. many finechemicals.

Modified or natural microbes can be used to work as "cell factories" to produce e.g. antibiotics, vitamins, vaccines, enzymes, and a diverse range of different chemicals like many food additives mentioned. As an alternative to some chemical processes, white biotechnology may provide cleaner solution for industry by consuming less water, raw materials and energy in production. Environmental impact is minimized while better products are offered at lower cost. As an example for this can be mentioned the production of vitamin B2 where the chemical multi step process was replaced by a one step fermentation process, which lowers production costs and saves natural resources by reducing associated carbon dioxide emissions by 80% and water emissions by 67% according to the manufacturer.

The economical and environmental aspects make white biotechnology based production processes an attractive alternative to replace many current production processes such as the production of D-EA. D-EA is a widely used antioxidant in the food industry; the world market is approximately 10 000 tonnes per year and estimated price according to market reports is 9 $/kg. It is produced by a two-step process, where glucose is first converted into D-2-keto-gluconic acid by fermentation followed by chemical lactonisation similar to the lactonisation of L-2-keto-gulonic acid in the Reichstein process. D-EA is a C-5 epimer of L-ascorbic acid having only 1/20 of its vitamin activity and is often used as a cheaper alternative to ascorbic acid, because it has the same antioxidant properties as ascorbic acid. It is used in meat products to maintain the red colour of cured meat by reducing up to 25% nitrites to nitric oxide enabling the formation of nitrosomyoglobin, the red colour forming molecule, and simultaneously preventing the formation of carcinogenic nitrosamines by reducing free nitrites. The residual quantity of D-EA in meat protects the final product against oxidation inhibiting the changes of flavour and colour by reacting with unwanted oxygen. Two forms of erythorbates, D-EA and its salt sodium erythorbate are used as an antioxidant particularly in processed meat and fruit products. Erythorbates are classified by the US Food and Drug Administration as generally recognised as safe (GRAS), and have been used in the food industry for more than 30 years.

TA is used as a food acidulant and is the key intermediate for the production of food emulsifiers. The processes used to produce TA have been based on the utilisation of by-products of wine industry. Tartaric acid is the molecule that makes unripe grapes taste sour and it is a principal flavour element in wine. It also has chemical and pharmaceutical applications and is considered one of the best acidulates with superior flavour to citric acid. The potassium salt of tartaric acid (potassium bitartrate or potassium hydrogen tartrate) is weakly acidic, and is known as "cream of tartar". Cream of tartar is used in baking powders (along with sodium bicarbonate) to produce carbon dioxide gas when water is added. TA is the precursor of the most common emulsifier used in frozen dough, diacetyl tartaric acid ester of monoglyceride (commercial name is DATEM). This emulsifier is composed of mixed esters of glycerol in which one or more of the hydroxyl groups of glycerol has been esterified by diacetyl tartaric acid and by fatty acids. This dough strengthener interacts with the hydrophobic parts of gluten to form a homogeneous network important to the bread-making process. This facilitates handling of the dough, as the dough will not stick to the equipment and also increases the bread volume. Tartaric acid and DATEM are also classified as GRAS. The yearly market of TA is currently approximately 60 000 tonnes and estimated price is 5 $/kg according to the market reports.

While the current production technologies of both TA and D-EA have been continued for a while, more cost effective production based on white biotechnology methods have raised interest. This is the case especially with the production of tartaric acid, which de-

pends markedly on wine crops, resulting in large variations in yearly production volumes of the acid. This has a strong effect on the market price of TA and thereby to the price of the products based on TA. Therefore, the production system for biologically produced tartaric acid with stable market prices would be valuable.

Biotechnological production of both ascorbic acid and TA has been intensively studied during the last decades. In the production of TA, most attention has been drawn to methods based on stereo-selective enzymatic hydrolysis of epoxysuccinic acid. Technically, this technology is relatively easy to realise but economically it is not most feasible because of the rather high price of raw material and multistage nature of the process. Several biotechnology companies have actively developed biotechnological production of vitamin C. All reported vitamin C production bioprocesses include at least two steps, fermentation of glucose into L-2-keto-gluconic acid and a chemical lactonisation. So far production of ascorbic acid directly from glucose by direct on-step fermentation remains elusive. Since conversion of glucose into D-EA can be done in a single step, this process has the potential of being the lowest cost technology for production of food antioxidants.

2 Literature review

2.1 Industrial use of biocatalysts

Enzymes control nearly all cellular-level chemical reactions that convert sunlight into carbohydrates, lipids, nucleic acids, amino acids, and other organic compounds. Enzymes work in a highly selective manner to accelerate the rate of chemical reactions in biological systems, usually limiting reactivity towards a particular substrate and converting it to a single desired product. For many substrates, enzyme-catalysed reactions exhibit rates that are many orders of magnitude faster than those of uncatalysed reactions. For some reactions, like the oxidation of hydrogen peroxide catalysed by catalase, the enzyme can act as "perfect catalyst", only the rate of diffusion of the substrate limiting the rate of reaction (Beers and Sizer 1952).

Most of the industrial uses of enzymes involve breaking down large molecules into simpler ones, e.g. the proteases in laundry detergents, and the many enzymes used in starch processing (Gupta *et al.* 2002 and Hoondal *et al.* 2002). Production of sweet corn syrups by amylases and glucose isomerase is one noteworthy example of how a chemical transformation can be carried out using enzymes on the industrial scale (Chaplin and Bucke 1990). However, the usage of enzyme applications in industry would be multiplied if the enzymes could be used for creating complex molecules from simple ones or transforming existing chemical structures into more useful compounds. For example, synthesis routes that can distinguish between chiral chemical structures are being pursued for pharmaceutical, food, and agricultural applications and the enzymes that carry out such chemical transformations have awakened growing interest.

Besides the enzymes can be used separately in many different applications, they can also be used together in more complex enzymatic networks designed for production of compounds of interest. One way to do this is to use the methods of metabolic engineering of living cells. Metabolic engineering aims at the directed modification of the enzymatic, regulatory, or transport activities of the cell to more suitable cellular properties for the production process (Bailey 1991 and Stephanopoulos *et al.* 1998). In other words the complex enzymatic networks mainly in microbial cells are used in production of useful metabolites in such quantities and rates that industrial production becomes feasible. The first applications of metabolic engineering have incorporated genes for metabolic pathway enzymes and transport proteins. In this manner, for example *E. coli* is made to produce vitamin C from galactose and 1,3-propanediol from glucose and vitamin B2 is commercially produced by two companies with genetically modified microorganism *Ashbya gossypii* and *Bacillus subtilis* (Lee *et al.* 1999, Perkins *et al.* 1999, Emptage *et al.* 2003, Jimenez *et al.* 2005, for a rewiev see Stahman *et al.* 2000 and Nakamura and Whited 2003.). In more complex processes separately produced enzymes and genetically engineered microbial strains can be combined for production purposes. A good example for this would be the production bioethanol. In the production process of bioethanol, biomass containing complex polymers is first liquefied enzymatically to sugar monomers using degrading enzymes such as cellulases, xylanases, proteases and amylases. The liberated sugars such as glucose and xylose are then further fermented to ethanol preliminary by using genetically engineered microbes like *Saccharomyces cerevisiae* and *Escherichia coli* (Ingram *et al.* 1987 and Ohta *et al.* 1991 for a rewievs see Aristidou and Penttilä 2000 and Jeffries and Jin 2004).

2.2 Glucose is commonly used carbon source in bioconversions

Plant and algae are the main sources of biomass, which is generated many million tonnes annually. Biomass includes organic polymeric material, such as lignin, starches, celluloses and hemicelluloses produced ultimately almost completely by photosynthesis. As mentioned earlier, biomass is a potential source to produce energy such as methane and bioethanol, and also other products such as fine chemicals, animal feed, and specialty products (e.g., flavours, enzymes and pigments). Biomass is an attractive alternative to petroleum-based sources because it is not only a low-cost substrate for production, but is also environmentally friendly; it is renewable, improve the biodegradability of the end products and reduce pollution during processing by decreasing the carbon liberated from the earth's crust to the atmosphere.

The components of plant biomass include mainly cellulose (polymer of glucose), hemicellulose (heterogeneous sugar polymer containing hexoses and pentoses) and lignin (complex molecule of phenylpropane units). Both cellulose and hemicellulose can be enzymatically hydrolysed to fermentable forms of sugar providing a cheap and renewable raw material that can be converted, via natural or metabolically engineered microbes, to more valuable products. In other words, sugar monomers and especially glucose are the most abundant renewable source of carbon on earth, and are currently in the form of biomass, which in many cases is still considered as waste in agriculture and forest industry.

Besides that glucose is common and rather cheap molecule, it is also the central molecule of the basic energy metabolism of the cell. Most organisms can utilize glucose making it an attractive starting material in many microbial bioconversions. Most aerobic microorganisms usually oxidize completely their carbon sources to carbon dioxide and water, producing at the same time energy and metabolites required for biosynthesis. However, under special circumstances the oxidation can be incomplete, accumulating intermediary or end products other than H_2O and CO_2. For example *Aspergillus* produces both organic acids and polyols depending on the growth conditions used. The acids include TCA-cycle related acids like citric acid and those directly derived from glucose like gluconic acid (Röhr *et al.* 1992). Also acetic acid bacteria are known for their rapid and incomplete oxidation of a wide range of sugars and alcohols and the near quantitative accumulation of the respective oxidation products in the growth medium (Deppenmeier *et al.* 2002). As examples of industrially important compounds produced by acetic acid bacteria are acetic acid a.k.a. vinegar, L-sorbose, 1-deoxynojirimycin, the diabetes drug miglitol (N-hydroxyethyl-1-deoxynojirimycin) and 2-keto-L-gulonic acid that is co-fermentation product with *Pseudomonas striata* (Greenshields 1978, Zinsheng *et al.* 1981, Scott and Tatterstall 1988, for reviews see Deppenmeier *et al.* 2002 and Keliang and Dongzhi 2006). L-sorbose and 2-keto-L-gulonic acid are used as precursors in the ascorbate synthesis by the classical Reichstein process (Reicstein 1941).

2.3 Tartaric acid

Tartaric acid (2,3-dihydroxysuccinic acid, Fig. 1) contains two chiral centres and therefore exists in several stereoisoforms, L-(+)-, D-(-)- and meso-tartaric acid, of which L-(+)-tartaric acid is the most widely distributed in nature.

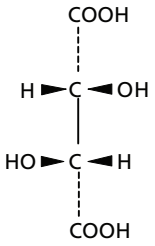

Figure 1 Stereochemical structure of L-(+)-TA

2.3.1 Biosynthesis of TA in plants

Biosynthesis of TA occurs in many plants e.g. in fruits and beans and it is formed in especially large amounts in grapes (Stafford 1959, Stafford 1961, Blair and DeFraties 1995 for a rewiev see Loewus 1999). The function of TA in plants is not completely understood. One possibility is that TA serves as an oxalic acid reserve or an intermediate/end product in oxalic acid synthesis. Oxalic acid is mainly a product of ascorbic acid metabolism and serves as a sink for excess calcium in plants forming calcium oxalate in specific plant vacuoles called crystal idioblasts (Kostman *et al.* 2001).

According to many experiments involving ^{14}C or ^{3}H labeled metabolites, TA biosynthesis is found to occur via three different pathways depending on the plant (Fig. 2). Many of the enzymes involved in the reactions have not yet been characterised. In grape berries TA is the metabolic product of ascorbic acid (Saito and Kasai 1969, Wagner *et al.* 1975). More specifically, the experiments indicated that ascorbic acid was converted via 2-keto-gluconic acid (2-KGA) and idonate to 5-KGA and further cleaved between carbons C4 and C5. The resulting unidentified four-carbon fragment was converted to tartaric acid and the unidentified two-carbon fragment recycled back to the metabolic carbon pool. The second pathway was discovered in a geraniaceous plant, *Pelargonium crispum* (Wagner and Loewus 1973, Saito K. *et al.* 1997). In contrast to the first pathway, here the ascorbate is cleaved between the second and third carbons. The cleavage products are oxalic acid and L-threonic acid of which the latter is further metabolised to tartaric acid. This pathway is probably the most significant in plants due to the formation of oxalic acid and is also believed to be the most common among plants. Oxalic acid, as mentioned earlier, is an important molecule in plant cells in maintaining the ionic balance by binding excess calcium. The third pathway to tartaric acid is found mainly in leguminous plants (Saito and Loewus 1989) and does not involve the ascorbic acid route. Instead, glucose is converted to gluconic acid via D-glucono-1,5-lactone and further to 5-KGA followed by a cleavage between carbons four and five similarly to pathway one. The resulting four-carbon fragment is further converted to tartaric acid.

2.3.2 5-KGA is the key-molecule in TA bioproduction

The role of 5-KGA as the key intermediate in TA biosynthesis in plants can be clearly seen in Fig. 2. However, the exact mechanism by which 5-KGA is converted into TA is not yet known. In industrial production perspectives it is interesting that 5-KGA is known to be synthesised from gluconic acid not only by plants but also by microbes, and 5-KGA can be used as precursor in TA production. It can also be used for the production of xylaric acid (Fleche 1998) and a number of flavors (Keliang and Dongzhi 2006).

Several strains of *Gluconobacter* and *Acetobacter* are reported to produce 5-KGA in to the culture medium (Adachi *et al.* 1979 and Moo-Young 1985). Further fermentation studies of 5-KGA production by some of these strains have been conducted (Table 1, Shinagawa *et al.* 1983, Weenk *et al.* 1984). According to these studies, *G. suboxydans* strain IFO12528 was reported to produce the highest yield of 5-KGA from glucose or gluconate. This strain was able to convert over 90% from initial carbon source to 5-KGA (Shinagawa *et al.* 1983). In these experiments the best yield and 5KGA:2KGA ratio was obtained at very low pH of 2.5–3.

All reported methods used to produce 5-KGA are based on fermentation of glucose with *G. suboxydans*. Such fermentations always lead to co-production of isomeric 2-keto-gluconic acid and often also 2,5-diketo-gluconic acid. Another problem in these fermentations is that 5-KGA is consumed by the production host during the later stages of fermentation.

G. oxydans gets its name from *oxys*; Latin for "sharp, acidic", and *dans*; "giving". *Gluconobacters* are historically interesting because they oxidize ethanol to acetic acid and vinegar production is over 6000 years old. Acetic acid bacteria in general are known to

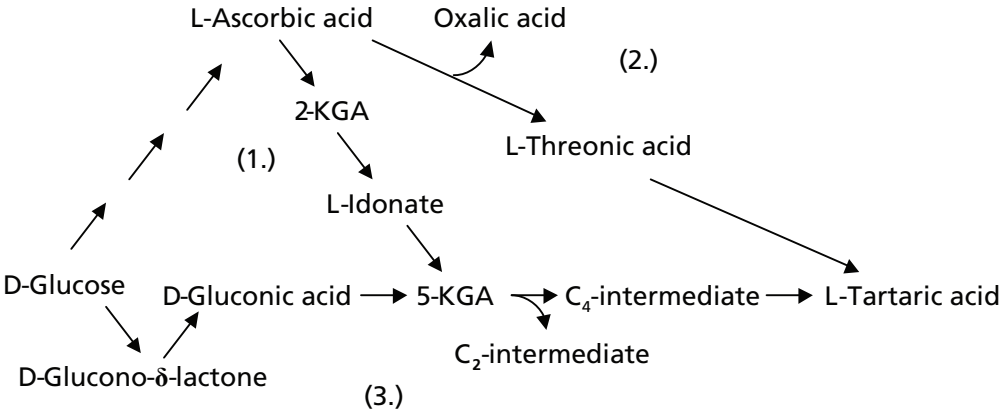

Figure 2 TA-synthesis in plants occurs via three alternative pathways depending on the plant. (1.) In grape berries ascorbic acid is converted via 2-KGA to 5-KGA and further cleaved resulting uncharacterised C4-fragment that is converted to TA. (2.) In geraniaceous plant ascorbate is cleaved to L-threonic acid and oxalic acid. L-threonic acid is further metabolised to TA. The third (3.) pathway to TA is present mainly in leguminous plants. Here glucose is converted to gluconic acid and further to 5-KGA, which is cleaved similarly than in pathway one to produce TA via an unknown intermediate.

Table 1 Production of ketogluconates by some *Gluconobacter* species (Weenk *et al*. 1984)

Strain	GLA[a]	2KGA[a]	5KGA[a]	GA 2-DH[b]	GA 5-DH[b]
G. oxydans sp. *melanogenes* ATCC15163	3	16	12	0.3	0.2
G. oxydans sp. *melanogenes* Org 13091	0	25	13	0.7	0.2
G. oxydans sp. *albidus* IFO3251 t2	0	97	8	0.5	0.03
G. oxydans sp. *cerinus* IFO3262 t1	0	93	10	0.3	0.03
G. oxydans sp. *suboxydans* ATCC621-H	20	13	30	0.4	0.2
G. oxydans deLey8131	6	4	4	0.03	0.06
G. oxydans deLey8624	30	26	7	0.09	0.04
G. oxydans deLey4943	60	37	6	0.2	0.01
G. oxydans sp. *suboxydans* ATCC23773	5	52	21	0.3	0.3
G. oxydans sp. *industris* IFO3260	0	35	35	0.3	0.2
G. suboxydans IFO12528	36	10	33	0.1	0.6

Abbreviations: GLA – gluconic acid, 2KGA – 2-keto-gluconic acid, 5-KGA – 5-keto-gluconic acid, GA 2-DH – gluconate 2-dehydrogenase, GA 5-DH – gluconate 5-dehydrogenase.
[a](%) yields of product from consumed glucose.
[b]U/mg.

oxidize for the production of energy, not only glucose and gluconate, but a great variety of carbohydrates and derivatives, including hexoses, pentoses, hexonic acids, primary and secondary alcohols, aldehydes, hydroxy acids, cyclitols, polyols, and aliphatic glycols (for rewiev, see Deppenmeier *et al.* 2002 and Adachi *et al.* 2003). Many of these oxidations including the oxidation of gluconate to 5-KGA follow the specific Bertrand-Hudson rule, which predicts the specificity of oxidation of polyols by acetic acid bacteria (Fig. 3): "polyols with a cis-arrangement of two secondary hydroxyl groups in D-configurations to the adjacent primary alcohol group are oxidized to the corresponding ketoses". The rule was first described by Bertrand (1904) in a work covering biochemical preparation of sorbose and then later expanded by Hann *et al.* (1938) to cover the oxidation of other sugar alcohols.

Several biotechnologically important molecules are produced by acetic acid bacteria according to the Bertrand-Hudson rule, the most interesting ones are L-sorbose and 5-KGA. L-sorbose is the oxidation product of D-sorbitol and is used as a key intermediate in ascorbate synthesis by the classical Reichstein process and by modern bioprocesses based on the biotechnological production of 2-keto-L-gulonic acid (Sugisawa *et al.* 1990).

2.3.3 Membrane bound oxidoreductases of *G. suboxydans*

Although acetic acid bacteria have for long been known to catalyze many important reactions, the enzymes involved in these catalysis have not been known until recently.

Information on the 5-KGA forming enzyme, GA 5-DH (EC 1.1.1.69), is scarce. A report by Shinagawa *et al.* (1999) described the extraction of GA 5-DH activity from the cell

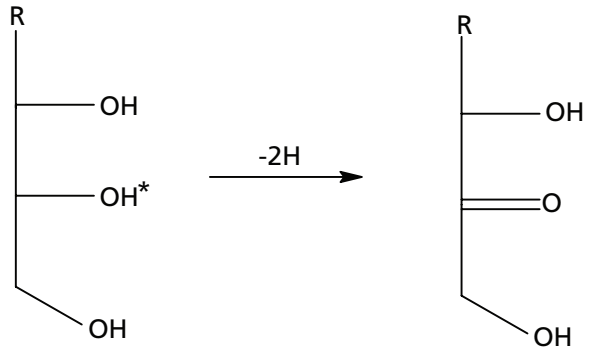

Figure 3 The definition of the preferred polyol oxidation route according to the Bertrand–Hudson rule. The hydroxy group oxidised according to the rule is marked with an asterisk.

membrane fraction of *G. suboxydans.* The main conclusion of this study was that GA 5-DH belongs to the class of pyrroloquinoline quinone (PQQ)–dependent membrane dehydrogenases.

During the last few years, two novel sugar alcohol oxidising enzymes of *Gluconobacter* sp. have been isolated and characterised, namely D-sorbitol dehydrogenase (EC 1.1.99.21) of *G. suboxydans* IFO3255 (Sugisawa and Hoshino 2002) and D-arabitol dehydrogenase of *G. suboxydans* IFO3257 (Adachi *et al.* 2001). Also the gene for D-sorbitol dehydrogenase has been cloned (Miyazaki *et al.* 2002). Both of these enzymes have been found to be PQQ-dependent membrane bound dehydrogenases with similar and rather broad substrate specificity. Some quite distinct properties of these two enzymes were also found, including subunit structure, the optimum pH and pH-stability. However, very recently the group behind the characterisation of these enzymes concluded based on immuno cross-reactivity studies that, despite the differences obtained with the two enzymes, they are actually the same enzyme encoded by one gene (Matsushita *et al.* 2003). At that time there was not enough genetic information of these enzymes available to support this hypothesis. They also suggested that this enzyme could be responsible for almost all sugar alcohol oxidation following the Bertrand-Hudson rule in *Gluconobacter* species including also the oxidation of gluconate to 5-KGA. It was also suggested, that glycerol dehydrogenase (Ameyama *et al.* 1985), polyol dehydrogenase (Cho *et al.* 1990) and mannitol dehydrogenase (Oikawa *et al.* 1997) isolated from *Gluconobacter* sp. all are the same major polyol dehydrogenase encoded by the same gene. The very recently published complete genome sequence of *G. oxydans* leaves still some question marks to this hypothesis (Prust *et al.* 2005). According to the genome data, there are several polypeptides in *G. oxydans* that are significantly homologous to the polyol/sorbitol dehydrogenase (AAW60628). According to the genome project, *G. oxydans* contains three other, still uncharacterised PQQ-dependent polypeptides encoded by chromosomal loci AAW60294 (44% identical to polyol/sorbitol dehydrogenase), AAW61191 (39% identical to polyol/sorbitol dehydrogenase), AAW61595 (37% identical to polyol/sorbitol dehydrogenase). Cloning and characterisation of these enzymes would finally show if the polyol/sorbitol dehydrogenase alone really is responsible for the bulk oxidation of sugar alcohols by *Gluconobacter* sp. or if more than one enzyme are indeed involved to these oxidations.

The gene structure of polyol/sorbitol dehydrogenase was first described by Miyazaki

et al. (2002). The enzyme is encoded by two adjacent open reading frames, a small and a large one, of which the larger is homologous to the glucose dehydrogenase, with 35% identity to the glucose dehydrogenase of *G. oxydans*. There is no non-coding DNA between the two coding areas since the stop codon (TGA) of the smaller ORF partially overlaps the initiator codon (ATG) of the larger ORF (…**TGA**TG…). Both subunits were found to be needed for the expression of an active enzyme. The smaller ORF was found to encode a hydrophobic polypeptide, with 29% identity to the N-terminal region of glucose dehydrogenase. This region is considered to be the membrane-spanning domain of glucose dehydrogenase and is involved in binding the enzyme to the membrane and for electron transfer across the membrane (Yamada *et al.* 1993). Based on the deduced amino acid sequence of the enzyme it was suggested that the electron acceptor of this enzyme is ubiquinone rather than cytochrome C similar to the glucose dehydrogenase. In support of this it was found based on sequence homology to *E. coli* glucose dehydrogenase that the two amino acids (Asp 466 and Lys 493), proposed to be related to the initiation of glucose oxidation and transfer of an electron from the reduced form of PQQ, were conserved (Asp 398 and Lys 426) also in polyol dehydrogenase (Miyazaki *et al.* 2002).

2.3.4 Biotechnological methods for TA production

The raw material available for the current manufacture of natural tartaric acid and its salts are by-products of wine making. Crude TA can be recovered in several steps during wine production; from grape juice filter cakes, from primary fermentation vat bottoms, and from the vat bottoms of secondary fermentation. The yearly variations in grape harvests and therefore in production volumes lead to large fluctuations in the market price of TA, limiting its use in industry.

Two different approaches for the production of TA utilising biotechnology have been the production of TA from maleic acid via epoxysuccinic acid and from glucose via 5-KGA. TA can be produced from maleic acid in a two-step process involving chemical oxidation and microbial conversion (Blair and DeFraties 1995). In the first step, epoxysuccinic acid is produced from maleic anhydride by oxidation with hydrogen peroxide using tungsten acid as a catalyst (Church and Blumberg 1951). TA can be subsequently produced from cis-epoxysuccinate by microbes (Tsurumi and Fujioka 1976, Miura *et al.* 1976, Miura *et al.* 1977, Willaert and De Vuyst 2005). The microbes used in the conversion are derived from the following genera: *Nocardia, Agrobacterium, Rhizobium, Pseudomonas, Acetobacter* and *Corynebacterium*. The enzyme catalysing this reaction is epoxide hydrolase that hydrolyses the epoxy ring of cis-epoxysuccinate (Miura *et al.* 1977, for a rewiev see Orru *et al.* 1999). The disadvantage of this technically rather simple process is the high price of the raw material, but it has still been economically profitable especially in China. The increase of production of TA from maleic anhydride in China has nearly doubled 2003–2005 from 8000 to 14 000 tonnes and is expected to increase steadily in future (China Chemical Reporter).

As mentioned earlier, 5-KGA can be produced from glucose using *Gluconobacter* species, although no commercial production of 5-KGA by fermentation has been reported to take place. The first 5-KGA production studies revealed that the 5-KGA was to some extent further oxidized to TA and glycolic acid during the microbial fermentations (Yamada *et al.* 1971). TA yield of 35% from glucose was obtained after six days of fermentation of randomly mutagenised *G. suboxydans* strains (Kodama *et al.* 1972, Kotera *et al.* 1972). The

mutagenisation was shown to diminish the 2-KGA pathway, allowing more carbon to be directed to the 5-KGA pathway. The mutated strains were also able to tolerate increased acidity and high concentrations of glycolic acid in particular, which was earlier shown to inhibit bacterial growth. However, it was later concluded that the formation of TA during the fermentation was actually caused by the oxidation of 5-KGA to TA by vanadate present in the fermentation broth (Klasen *et al.* 1992). Enzymes were not found to be involved in the 5-KGA-oxidation reaction to TA in these cultivations. The vanadate-catalysed oxidation of 5-KGA to TA during the cultivations was later studied by the same group (Matzerath *et al.* 1995). A concomitant study demonstrated that noble metals can also be used to catalyse the oxidation of 5-KGA to TA (Miasnikov and Jacobsen 2003).

The enzymes converting 5-KGA to TA in plants have yet to be identified. Speculatively a ketolase (such as transketolase or phosphoketolase) can remove the keto moiety of 5-KGA generating a tartaric acid semialdehyde. Secondly, the semialdehyde can be oxidized into tartaric acid by a dehydrogenase.

Also NADP-dependent gluconate 5-dehydrogenase has been proposed to be responsible for 5-KGA bulk-production by *Gluconobacter*. This enzyme was cloned from *G. oxydans* DSM3503 and expressed homologously (Klasen *et al.* 1995). It is a cytoplasmic soluble dehydrogenase with an optimum pH for D-gluconate oxidation around 10. The conclusion made by the authors, that NADP-dependent gluconate 5-dehydrogenase would be the enzyme responsible for the bulk oxidation of gluconate, was apparently wrong. The cytoplasmic location of this enzyme makes its use for the oxidation of extracellular gluconate to extracellular 5-KGA energetically highly unfavorable. *Gluconobacter* strains overexpressing NADP-dependent gluconate 5-dehydrogenase to over 80-fold lead to only 11% increase in the accumulated 5-KGA in the culture medium compared with the wild type strain (Klasen *et al.* 1998). This conversion corresponded to a yield of 14% from glucose.

2.4 D-Erythorbic acid

D-EA (araboascorbic acid, isovitamin C, Fig. 4) is a C-5 epimer of L-ascorbic acid (vitamin C). While ascorbic acid (L-AA) is widespread in the animal and plant kingdoms (Chatterjee 1973), it is replaced in microorganisms by its analogues, D-EA and a five-carbon analogue D-erythroascorbic acid. D-erythroascorbic acid is prevalent among yeasts (Leung and Loewus 1985, Nick *et al.* 1986, Dumbrava and Pall 1987, Loewus *et al.* 1995) while D-EA is found only in the filamentous fungus *Penicillium* (Takahashi *et al.* 1960, Yagi *et al.* 1967).

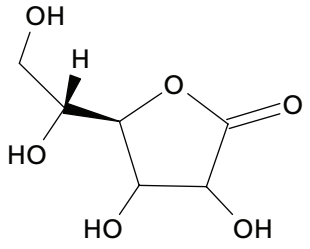

Figure 4 Stereochemical structure of D-EA

2.4.1 Physiology and functions of L-AA and its microbial analogues

L-AA is named for its anti-scorbutic properties in humans. However, biological functions of L-AA and its analogues are incompletely understood. In plants L-AA is thought to accumulate as a storage molecule that can be rapidly converted to different sugars, and its functions have been connected to respiration, growth, and carbon balance maintenance (Jaffe 1983, Loewus 1999). In animals the biochemical functions of L-AA include, for example, collagen synthesis, immune functions, drug-, folate-, cholesterol-, and iron metabolism, and carnitine biosynthesis (Blair and DeFraties 1995). In addition, L-AA can act as a reducing agent and as an effective antioxidant protecting cells from free radical damage. It can also interfere with nitrosamine formation and consequently may potentially reduce cancer risk. While L-AA is a well-characterised molecule, very little is known of its analogues D-EA and D-erythroascorbic acid. These L-AA analogues are believed to play similar roles in microbes as L-AA does in plants and animals.

Unlike L-AA, D-EA does not appear to exert vitamin C activity as for example, it did not prevent scurvy in guinea pigs (one of the few animal species other than humans that does not synthesize L-AA) at the same dosage (Reiff and Free 1959). However, increasing the D-EA dosage 20 fold was found to be sufficient to replace the natural L-AA requirement in these animals (Goldman *et al.* 1981). In studies with young women was found that up to 1000 mg/day of D-EA for as long as 40 days was rapidly cleared from the body and had only little effect on the bioavailability of L-AA, indicating that D-EA does not diminish the bioavailability of L-AA in humans at nutritionally relevant levels (Sauberlich *et al.* 1996). D-EA is much more poorly absorbed and retained in the tissues than L-AA, and is also poorly reabsorbed in the kidney and rapidly excreted. As a result, it has low anti-scorbutic activity also in humans and interferes significantly only with L-AA uptake and retention in the tissues when concentrations are at least an order of magnitude higher than concentrations of L-AA.

However, D-EA acts similarly to L-AA in molecular functions of human cells. For example the protective effects of L-AA and D-EA against lipid peroxidation were investigated in guinea pigs (Kunert and Tappel 1983). The authors concluded that the antioxidant function of L-AA is relatively non-specific and the two stereoisomers did not differ with regard to their antioxidant properties *in vivo*. In addition, L-AA and D-EA demonstrated similar activity in promoting the hydroxylation of peptidyl proline of carnitine biosynthesis in a cell-free system (Hutton *et al.* 1967, Kutnik *et al.* 1969).

The effects of L-AA and D-EA on collagen synthesis were studied in cultured human skin fibroblasts (Murad *et al.* 1981). At the high concentrations used, both L-AA and D-EA increased collagen synthesis about eightfold with no significant change in synthesis of non-collagen protein. The results were taken to indicate that collagen polypeptide synthesis and posttranslational hydroxylation of collagen are independently regulated by L-AA, and with D-EA having similar effects. The effect of L-AA on collagen synthesis was maximal at a concentration of 30 μM while D-EA stimulated the collagen synthesis at ten-fold higher concentration (Murad *et al.* 1983).

2.4.2 Biosynthesis of L-AA and its analogues

L-AA biosynthesis in animals and plants is extensively studied (for reviews, see Loewus 1999, Smirnoff 2001, Valpuesta and Botella 2004). In animals L-AA is synthesised from

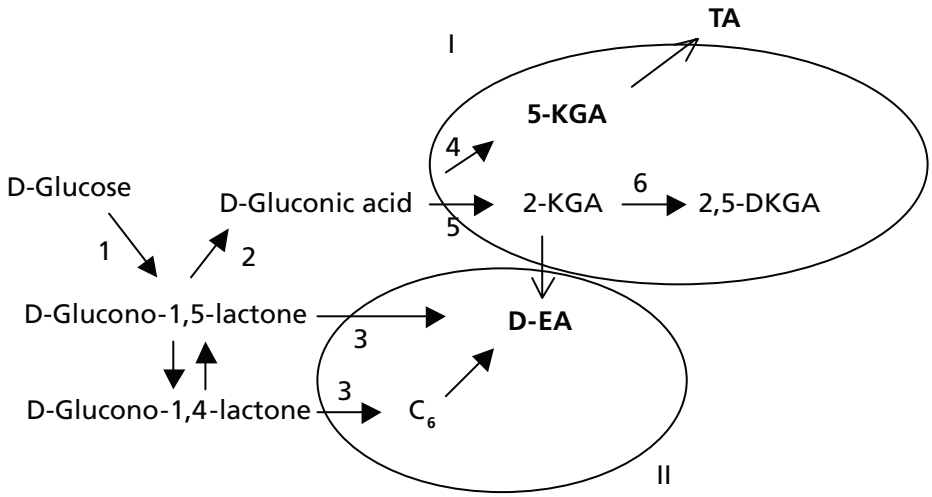

Figure 5 Metabolic pathways and chemical conversions necessary for the production of D-EA and 5-KGA. The circled area I represents the enzyme activities present in *G. suboxydans* and area II represents the enzyme activities present in *P. cyaneo-fulvum.* Other pathways are similar in both microbes. Conversion of 5-KGA to TA and 2-KGA to D-EA can be done by chemical conversion reactions used in production processes (marked with an open arrow →). The numbered reactions are catalysed by the following enzymes: 1; glucose oxidase, 2; gluconolactonase (or spontaneous), 3; GLO, 4; GA 5-DH, 5; gluconate 2-dehydrogenase, 6; 2-keto-gluconate dehydrogenase.

glucose and is one end product of the glucuronic acid pathway. In plants it is also a product of hexose phosphate metabolism, but the biosynthesis is more complicated. Furthermore, some plants are capable of using alternative L-AA biosynthetic route from glucose via L-sorbsone using NADP-dependent dehydrogenase (Loewus *et al.* 1990).

The D-erythroascorbic acid produced in yeast is synthesised from D-arabinose (Murakawa *et al.* 1977). D-arabinose is first oxidized to its 1,5-lactone, which is further converted to arabinono-1,4-lactone. In the last step the arabinono-1,4-lactone is oxidized to D-erythroascorbic acid by D-arabinono-1,4-lactone oxidase. The product of the oxidation reaction catalysed by this enzyme is L-AA, if L-galactono-1,4-lactone is used as substrate instead of D-arabinono-1,4-lactone (Huh *et al.* 1994).

The D-EA synthesis pathway of *Penicillium* is also known (Fig. 5), and it is similar to the yeast pathway described above, comprising just two steps (Takahashi *et al.* 1976, Murakawa and Takahashi 1977). Glucose is first oxidized into D-glucono-1,5-lactone by GOD. The conversion of D-glucono-1,5-lactone to D-glucono-1,4-lactone is believed to be a nonenzymatic equilibrium reaction (Isbell and Frush 1933, Mitchell and Duke 1970, Shimahara and Takahashi 1970, Combes and Birch 1988). Glucono-1,5-lactone is rather unstable at neutral pH and the nearly irreversible hydrolysis of the 1,5-lactone ring to gluconic acid may occur spontaneously but is also catalysed by a lactonase (EC 3.1.1.17, Bruchmana *et al.* 1987, Kobayashi *et al.* 1998). In the second step, both D-gluconolactones are further oxidized by GLO (E.C. 1.1.3.X). While GOD is a well-known enzyme and is widely distributed among fungi, GLO is so far found only from *Penicillium* sp. (Yagi *et al.* 1967). GLO can use as substrate 1,4- and 1,5-lactones even though D-EA itself is a 1,4-lactone. The oxida-

tion product of D-glucono-1,4-lactone is D-EA. D-glucono-1,5-lactone is oxidized by GLO to an unknown intermediate having a 1,5-lactone ring, which is rapidly non-enzymatically further converted to D-EA (Murakawa and Takahashi 1977).

2.4.3 Sugar lactone oxidases

Sugar lactone oxidases are a group of enzymes responsible for the final step of biosynthesis of L-AA and its analogues in plants, animals and microbes. In the major L-AA synthesis route in plants, the oxidation of L-galactono-1,4-lactone to L-AA catalysed by L-galactono-1,4-lactone dehydrogenase is the final step. In animals, the corresponding step involves the oxidation of L-gulono-1,4-lactone and is catalysed by L-gulono-1,4-lactone oxidase. It is noteworthy that all characterised sugar lactone oxidases acting on aldono lactones are specific only towards 1,4-lactones, except GLO of *P. cyaneo-fulvum*, which can use both D-glucono-1,4-lactone and D-glucono-1,5-lactone as its substrate.

Sugar lactone oxidases share also other common features, e.g. they are flavoproteins having FAD as the prosthetic group and most of them are membrane bound. Of the enzymes listed in Table 2, only GLO of *P. cyaneo-fulvum* was isolated as soluble protein from cell lysate (Takahashi *et al.* 1976). Accordingly, all the other isolated enzymes have been reported to form aggregates during purification, which is typical for the hydrophobic membrane bound proteins. However, it has been found that in animal cells the subcellular localisation is different than in plant and yeast cells. The animal L-gulonolactone oxidases are integral membrane proteins of the endoplasmic reticulum and the active site of the enzyme is located in the ER lumen (Eliceiri *et al.* 1969, Kiuchi *et al.* 1982, Koshizaka *et al.* 1988, Puskas *et al.* 1998) whereas the plant L-galactonolactone oxidases are localised in the inner mitochondrial membrane (Mapson and Breslow 1958, Oba *et al.* 1995, Siendones *et al.* 1999). Also the yeast D-arabinonolactone oxidase is a membrane-bound mitochondrial enzyme (Huh *et al.* 1994).

The inhibition pattern of sugar lactone oxidases seems to be similar. Divalent cations and p-chloromercuribenzoate inhibit all tested D-gluconolactone oxidases. The inhibition of these enzymes by p-chloromercuribenzoate indicates that a -SH group is required for the activity of the enzymes.

The properties of L-galactono-1,4-lactone oxidase isolated from yeast by two groups are reported to be rather controversial (Nishikimi *et al.* 1978, Bleeg and Christensen 1982). Both enzymes were isolated as mitochondrial membrane proteins, but the substrate specificities, optimum pHs and particularly the subunit structures have been reported to show significant differences as can be seen in Table 2.

The enzyme isolated by Bleeg and Christensen (1982) was found to be a homotetramer of 18 kDa monomers, while the L-galactono-1,4-lactone oxidase isolated by Nishikimi *et al.* (1978) was found to be a monomer of 56 kDa. The genome sequence of *S. cerevisiae* revealed only one gene encoding a isoenzyme of 59 493 Da. A *C. albicans* homologue for this enzyme was cloned and, after further characterisation, was renamed as D-arabinono-1,4-lactone oxidase (Huh *et al.* 1994). The enzyme can oxidize both L-galactono- and D-arabinono-1,4-lactone with equal efficiency.

The genes encoding animal, plant and yeast sugar lactone oxidases have been cloned and characterised, e.g. the genes of L-gulono-1,4-lactone oxidase from rat (Koshizaka *et al.* 1988) and L-galactono-1,4-lactone dehydrogenase from cauliflower (Östergaard *et al.* 1997). The typical domain for covalently bound FAD (PROSITE pattern PS00862: P-x(10)-

Table 2 Biochemical properties of sugar lactone oxidases

Organism	**Cauliflower**	**Sweet potato**	***S. cerevisiae***	***C. albicans***
Enzyme	L-galactono-1,4-lactone DH	L-galactono-1,4-lactone DH	D-arabinono-1,4-lactone oxidase	D-arabinono-1,4-lactone oxidase
Localisation	Mitochondria	Mitochondria	Mitochondria	Mitochondria
Native size, kDa	56	56	290 and 70–74	110 and 84
Subunit size, kDa	57.837	56	56 and 18	66.7
Prosthetic group	Flavine	Flavine	FAD	Flavine
Substrate specificity	L-galactono- and D-altrono-γ-lactone	L-galactono-γ-lactone	L-galactono-, L-gulono- and D-altrono-γ-lactone	D-arabinono-, L-galactono-, L-gulono- and L-xylono-γ-lactone
Inhibitors	Riboflavine, atebrine, Cu^{2+}	Akriflavine, PCMB	PCMB, S^{2-}, SO_3^{2}, iodoacetamide	Divalent cations, PCMB
pH optimum	7.4–7.9, 8.0–8.5	7.9	6.8 and 8.9	6.1
K_m, mM	2 and 3.3	0.12	n.d./0.3	44.1
Organism	**Rat**	**Goat**	**Chicken**	***P. cyaneo-fulvum***
Enzyme	L-gulono-1,4-lactone oxidase	L-gulono-1,4-lactone oxidase	L-gulono-1,4-lactone oxidase	GLO
Localisation	Microsome	Microsome	Microsome	Cytosol (soluble)
Native size, kDa	500, 450 and 100	500	400	150
Subunit size, kDa	50.474	51	50	n/d
Prosthetic group	FAD	Flavine	FAD	Flavine
Substrate specificity	L-gulono-γ-lactone, L-galactono-γ-lactone	L-gulono-γ-lactone	L-gulono-, L-galactono-, D-mannono- and D-altrono-γ-lactone	D-glucono-γ-lactone and D-glucono-δ-lactone
Inhibitors	Hg^{2+}, S^{2-}, SO_3^{2-}, p-nitrothiophenol	n.d.	n.d.	Fe^{3+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Ag^{2+}
pH optimum	7.5	7.5	7.0	5.6–6.0
K_m, mM	0.066 and 0.4	0.15	0.007	1.7

Data obtained from: C. albicans (Huh *et al*. 1994), Cauliflower (Mapson and Breslow 1958, Östergaard *et al*. 1997), Chicken (Kiuchi *et al*. 1982), Goat (Chatterjee *et al*. 1960), *P. cyaneo-fulvum* (Takahashi *et al*. 1976), Rat (Eliceiri *et al*. 1969, Nakagawa and Asano 1970, Nakagawa *et al*. 1975, Nishikimi *et al*. 1976), *S.cerevisiae* (Nishikimi *et al*. 1978, Bleeg and Christensen 1982), Sweet potato (Oba *et al*. 1995).

[DE]-[LIVM]-x(3)-[LIVM]-x(9,12)-[LIVM]-x(3)-[GSA]-[GSTCHRQ]-G-H) can be found from the deduced protein sequences of these enzymes. The last histidine of the pattern is thought to bind the FAD covalently. Curiously it is absent from the otherwise homologous domain

of sweet potato and cauliflower enzymes (although the histidine is present in *A. thaliana* enzyme) and as result, the binding of the FAD co-factor to the sweet potato L-galactono-1,4-lactone dehydrogenase was found to be noncovalent (Imai *et al.* 1998).

Cauliflower and yeast L-galactono-1,4-lactone dehydrogenase and D-arabinono-1,4-lactone oxidase genes include a putative mitochondrial targeting signal (Östergaard *et al.* 1997, Lee *et al.* 1999), which is removed from the mature enzyme. The targeting signal in the yeast enzyme has been shown to direct the recombinant enzyme to the cell membrane fraction of *E. coli* cell lysate (Lee *et al.* 1999).

Similarly the secretion signal found in L-gulono-1,4-lactone oxidase has been shown to target the recombinant enzyme to the ER membrane of monkey COS-1 cells (Yagi *et al.* 1991). The membrane association of the rat L-gulono-1,4-lactone oxidase is also supported by the Kyte-Doolittle hydropathy plot analysis of the enzymes deduced amino acid sequence (Koshizaka *et al.* 1988). The analysis reveals five strong hydrophobic regions forming a β-sheet structure in the polypeptide, suggesting the putative membrane anchoring sites.

The primates, guinea pig and some birds have lost the ability to produce L-AA and they require L-AA in their diet (Chatterjee 1973). However, the pseudogene encoding the corresponding sugar lactone oxidase is present in the human genome (Nishikimi *et al.* 1994). Based on the mutation frequencies it has been estimated, that the gene of primates has been inactivated some 40 million years ago before the divergence of the old and new world monkeys. One explanation for the gene loss could be that the selective pressure to maintain the gene was lost due to apparent changes in diet.

It is obvious from Table 2 that GLO of *P. cyaneo-fulvum* is quite different from the other characterised sugar lactone oxidases. While the other enzymes were found to be membrane bound proteins, GLO was purified as a soluble enzyme from the cell extract of *P. cyaneo-fulvum* (Takahashi *et al.* 1976). The size of the native enzyme is reported to be 150 kDa while the apparent sizes of the other sugar lactone oxidases are around 50–60 kDa. This could be due to the multimeric structure of GLO, while the other sugar lactone oxidases are thought to function as monomers. The pH-optimum of GLO is reported to be pH 5.6–6, over one pH unit lower than the optimum pH 7–8 of the animal, plant and *S. cerevisiae* enzymes. Only the *C. albicans* enzyme's optimum pH is quite similar to GLO. Also the substrate specificity of GLO is significantly different, while the other sugar lactone oxidases are reported to accept only the corresponding 1,4-lactones as substrate, GLO can use both the D-glucono-1,4- and D-glucono-1,5-lactones as its substrate (Murakawa and Takahashi 1977).

The GenBank reveals a large number of uncharacterised homologues of the sugar lactone oxidases and some of them are of prokaryotic origin, such as *Streptomyces coelicolor* NP629920.1, *Oceanobacillus iheyensis* NP692632.2 and *Bacillus cereus* YP082197.1. However, there are yet no reports on identification of prokaryotic lactone oxidase activity.

2.4.4 Biotechnological methods for D-EA production

D-EA is produced in industry by a two-step process (Fig. 6). Glucose is first converted into D-2-keto-gluconic acid by fermentation, using usually *Pseudomonas* sp., which converts the glucose to 2-KGA with high yield (Rose 1978). The second step in the process is chemical lactonisation, similar to the lactonisation of L-2-keto-gluconic acid in the traditional Reichstein process still used in L-AA production (Reichstein and Grüssner 1934, Reichstein 1941).

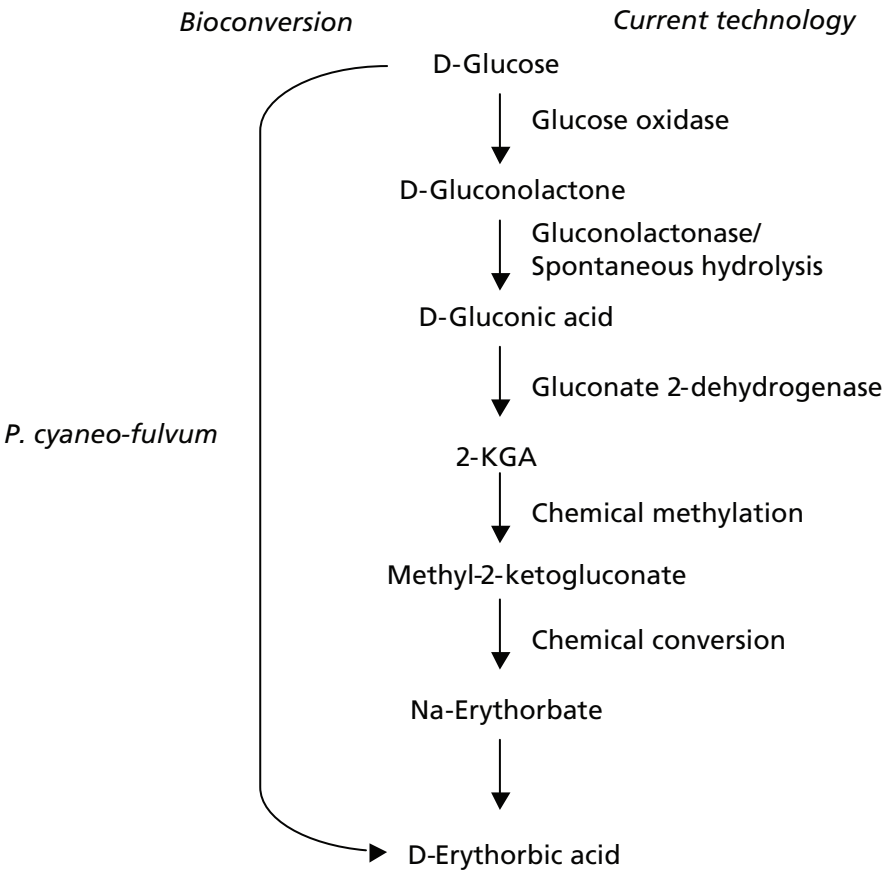

Figure 6 Method for the production of D-EA. Current D-EA production process consists of production of 2-KGA from glucose by fermentation followed by a chemical conversion of 2-KGA to D-EA. *P. cyaneo-fulvum* converts glucose directly to D-EA, but with insufficient production parameters for commercial purposes (Yagi *et al.* 1967, Shimizu *et al.* 1967, Takahashi 1969).

The possibility to produce D-EA by direct fermentation of glucose was studied in the 1960's, when direct conversion of glucose into D-EA by *Penicillium*-fungi was discovered (Takahashi *et al.* 1960). Despite the screening of 5000 fungal and bacterial strains, only *Penicillium* sp. were found to produce D-EA (Yagi *et al.* 1967). This screening was followed by an extensive mutagenesis/selection program resulting in strains capable of converting glucose into D-EA in about 40% yield over a week-long fermentation (Shimizu *et al.* 1967). The obtained process parameters were not sufficient to make the direct fermentation of glucose into D-EA economically feasible using these strains.

In later studies it was found that induction conditions for the glucose oxidation system necessary for the D-EA production, consisting of glucose oxidase, catalase and gluconolactonase, were very similar to the conditions of the failed D-EA production experiments during the 1960's (Bruchmana *et al.* 1987). According to the GenBank, it is highly probable that the lactonases are rather widely spread among fungi. Both *Aspergillus nidulans* and

Neurospora crassa possess uncharacterised hypothetical proteins (GenBank accessions XP_682246 and CAE81965 respectively) that are homologous to *Fusarium oxysporum* lactonohydrolase amino acid sequence encoded by chromosomal locus BAD54840 (Kobayashi *et al.* 1998). When *A. niger* is grown aerobically on glucose-containing media, extremely high levels (about 40 U/mg dw) of D-gluconolactonase are produced (Witteveen *et al.* 1993). The fungal glucose oxidation system is induced optimally at pH of 5.5, high aeration and high glucose concentrations. It was found in D-EA production trials by Shimizu *et al.* (1967) that moderate aeration and glucose feeding were critical factors for the high D-EA yield and good productivity. The conditions, which induced induction of glucose oxidation system, lower or higher aeration and higher glucose concentrations, prolonged the fermentation time and lowered D-EA yields. Strong accumulation of gluconic acid was observed even in optimal D-EA production conditions (Takahashi 1969) indicating the presence of lactonase activity in the cultivation broth. It is tempting to speculate that the main reason for the insufficient D-EA production parameters was the co-induction of lactonase expression during the D-EA production experiments, and therefore the loss of carbon via the practically irreversible hydrolysis of the D-glucono-1,5-lactone to gluconic acid (Fig. 5). The highest D-EA yields from glucose were obtained when washed mycelium of *P. cyaneofulvum* was incubated with dilute glucose solution. The conversion rate, and therefore productivity were low in these conditions.

3 Aims of the study

The aims of this study were to produce two gluconate related organic acids, D-EA and 5-KGA by enzymatic conversion or direct fermentation on glucose using metabolically engineered microbes as production hosts. The final goal was to show, at a proof-of-concept level, the functionality of the enzymatic conversion or one-step fermentation of glucose to these acids. The further development, scale up and optimisations of the enzyme or fermentation based production processes would be done later after evaluation of the feasibility of plan.

The specific aims of the study were:

- To purify the GLO of *P. cyaneo-fulvum* and GA 5-DH of *G. suboxydans*
- To clone the genes encoding these two enzymes and their over-expression in a suitable heterologous host
- To characterize the expressed enzymes and to evaluate the possibility to use them in an enzyme based production process
- Based on the new genome data, to discover novel enzymes with the same specific activities and possibly with new and better features for the industrial processes
- To use recombinant microbes overexpressing the necessary enzymes for efficient one-step fermentation of glucose to the end products

4 Materials and methods

The materials and methods of this study are described in detail in the original publications I–III. Here only the most essential and previously unpublished materials and methods are presented.

4.1 Microbial strains and growth media (I, II, III, unpublished)

Host strains of the isolated enzymes and the strains used for the expression and cloning are summarised in Table 3.

G. suboxydans IFO12528 growth medium consisted of 10 g Na-gluconate, 10 g glucose, 3 g yeast extract and 2 g peptone in 1 l of H_2O (pH 6.5). This strain was cultured at 30°C and 200 rpm. For large-scale production of GA 5-DH in fermentor, glycerol (3 g/l) was also added to the medium. *X. campestris* DSM3586 was grown in medium containing 10 g yeast extract, 20 g glucose, 20 g $CaCO_3$ in 1 l of water and at 30°C and 200 rpm. *P. cyaneofulvum* ATCC10431 was cultivated in a mineral medium described by Yagi *et al.* (1967).

4.2 Enzyme activity assays

4.2.1 GLO assay (I)

GLO activity was measured spectrophotometrically by following the reduction of 2,6, -dichlorophenolindophenol (DCIP) by D-EA at 600 nm and 30°C. The reaction mixture, freshly prepared for each experiment, consisted of 50 mM potassium biphthalate buffer

Table 3 Strains used in this study

Strain	Code/Source	Use in the study
Penicillium cyaneo-fulvum[a]	ATCC10431	Source of GLO and its gene
Gluconobacter suboxydans	IFO12528	Source of GA 5-DH and its gene
Xanthomonas campestris	DSM3586	Source of GA 5-DH gene
Aspergillus niger	N592	Source of GOD gene
Pichia pastoris GS115	Invitrogen	Expression host
Saccharomyces cerevisiae GRF18	ATCC6466	Expression host
Escherichia coli XL1-Blue MRF'	Stratagene	Expression host, cloning
Escherichia coli AS11	ATCC47069	Source of tktA- and tktB-genes
Escherichia coli XLOLR	Stratagene	Cloning

[a] Renamed later as *P. griseoroseum.*

pH 6.2, containing 2 mM hydroxyquinoline, 12 μM DCIP and 70 mM of D-glucono-1,5-lactone added as a form of 10% stock solution in dimethylformamide. A calibration curve was obtained by adding D-EA to the reaction mixture. One unit of activity was defined as one μmole of D-EA produced per minute under the enzyme assay conditions.

4.2.2 GOD assay (I)

GOD activity was determined by measuring the formation of H_2O_2 using the method described by Witteveen *et al.* (1990) following the oxidation of o-dianisidine by H_2O_2 at OD 450 nm. One unit of GOD activity was defined as one μmole of o-dianisidine oxidized per minute under the reaction conditions.

4.2.3 GA 5-DH assays (II, III)

During GA 5-DH purification the ferricyanide-based activity assay of Wood *et al.* (1962) was used. For characterisation of recombinant GA 5-DH, a 2,6-dichloroindophenol-based assay was used. The reaction mixture contained: 280 μl of 100 mM Na-acetate buffer pH 5.5, 2 μl of 1 mg/ml PQQ, 3 μl of 1 M $MgCl_2$, 3 μl of 1 M $CaCl_2$, 7 μl of 25 mM DCIP, 20 μl of substrate solution (0.5 M, pH 5.5) and 5 μl of cell suspension or crude extract. The reaction was carried out at 30ºC and followed spectrophotometrically at 600 nm.

In experiments using crude extracts or cell suspensions of the recombinant *E. coli* strains, similarly treated *E. coli* strain, lacking the GA 5-DH gene, was used as negative control. The following ε_{600} values for DCIP at different pHs were used for calculation of activity: pH 5.5–9.45 mM^{-1}, pH 6.0–10.8 mM^{-1}, pH 6.5–13.0 mM^{-1}, pH 7.0–15.0 mM^{-1} (Truesdell *et al.* 1991). One unit of GA 5-DH activity was defined as one μmole of the DCIP reduced per minute under the reaction conditions.

4.2.4 Lactonase assay (unpublished)

To measure the acid forming effect of lactonases on D-glucono-1,5-lactone, the change of pH under GLO-assay conditions was measured at room temperature after addition of substrate (10% D-glucono-δ-lactone in DMSO) and compared to the effect of added gluconic acid (0–90 mM) in the control reaction (Witteveen *et al.* 1992). One unit of activity was defined as one μmole of gluconic acid formed per minute at reaction conditions.

4.3 Analytical methods

4.3.1 HPLC (II, III)

The products of gluconate oxidation by GA 5-DH were identified by HPLC on a Toyopearl TSK DEAE 2SW column, 4.6 x 250 mm (Pharmacia, Sweden) equilibrated and eluted with 10 mM phosphoric acid (pH 2.55) using refractometric detection.

The products of oxidation of polyols by GA 5-DH were analysed by HPLC on Aminex

HPX-87P column (Bio-Rad, USA) equilibrated and eluted with H_2O using refractometric detection.

Tartaric acid-semialdehyde was analysed using Fertigsäule Polyspher OA KC-column (Merck, USA) at 30°C with 5 mM H_2SO_4 as a mobile phase. UV detector at 215 nm was used in the detection.

4.3.2 TLC (I, II, III)

5-KGA and 2-KGA were analyzed on Kieselgel 60 TLC-plates (Merck, USA). Mobile phase consisted of 1-Butanol:acetic acid:water (3:2:1). Following chromatographic separation the plates were first dried, and then dipped in 5% H_2SO_4 solution in ethanol and incubated at 140°C for 5 min for detection.

D-EA was analysed on Silica gel 60-plates (Merck, USA). Before use the TLC-plates were first impregnated with 0.3 M NaH_2PO_4 and then dried. The mobile phase consisted of Acetone:1-Butanol:H_2O (8:1:1) (Ghebregzabher *et al.* 1976). D-EA was detected by spraying the plates with 0.08% DCIP-solution.

4.4 Purification of enzymes

4.4.1 Purification of GLO (I)

GLO was purified by using a modification of a procedure described earlier (Takahashi *et al.* 1976). Briefly, *P. cyaneo-fulvum* ATCC 10431 was cultivated for 60 hours in a 15 l fermentor in mineral medium. The mycelium was collected by filtration, washed and homogenized in a bead-beater. The homogenate was centrifuged and 80 ml of DEAE-Sepharose FF (Pharmacia) was mixed per litre of supernatant (conductivity 2.5 mS/cm). The resin was removed by filtration and the enzyme precipitated by adding ammonium sulphate to 60% (w/v) saturation. Following dialysis, the crude GLO preparation (conductivity 1.2 mS/cm) was fractionated on DEAE-Sepharose FF using a linear NaCl elution gradient. The active fractions were pooled, concentrated by ultra-filtration and applied to a Sephacryl S-300 HR column (Pharmacia). A peak containing the GLO activity was collected, applied to a Bio-Gel HT hydroxyapatite column (BIO-RAD, USA) and eluted with a linear gradient of ammonium sulphate. The fractions with highest specific activity contained essentially homogenous GLO and were used for protein sequencing.

4.4.2 Purification of GA 5-DH (II)

The fermentation of *G. suboxydans* IFO12528 was carried out at 30°C for 20–24 h. The cells from 10 l culture were collected by centrifugation, washed and resuspended in 5 ml of buffer. The suspension, containing about 20% (w/v) of wet cell mass, was sonicated at 4°C until no intact cells could be observed under the microscope. The lysate was centrifuged. The pellet was washed, centrifuged and re-suspended in 5 ml of buffer containing 1% *n*-octyl-β-D-glucoside. The mixture was allowed to stand at 4°C for 6 hours followed by an-

other centrifugation. The pellet containing most of the GA 5-DH activity was re-suspended in 5 ml of buffer containing 10% *n*-octyl-β-D-glucoside. The extraction of GA 5-DH was allowed to proceed at 4°C overnight. After centrifugation, the supernatant was applied onto a Mono S HR 5/5 column (Pharmacia). GA 5-DH was eluted by a linear gradient of NaCl. Active fractions were pooled and further purified by discontinuous PAGE. After running the gel it was cut to approximately 2 mm slices, which were crushed and extracted overnight. The extracts containing GA 5-DH activity were subjected to SDS-PAGE. The major polypeptide (approximately 80 kDa) in this preparation was used for protein sequencing.

4.5 Cloning and heterologous expression of genes

4.5.1 Construction and screening of cDNA-library of *P. cyaneo-fulvum* (I)

mRNA was isolated from the total RNA of *P. cyaneo-fulvum*, converted to cDNA, and a cDNA-library was constructed in phage λ. The phage library was screened by hybridisation, using as a probe a PCR-fragment obtained with degenerate oligo nucleotides planned according to the peptide sequences. The hybridisation positive phage clones were purified, converted to a phagemid form, and analysed by restriction mapping. One representative clone was sequenced.

4.5.2 Expression of the *P. cyaneo-fulvum* GLO gene in *S. cerevisiae* (unpublished)

The expression vector used for the expression of GLO in *S. cerevisiae* was based on the yeast/*E. coli*-shuttle vector pJDB207 (Beggs 1981). Construction of the expression vector was accomplished in two stages. Firstly, pAC109 was constructed by simultaneously ligating three DNA fragments containing the following functional elements; (1) the 0.45 kb *Bam*HI–*Eco*47III fragment of the promoter area of the *S. cerevisiae PHO5* gene, (2) the 0.38 kb *Hae*III–*Hind*III fragment of the *S. cerevisiae MFα1* gene containing a part of coding area corresponding to the sequence of the prepropeptide of the yeast α-factor precursor protein (MFα1-prepropeptide) and (3) the 6.5 kb *BamH*I–*Hind*III fragment of pJDB207 containing the selection markers and the fragments necessary for the plasmid replication. Secondly, a synthetic polylinker was inserted into pAC109, giving rise to plasmid pGTY. The polylinker was composed of two oligonucleotides oTOP 5′-AGCTCTCGAGATCTCCCGGGA-3′ and oBOT 5′-AGCTTCCCGGGAGATCTCGAG-3′. The plasmid that has the polylinker inserted in such an orientation that the *Hind*III site is located proximally to the MFα1-prepro area was selected and named pGTY.

The DNA sequence of the cloned GLO gene was modified by PCR using oligonucleotides oMGLO5 5′-GAAGAAGCTTACCGGTGGTTCAATTGGCAGTTTTTGGT-3′ and oMGLO3 5′-CACGACGTTGTAAAACGACGGCCAG-3′ into a form suitable for constructing a fusion with the MFα1-prepropeptide. All of the noncoding sequence and the sequence corresponding to amino acid residues 1–20 of the deduced GLO coding sequence were deleted and a *Hind*III site was introduced in a position allowing an in-frame fusion of the MFα1-prepropeptide and mature GLO. After restriction of the PCR-product and pGTY with

*Hind*III and *Xho*I, the two fragments were ligated resulting plasmid pGTY(GLO). This plasmid codes for a fusion protein composed of the MFα1-prepropeptide and the presumptive mature part of the *P. cyaneo-fulvum* GLO.

The genomic GLO was cloned by constructing an additional expression vector containing an additional 249 non-coding nucleotides after the stop-codon with the natural transcription terminator of GLO using oGLOCD53 5'-GAAGAAGCTTACCG-GTGGTTCAATTGGCAGTTTTTGGT-3' as sense oligonucleotide, oGLOCD3C 5'-GGTA-AGCTTCTCGAGCCTAGGAACTGGTGGTAGATGAATTGGTCA-3' as antisense oligonucleotide and *P. cyaneo-fulvum* genomic DNA as template in PCR. Otherwise the vector was identical to pGTY(GLO). This vector was called pGTYG(GLO).

S. cerevisiae strain GRF18 was transformed using the lithium acetate method (Sherman *et al.* 1986). One of the transformed clones and the recipient strain used as a control were grown until early stationary phase in a rotary shaker at 180 rpm and 30ºC in 0.3 l SC-his medium containing 0.67% Yeast Nitrogen Base w/o amino acids (Difco, USA), 2% glucose and 100 mg/l histidine (for the control strain leucine was also added at 100 mg/l). The yeast cells from these cultures were used to inoculate two identical 15 1 fermentors each containing 10 l of a low-phosphate medium. To prepare the low-phosphate medium, an 8% solution of Bacto-peptone (Difco, USA) was treated with $CaCl_2$ (added to 0.4 M concentration) at pH 11 and 100ºC for 5 min. The peptone solution was cooled to room temperature on ice, adjusted to pH 5.5, filtered through paper and 0.4 µm pore-size membrane and used as the stock solution of phosphate-depleted peptone. Low-phosphate medium contained 2% phosphate-depleted peptone and 5% glucose. The fermentation conditions used in cultivation were following: stirring 300 rpm, aeration 5 l/min and pH 5.5 (maintained by addition of 4 M NaOH). Samples of the cultures were taken at constant intervals and cell density was followed by measuring the absorbance at 600 nm. The GLO activity was measured after removing the cells from the samples by centrifugation, concentrating the samples about 500-fold using Centriplus membranes (Amicon, USA) and removing the low molecular weight components of the fermentation medium by gel filtration using disposable Econo Pack 10 DG mini-columns (Bio Rad, USA).

4.5.3 Over-expression of the GLO gene in *P. pastoris* (I, unpublished)

Two different sets of expression vectors were planned for the expression of GLO in *P. pastoris*. pPIC3.5K(GLO) containing the complete coding region of the GLO gene under the control of the *P. pastoris AOX1* promoter and pPIC9K(GLO) containing the coding sequence of the mature GLO preceded by the MFα1-prepropeptide in an otherwise similar vector as pPIC3.5K (Invitrogen, USA). *P. pastoris* was transformed by electroporation using the method of Scorer *et al.* (1994).

Recombinant strains of *P. pastoris* expressing the GLO under control of the *AOX1*-promoter were grown in a rotary shaker at 30 °C and 200 rpm in BMGY-media (10 g/l yeast extract, 20 g/l peptone, 100 mM potassium phosphate, pH 6, 13.4 g/l Yeast Nitrogen Base (Difco, USA), 10 g/l glycerol, 4×10^{-4} g/l biotin). After reaching early stationary phase the cells were pelleted and resuspended in BMMY-media (similar to BMGY, except that glycerol was replaced with 5 ml/l methanol). Cultivation was continued for 96 hours during which time additional methanol (5 ml/l) was added to the culture and aliquots were withdrawn for testing GLO activity after every 24 hours of fermentation.

4.5.4 Co-expression of GOD and GLO genes in *P. pastoris* (I)

For production of D-EA in *P. pastoris,* two vectors were created for simultaneous expression of GLO and GOD. DNA fragment carrying the GLO gene from one of the active pPIC3.5K(GLO) vectors was transferred under control of the GAP promoter in pGAPZA-vector (Invitrogen, USA) resulting in the expression vector pGAPZA(GLO). The selection marker *HIS4* of pPICK3.5K was also transferred to pGAPZA(GLO) and the resulting vector was named pGIC(GLO).

GLO expression in *P. pastoris* under the control of the *GAP*-promoter was tested in three different media (YPD, YPD buffered with 20 g/l $CaCO_3$ and BMGY). The fermentation was done in 250 ml Erlenmeyer flasks containing 50 ml of medium at 30°C and 200 rpm. Cultivations were continued for 10 days during which time aliquots were withdrawn for testing GLO activity after every 24 hours.

The coding region of *A. niger* GOD gene was amplified by PCR using *A. niger* genomic DNA as template and the PCR product was transferred to the plasmid pGAPZB. The resulting expression vector, pGAPZB(GOD), contained the complete coding region of the GOD gene under control of the *P. pastoris GAP* promoter.

The *P. pastoris* strain GS115 was transformed with pGIC(GLO) and about 130 transformants were screened for clones expressing the highest levels of GLO activity. One such clone was further transformed with pGAPZB(GOD) and both GOD and GLO activities were assayed in a random set of about 10 transformants. One clone producing both GOD and GLO activities was selected to study the fermentation of glucose into D-EA.

4.5.5 Expression of *P. cyaneo-fulvum* GLO gene in *E. coli* (unpublished)

To test whether GLO could be expressed in *E. coli*, two expression vectors were constructed. pTAC(GLOP) was constructed by ligating the PCR product of sense oligonucleotide oGLOCD51 5'-CCAACAATTGATGCTGAGCCCTAAGCCGGCTTTCCTGC-3' and anti-sense oligonucleotide oGLOCD3 5'-CAAAGCTTCTAGAGCCTCAGACCACTCATATCACATC-3' digested with *Mfe*I and *Hind*III with the pUC19 based plasmid pTAC (pTAC is described in detail in the original publication II) digested with *Eco*RI and *Hind*III. The whole open reading frame of preGLO was cloned under the *tac* promoter. The oligonucleotide primer oGLOCD52 5'-CCAACAATTGATGTACCGGTGGTTCAACTGGCAGTTTTTGG-3' was used as the forward primer in PCR to clone an otherwise similar ORF of mature GLO under the *tac* promoter. The resulting vector pTAC(GLOM), was transformed into the *E. coli* strain XL1-Blue MRF' and induction was carried out as described by Sambrook *et al.* (1989).

4.5.6 Expression of GA 5-DH genes in *E. coli* (II, III)

The DNA-fragments containing both the small and the large open reading frames of both *G. suboxydans* and *X. campestris* GA 5-DH were amplified by PCR and ligated with pTAC. The resulting vectors were named pTAC(GAD) and pTAC(GADX) respectively.

The expression vectors were used to transform the *E. coli* strain XL1-Blue MRF', the transformants were grown in LB containing 1 mg/l of PQQ and 100 mg/l ampicillin at 30°C and 200 rpm to OD_{600} 1.5–2. The induction of expression was done by adding 25 mg/l

Table 4 Expression vectors constructed in this study

Vector (host)	Promoter	Expressed enzyme
pPIC3.5K(GLO) (*P. pastoris*)	*AOX1*	GLO
pPIC9K(GLO) (*P. pastoris*)	*AOX1*	GLO
pGIC(GLO) (*P. pastoris*)	*GAP*	GLO
pGAPZB(GOD) (*P. pastoris*)	*GAP*	GOD
pGTY(GLO) (*S. cerevisiae*)	*PHO5*	GLO
pGTYG(GLO) (*S. cerevisiae*)	*PHO5*	GLO
pTAC(GLOP) (*E. coli*)	*TAC*	GLO
pTAC(GLOM) (*E. coli*)	*TAC*	GLO
pTAC(GAD) (*E. coli*)	*TAC*	GA 5-DH
pTAC(GADX) (*E. coli*)	*TAC*	GA 5-DH
pGTK74(tktA) (*E. coli*)	*DEGQ36*	Transketolase A
pTAC(tktB) (*E. coli*)	*TAC*	Transketolase B

of isopropyl-β-thiogalactoside and continuing the incubation additionally 2 hours in same conditions, after which the cells were collected by centrifugation, washed twice and resuspended in a smallest possible volume of the resuspension buffer.

4.5.7 Construction of vectors for the expression of *E. coli* transketolases (unpublished)

The *E. coli* transketolase genes were amplified from *E. coli* AS11 genomic DNA. Transketolase A gene was amplified by using oligonucleotide primers oTktA5 5′-CCTCAATTGATGTCCTCACGTAAAGAGCTT-3′ and oTktA3 5′-TGCTGGATCCGCAAACGGACATTATCAAGG-3′. The PCR product was digested with *Mfe*I and *Bam*HI and ligated to pGTK74 (Povelainen *et al.* 2003) digested with *Eco*RI/*Bam*HI to generate pGTK74(tktA). In this plasmid the tktA is under control of the constitutive *degQ36* promoter. The transketolase B gene was amplified using oligonucleotide primers EcoTKTB52 5′-GCCGAATTCATGTCCCGAAAAGACCTTGCCA-3′ and EcoTKTB3 5′-GCCAAGCTTTGACGGTCAGCGTTTTCGCC-3′. This PCR product was digested with *Eco*RI and *Hind*III and ligated to pTAC digested with the same restriction enzymes. The plasmid constructed was named as pTAC(tktB), where the tktB gene is under *tac* promoter control. The vectors constructed in this study are summarised in Table 4.

4.6 Characterisation of the enzymes

4.6.1 Enzymatic properties of recombinant GLO (I)

The experiments described in this section were performed using purified recombinant GLO.

pH-activity profile of GLO was determined by using 100 mM concentrations of the following buffers: sodium acetate, pH 5–6.5; potassium biphthalate, pH 5–6.5; sodium phosphate, pH 6.5–7.5 in an otherwise standard GLO assay.

pH-stability profile was determined by incubating the enzyme overnight at room temperature in the following buffers (all 100 mM): glycine, pH 2.5–4.5; sodium acetate, pH 4.5–6.5; potassium phosphate, pH 6.5–8.5 and Tris-HCl, pH 8.5–9.5 and by measuring the residual activity.

Thermostability and temperature activity profiles were determined in 50 mM potassium biphthalate buffer, pH 6.2 containing 2 mM hydroxyquinoline. To study thermostability, the enzyme was incubated 30 minutes at the indicated temperature (between 25°C and 95°C), chilled in an ice-water bath and assayed immediately. The temperature activity profile was elucidated by running the standard GLO assay at defined temperature for five minutes.

The K_m for recombinant GLO was calculated using the Lineweaver-Burk plot (Dixon and Webb 1979).

Endoglycosidase H treatment was performed by incubating the GLO with endoglycosidase H (Sigma, USA) in 10 mM sodium phosphate buffer pH 6.5, 0.1 mM EDTA, 0.05% SDS and 25 mM β-mercaptoethanol at 37°C overnight.

4.6.2 Enzymatic properties of recombinant GA 5-DHs (II, III)

The experiments described in this section were performed using washed cells of the recombinant *E. coli* as crude GA 5-DH preparations. The parent *E. coli* strain transformed with pTAC and not expressing the GA 5-DH operon served as a control.

pH-activity profile of GA 5-DH was determined using 100 mM concentrations of the following buffers: glycine chloride, pH 2.5–4.5; sodium acetate, pH 4.5–6.5; PIPES-HCl, pH 6.0–7.5 and Tris-HCl, pH 7.5–9.0. The pH of the assay mixtures was adjusted after the addition of substrates. At a pH below 5.5 the potassium ferricyanate-based assay of Wood *et al.* (1962) was used.

pH-stability profile was determined by incubating the enzyme 48 hours at room temperature in the following buffers (all 100 mM and containing also 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and 6 µg/ml PQQ): glycine, pH 2.5–4.5; sodium acetate, pH 4.5–6.5; PIPES-HCl, pH 6.0–7.5; Tris-HCl, pH 7.5–9.0 and measuring the residual activity.

To study thermostability, the enzyme was incubated in an indicated temperature for 20 min, chilled on ice-water bath and assayed immediately.

The temperature-activity profile was elucidated by running the standard GA 5-DH assay at the indicated temperatures for 5 minutes.

To study the reconstitution of GA 5-DH with divalent cations, the enzyme preparation was first inactivated by adding EDTA and then extensively dialysed. Unless indicated otherwise, salts of divalent metals were added to the apo-enzyme preparations to 5 mM concentration, and the reconstitution was allowed to proceed for 30 min at room temperature followed by the standard activity assay.

The kinetic constants for GA 5-DH were calculated using Lineweaver-Burk plots (Dixon and Webb 1979). Since measurements were made with crude enzyme preparations V_{max} values could only be expressed in relative terms.

4.7 Production of D-EA and 5-KGA using recombinant enzymes

4.7.1 Production of D-EA from glucose by fermentation (I)

The direct conversion of glucose to D-EA using a *P. pastoris* strain co-expressing both GLO and GOD was studied in YPD (containing 2% and 5% glucose) and BMDY (similar to BMGY, except that glycerol is replaced with 2% glucose). The fermentation was done in 250 ml Erlenmeyer flasks containing 50 ml of medium at 30°C and 200 rpm. Cultivation was continued for seven days during which time aliquots were withdrawn and the D-EA content and the enzyme activity of both enzymes were analysed daily.

4.7.2 Conversion of glucose and sodium gluconate to 5-KGA by recombinant *E. coli* (II, III)

The *E. coli* transformants expressing GA 5-DH were grown in 100 ml of LB containing 1% glucose to OD_{600} 0.4. Cells were collected by centrifugation and used to inoculate the same volume of fresh LB medium (without glucose) containing 30 mg/l of isopropyl-β-thio-galactoside and 15 µM of PQQ and further incubated at 30°C with shaking for 2 hours. The cells were collected by centrifugation, washed with 100 mM sodium acetate buffer, pH 5.5 containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and resuspended in 5 ml of the same buffer containing 15 µM of PQQ and disrupted by sonication. Sodium gluconate or glucose was added to a final concentration of 2% and the resulting reaction mixture was incubated overnight at room temperature. An identical reaction mixture containing *E. coli* XL1-Blue MRF' cells transformed with pTAC was used as a control.

The pTAC(GADX) transformants were grown in shake flasks in LB over night. Non-induced cells from 200 ml of overnight culture, without glucose or sodium gluconate, were pelleted and resuspended to 20 ml of LB. 1 ml of this suspension was added to 9 ml of LB containing 1 mg/l PQQ, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 1% or 5% glucose or sodium gluconate, pH 4, 5 and 6. These cultures were then incubated at 30°C and 200 rpm overnight and analyzed.

4.7.3 Production of tartaric acid semialdehyde *in vitro* (III)

A suitable amount of *E. coli* pGTK74(tktA) cell extract was added into the reaction mixture (100 mM glysyl-glysine buffer, pH 7.6, 9 mM $MgCl_2$, 0.5 g/l BSA, 0.25 mM thiamine pyrophosphate, 0.1 mM sodium arsenate, 0.2 mM NADH, 2 mM glyceraldehyde 3-phosphate, 0.2 U/ml arabitol phosphate dehydrogenase), incubated overnight at 37°C and analysed by HPLC.

5 Results and discussion

5.1 Feasibility of D-EA production using recombinant GLO

5.1.1 Purification of *P. cyaneo-fulvum* GLO (I)

A slightly modified procedure of purification of GLO from the method used by Takahashi *et al.* (1976) was applied to obtain homogenous enzyme from extracts of *P. cyaneo-fulvum* mycelia. The purification procedure is summarised in Table 5. Purified GLO appeared on SDS-PAGE as a diffuse band of 66–90 kDa and this pattern changed to a single sharp band of 56 kDa after treating with endoglycosidase H. The molecular weight of native GLO was estimated by gel filtration to be 160 kDa, which is similar to the earlier estimate of 150 kDa (Takahashi *et al.* 1976). Comparison of the molecular weight of native GLO with the SDS-PAGE data clearly suggests that GLO is a homodimer. This is an unexpected property for a lactone oxidase since all other known enzymes of this class are monomers (Huh *et al.* 1994, Östergaard *et al.* 1997, Imai *et al.* 1998). The purified enzyme had a specific activity towards D-glucono-1,5-lactone of 6.1 U/mg, similar to the previously reported value of 5.6 U/mg (Takahashi *et al.* 1976).

5.1.2 Cloning of *P. cyaneo-fulvum* GLO gene and analysis of the gene sequence (I)

A *P. cyaneo-fulvum* cDNA-library was constructed and screened by hybridisation. The probe used for hybridisation was obtained by PCR, using degenerate oligonucleotides planned by reverse translation of the peptide sequences obtained from protein sequencing. The N-terminal amino acid sequence of GLO was obtained by automated Edman-degradation of the purified enzyme after a preparative SDS-PAGE and subsequent in gel digestion with Lys-C. Degenerate oligonucleotides (all together three different from N-terminus

Table 5 Summary of the GLO purification

Purification step	Total activity, U	Total protein, mg	Yield, %	Specific activity, U/mg
Cell extract	322	4034	100	0.08
1. DEAE-sepharose FF[a]	289	1105	90	0.26
$(NH_4)_2SO_4$	118	196	50	0.6
2. DEAE- sepharose FF[b]	72	60	22	1.2
Sephacryl S-300	31	8	10	4.0
Hydroxyapatite	20	3	6	6.1

[a]Conductivity of sample 2.5 mS/cm.
[b]Conductivity of sample 1.2 mS/cm.

and five from internal peptides were planned) designed by reverse translation of the four amino acid sequences obtained from the protein sequencing were used to prime PCR with chromosomal DNA of *P. cyaneo-fulvum* as template. The largest PCR product obtained was approximately 1.2 kb in size and encoded, according to a GenBank BLAST-search, a protein homologous to FAD-dependent dehydrogenases. The corresponding cloned cDNA of 1.8 kb (EBI accession number AY576053) was well in accordance with the size of GLO mRNA estimated by Northern blot analysis.

The cloned cDNA fragment contained a 1443 bp open reading frame encoding a polypeptide of 480 amino acid residues. The calculated molecular weight of this polypeptide (54.3 kDa) matches with the experimental estimate of the molecular weight of deglycosylated GLO (56 kDa). The open reading frame is preceded by a 5'-non-coding region of 79 nucleotides and followed by a 3'-non-coding region of 267 nucleotides.

In silico analysis of the sequence features of the cloned cDNA indicated that it contains a full-length coding sequence of the GLO gene. The deduced amino acid sequence contained the sequenced internal peptides and the sequenced NH_2-terminus of mature GLO. The open reading frame was followed by a non-coding region ending in a polyA-tail and included a putative fungal polyadenylation signal TATATA (Helden *et al.* 2000) at the expected position of 43 nucleotides before the polyA-tail. Comparison of the predicted amino acid sequence of GLO to the sequences of other aldonic acid lactone oxidases suggested that GLO is only a distant relative of L-gulono- (22% identical), L-galactono- (26% identical) and D-arabinonolactone (26% identical) oxidases (Fig. 7). Notably, there is no indication of a fungal lactone oxidase family. The sequences of the two known fungal lactone oxidases (D-arabinonolactone oxidases from *C. albicans* and *S. cerevisiae*) are no more homologous to the GLO sequence than to the sequence of the rat L-gulonolactone oxidase.

The phylogenetic tree presented in Fig. 7 also includes the sequences of one presumptive fungal (*Aspergillus nidulans*) and three presumptive prokaryotic (from *Oceanobacillus iheyensis*, *Bacillus cereus* and *Streptomyces avermitilis*) lactone oxidases. These sequences were identified by a BLAST-search of the GenBank sequences as the closest homologues of GLO. There is no information in the literature about the production of ascorbic acid analogues by prokaryotes. It would be interesting to investigate whether the oxidases from *O. iheyensis*, *S. avermitilis* or *B. cereus* are indeed aldonic acid lactone-specific and do produce ascorbic acid analogues like the sequence homology suggests.

Earlier studies indicated that FAD is covalently attached to GLO (Harada *et al.* 1979). According to a search of the NCBI conserved domain database (Marchler-Bauer *et al.* 2003), the deduced amino acid sequence of GLO had a conserved putative binding site for covalently bound FAD of oxygen-dependent oxidoreductases (PROSITE pattern: PS00862). This domain corresponded to amino acid residues 34–196 in GLO and can also be found from D-arabinono-1,4-lactone oxidase and L-gulonolactone oxidase. In the domains of D-arabinono-1,4-lactone oxidase and L-gulonolactone oxidase, histidine residue 56 and 54 respectively were thought to be the site of co-factor attachment (Huh *et al.* 1994 and Imai *et al.* 1998). This histidine (His-67) is also present in the putative FAD-binding domain of GLO.

Based on the experimentally observed amount of N-linked oligosaccharides in GLO (20–30%), the predicted amino acid sequence of GLO was expected to contain multiple consensus sequences for N-glycosylation sites (Asn-X-Ser/Thr), eight such sites were found within the GLO deduced amino acid sequence.

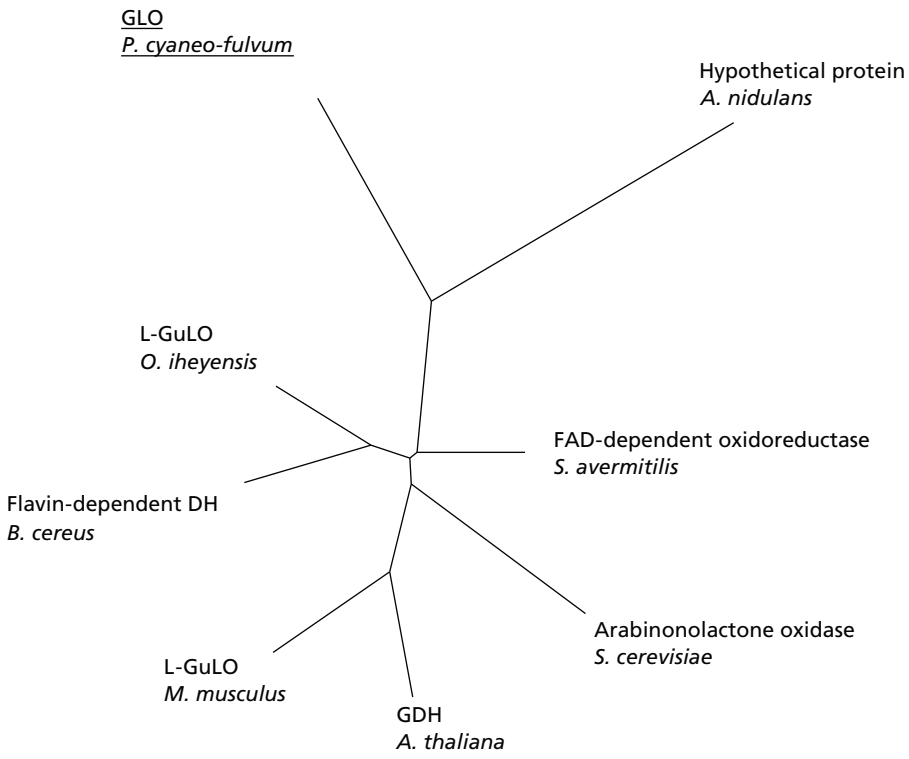

Figure 7 Phylogenetic relationships between amino acid sequences of several aldonic acid lactone oxidases and putative FAD-dependent oxidases. L-GuLO – L-gulono-1,4-lactone oxidase, GDH – L-galactono-1,4-lactone dehydrogenase, GLO –gluconolactone oxidase. NCBI accession numbers for the sequences used in phylogenetic comparison: AAR15891 *M. muculus* L-GuLO, NP_830486 *B. cereus* Flavin-dependent dehydrogenase, NP_823585 *S. avermitilis* putative FAD-dependent oxioreductases, NP_692632 *O. iheyensis* L-gulonolactone oxidase, EAA64236 *A. nidulans* hypothetical protein, BAA23804 *S. cerevisiae* L-galactono-gamma-lactone oxidase, BAC42562 *A. thaliana* putative L-galactone-1,4-lactone dehydrogenase.

5.1.3 Cellular localisation of *P. cyaneo-fulvum* GLO (I)

The amino acid analysis of the deduced protein sequence revealed that the experimentally determined N-terminal amino acid sequence of the mature enzyme was located within the coding sequence of GLO starting at position 21. The analysis of the deduced amino acid sequence of pre-GLO using SignalP-HMM model (http://www.cbs.dtu.dk/services/SignalP, Nielsen *et al.* 1999) supported this observation predicting the presence of a targeting signal with a probability of 0.999. The most probable location of the cleavage site is suggested between residues 20 and 21. No additional sorting or targeting signals were found using *in silico* analysis.

The Kyte–Doolittle hydropathy plot (Kyte and Doolittle 1982) using a standard transmembrane protein window size of 17–21 amino acids did not reveal any transmembrane

regions in the mature GLO protein. For the known transmembrane proteins D-arabinonolactone oxidases and L-gulonolactone oxidases the same analysis clearly indicated the presence of transmembrane regions, as was the case also with other transmembrane domain predicting models such as TMpred. TMpred is is based on TMbase, which is a database of naturally occurring transmembrane proteins (http://www.ch.embnet.org/software/TMPRED_form.html, Hofmann and Stoffel 1993). The results of the *in silico* sequence analysis suggested that GLO is a secreted enzyme. This conclusion was also supported by recombinant expression of GLO in *P. pastoris* where the native signal sequence was sufficient for complete secretion of GLO. These findings were somewhat surprising, since both in this study and by Takahashi *et al.* (1976), GLO was isolated as a soluble enzyme from cell extracts of *P. cyaneo-fulvum*. Moreover, all other known aldonic acid lactone oxidases show completely different targeting patterns. Animal L-gulonolactone oxidases are integral membrane proteins of the endoplasmic reticulum (Eliceiri *et al.* 1969, Kiuchi *et al.* 1982 and Puskas *et al.* 1998), plant L-galactonolactone oxidases are localised in the inner mitochondrial membrane (Mapson and Breslow 1958, Oba *et al.* 1995, Siendones *et al.* 1999) and yeast D-arabinonolactone oxidases are membrane-bound mitochondrial enzymes as well (Huh *et al.* 1994). No evidence could be obtained of membrane association for GLO. In the purification of the other sugar lactone oxidases and dehydrogenases detergents have been used to solubilise the enzyme from the membranes, but both in this study and by Takahashi *et al.* (1976) GLO was purified without detergents. Extraction of the membranes with detergents did not influence to the yield of GLO activity.

Secreted fungal enzymes tend to be highly glycosylated and this was also the case with GLO containing 20–30% of carbohydrate as mentioned earlier. However, only low (0.01–0.03 U/l) GLO activity was found in the concentrated culture broth of *P. cyaneo-fulvum*. Approximately 10% of the total GLO activity was secreted to the culture medium under the shake flask fermentation conditions. This is not unique property of a secreted fungal flavoenzyme belonging to the fungal glucose oxidation system. GOD of *Aspergillus niger* was for a long time believed to be an intracellular (Pazur 1966) or peroxisomal (van Dijken and Veenhuis 1980) enzyme, while a similar enzyme from *Penicillium* was found to be secreted (Kusai *et al.* 1960). However, later studies concluded that a variable (about 20–70%) fraction of *A. niger* GOD is also secreted to the culture medium depending on e.g. the composition of the culture medium used. The remaining fraction of the enzyme is distributed between cell wall and intracellular locations (Mischak *et al.* 1985 and Clarke *et al.* 2006). Enzyme kinetics and substrate chemistry also suggest that the whole D-EA pathway in *Penicillium* is located extracellularly. Unlike gulono-1,4-lactone and galactono-1,4-lactone, D-glucono-1,5-lactone is not stable at neutral or slightly alkaline intracellular pH (Mitchell and Duke 1970). GOD forms D-glucono-1,5-lactone outside of the fungal cell and it would be difficult to imagine a mechanism by which it could be transported into the cells without being irreversibly hydrolysed spontaneously or by the action of D-gluconolactonases (EC 3.1.1.17). Taken together, the data indicates that the most probable location of the bulk of GLO in *P. cyaneo-fulvum* is within the cell wall similarly to *A. niger* GOD and any prospective recombinant host for fermentative production of D-EA should produce GLO in secreted form.

The physiological role of GLO in *Penicillium* still remains unclear, but the location of the enzyme suggests it to differ from that of other sugar lactone oxidases. Among the filamentous fungi overproduction of primary metabolites is common. For example *Aspergillus* and *Penicillium* can rapidly convert glucose to glucono-1,5-lactone extracellularly producing hydrogen peroxide in this reaction. However, the fungus cannot utilise energy formed in

the reaction and therefore it must serve for other purposes. One possible function for this reaction is that it contributes to the competitiveness of the fungi. The reaction removes rapidly excess glucose in the nearby surrounding simultaneously acidifying the environment and producing toxic hydrogen peroxide to which itself is highly resistant. The role for GLO could be to increase the production of hydrogen peroxide even further without decreasing the amount of acid formed. Perhaps the GLO in *Penicillium* has a protective function and is involved in the glucose oxidation defense system of *Penicillium*.

5.1.4 Determination of functionality of GLO gene in *E. coli* and *S. cerevisiae* (unpublished)

To confirm the activity of the cloned *P. cyaneo-fulvum* GLO gene, it was first expressed in host organisms with well-established expression systems, *E. coli* and *S. cerevisiae*.

No GLO activity was obtained from the *E. coli* clones with the GLO-expression vector pTAC(GLOP) expressing preGLO or pTAC(GLOM) expressing mature GLO. The expression of mature and preGLO under the *tac* promoter failed and neither the cell lysate nor the concentrated cultivation medium was found to contain an active form of GLO. This was not unexpected remembering, that GLO is a secreted eukaryotic protein. Amino acid sequence of GLO contains 11 cysteine residues and therefore correct folding of GLO is possibly dependent on the more oxidative environment in ER-lumen of eukaryotes and cannot take place in reducing conditions of *E. coli* cytosol. Internal disulfide bridges may possibly be essential for formation of an active GLO. Neither of the expression vectors constructed contained a specific *E. coli* secretion signal. The secretion signal of GLO used to construct pTAC(GLOP) did not necessarily work as functional secretion signal in a prokaryotic host. Another possibility is that the flavine cofactor necessary for the activity of GLO is not correctly incorporated to GLO in *E. coli*. When *S. cerevisiae* D-arabinono-1,4-lactone oxidase was expressed in *E. coli* (Lee *et al.* 1999) FAD was found to be noncovalently attached to the enzyme while in yeast the attachment of FAD to the enzyme is covalent. This could also be the case with GLO.

No further *E. coli* expression vectors were constructed to examine these hypotheses due to the decision to move on to use eukaryotic expression host instead bacterial host. The secretion potential of *E. coli* was not considered to be strong enough for a secreted eukaryotic enzyme with posttranslational modifications.

Since the deduced amino acid sequence of GLO displayed many features of a secreted protein and was of eukaryotic origin, a yeast secretory expression system seemed to be more suitable for testing the functionality of the cloned GLO cDNA. The confirmation of the functionality of the cloned GLO gene was obtained in *S. cerevisiae* when the gene corresponding the mature GLO was cloned into the pGTY-vector in fusion with MFα1 secretion signal under control of the *PHO5* promoter. The transformed yeast was grown in phosphate depleted medium. The active GLO was found from the culture medium with an activity of 58 mU/l (Table 6) and the highest yields were obtained typically after 70 hours of growth in the fermentor (Fig 8). No GLO activity was found from the samples of cell lysate at any time point. The genomic DNA encoding the GLO gene was cloned in the same expression vector and found to be similarly active in yeast. The PCR-products of both genomic DNA and cDNA were analysed by restriction mapping and no differences were seen in the restriction profiles of these two different PCR-fragments, indicating absence of introns.

The expression level obtained was only approximately one tenth of the GLO expression

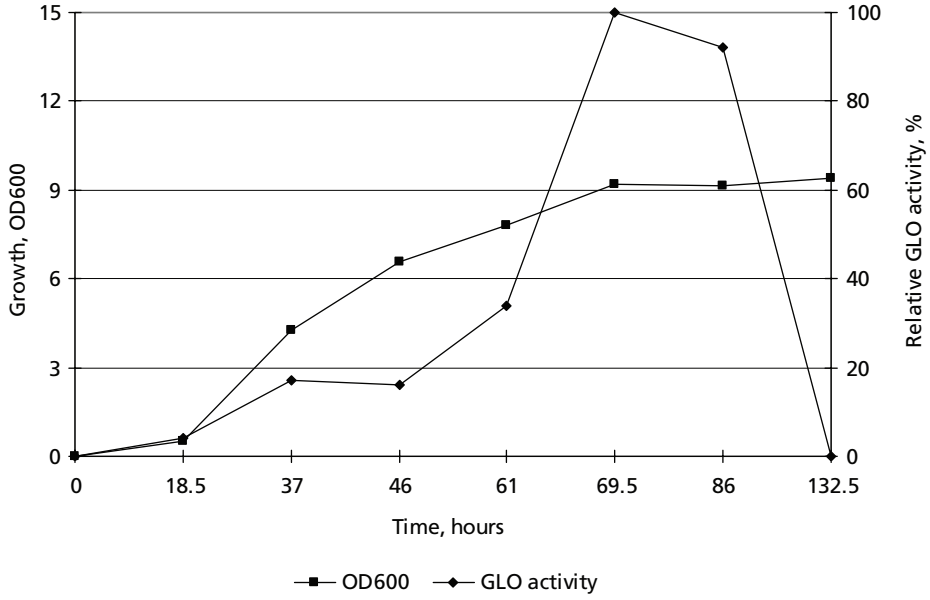

Figure 8 Induction of expression of GLO in recombinant *S. cerevisiae.* No GLO activity was observed in the control strain (GRF-18)

levels obtained using wild-type *Penicillium*. However, this result indicated that the screened cDNA-library clone of the GLO gene is indeed functional, but expression of the gene at a higher level would require a more efficient expression system. Compared to some other yeast strains, *S. cerevisiae* is considered relatively inefficient host for secretion of heterologous proteins. Higher expression levels of recombinant GLO may be expected when the GLO gene is introduced into other yeast with higher secretory potential, such as *Pichia pastoris*.

5.1.5 Overexpression of GLO and GOD in *P. pastoris* (I)

For the efficient production of secreted recombinant enzymes of fungal origin, *Pichia pastoris* is a commonly used expression host (for review, see Cereghino and Cregg 2000). The results of the studies of expression of both GLO and *A. niger* GOD genes under different promoters and secretion signals in *P. pastoris* are summarised in the Table 6.

The expression level of GLO under the *AOX1*-promoter in a shake flask cultivation compared to expression level of GLO in fermentor cultivation of the *P. cyaneo-fulvum* was 72-fold higher, about 25 mg/l. Both extra- and intracellular GLO activities were determined and the activity was found only to exist in the culture medium. No GLO activity was found from the fraction of lysed cells of recombinant *P. pastoris* containing both intracellular protein and protein from the cell wall. The efficient yeast secretion signal MFα1 did not increase the expression levels of GLO compared to the native secretion signal of GLO. Therefore the GLO secretion signal was considered to be fully functional in *P. pastoris.*

The *AOX1* promoter is repressed by glucose and cannot be used in hosts designed for

Table 6 GLO and GOD activities obtained with recombinant and wild type strains

Strain	Construct	Promoter	Signal	Enzyme	Activity, U/l
P. cyaneo-fulvum	wt	wt	wt	GLO	5.0
P. pastoris	pPICK3.5K(GLO)	*AOX1*	wt	GLO	360
P. pastoris	pGIC(GLO)	*GAP*	wt	GLO	65
S. cerevisiae	pGTY(GLO)	*PHO5*	MFα1	GLO	0.06
P. pastoris	pGAPZB(GOD)	*GAP*	wt	GOD	12200

efficient conversion of glucose into D-EA by fermentation. The glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter has been reported to be induced well by glucose containing media and to be comparable in strength to the *AOX1*-promoter in *P. pastoris* (Waterham *et al.* 1997 and Sears *et al.* 1998). However, in the case of the expression of GLO, the obtained expression level under the *GAP*-promoter was 5–6 times lower than under the *AOX1*-promoter (Table 6). The expression level obtained with the *GAP* promoter using glycerol as carbon source was approximately ten times higher compared to the expression level obtained using glucose as carbon source in an otherwise similar medium. The effect of glucose or glycerol to the induction of *GAP* promoter was not tested.

The GOD gene was expressed to almost ten-fold higher levels (30–40 mg/l) than the GLO (4–5 mg/l) when the two genes were under the same promoter (*GAP*) and cultivated similarly. The imbalance in the activities of these two enzymes is even larger due to the higher specific activity of GOD (app. 360 U/mg) than GLO (14.4 U/mg for the yeast-derived GLO, see below chapter Properties of recombinant GLO). Therefore GOD activity obtained in recombinant *P. pastoris* was over 185-fold higher compared to the activity of GLO. The reason for this can only be speculated here. One obvious reason for this could be the not optimal codon usage of GLO for heterologous expression in *P. pastoris*. Comparing the codon usage of *P. pastoris* (Nakamura *et al.* 2000) to the codons of GLO can be noted that several codons present in GLO gene are rarely used by *P. pastoris*. For example the codon GCG for alanine in GLO (four such alanines out of 34 are found in GLO coding sequence) is only very rarely used by *P. pastoris.* Less than 6% of all alanine-encoding codons in *P. pastoris* are encoded by this codon. Nearly half of the alanines in *P. pastoris* are encoded by codon GCT. Similarly analysing every codon of GLO gene, and changing them to more commonly used ones by *P. pastoris*, could improve the expressability of GLO in *P. pastoris*. Another reasons for the low expression level of GLO could be that the folding of GLO is more complicated compared to the folding of GOD or the mRNA of GLO is degraded more rapidly than the mRNA of GOD. The half-life of different mRNAs in yeast is known to vary widely, ranging from approximately 3 minutes to more than 90 minutes (Wang *et al.* 2002, for rewiev see Hieronymus and Silver 2004). However, the reason for this not yet completely understood. One possible approach to overcome the low-level expression problems of GLO could be the use of random mutagenesis followed by high throughput screening for the search of mutants with more suitable character for expression.

5.1.6 Properties of recombinant GLO (I)

Recombinant GLO produced by *P. pastoris* pPIC3.5K(GLO) was purified to homogeneity by a combination of DEAE-Sepharose and hydroxyapatite chromatography and several properties of the recombinant enzyme were studied.

Recombinant heterologous (especially fungal) enzymes expressed in yeast are commonly known to be over-glycosylated. It was also expected to be the case for GLO, which is extensively glycosylated in its native host and has eight consensus sites for attachment of N-linked oligosaccharides. However, the glycosylation level of recombinant GLO produced by *P. pastoris* was similar to that of native GLO. The molecular weight of the recombinant GLO on SDS-PAGE was found to be 66–80 kDa and after endoglycosidase H treatment approximately 54 kDa, which corresponded to the molecular weight of the mature GLO (52.2 kDa) predicted on the basis of deduced amino acid sequence.

pH-stability profile of recombinant GLO (Fig. 9) was found to be essentially similar to the native GLO described by Takahashi *et al.* (1976). The pH-optimum of both recombinant and native GLO was found to be 6.25, which differs slightly from the earlier reported optimum pH of 5.6–6.

The temperature stability of recombinant GLO was measured and found to be rather typical for an enzyme derived from a mesophilic microorganism, having a temperature optimum at 42°C. Above 45°C the activity was quickly lost. This is in line with the result of Takahashi *et al.* (1976) that the activity of native GLO was completely lost after 30 min incubation at 55°C.

The specific activity of recombinant GLO (approx. 14.4 U/mg protein) was found to be over two times higher than that of the native enzyme. K_m values for D-gluconolactone were very similar with both forms of the enzyme (2.2 mM recombinant, 1.7 mM native by Takahashi *et al.* (1976)). This could be due to the inactivation of native GLO by hydrogen peroxide that is produced during fermentation of *P. cyaneo-fulvum* by GOD and GLO itself. Hydrogen peroxide is not similarly produced to the culture medium by *P. pastoris*, because it does not have GOD and therefore also substrate for GLO is absent.

The FAD in *S. cerevisiae* D-arabinono-1,4-lactone oxidase is covalently attached while the same enzyme produced in *E. coli* contains non-covalently bound FAD (Lee *et al.* 1999). The attachment mode of FAD in both native and recombinant GLO was compared using a fluorescence-based method of Nishikimi *et al.* (1977), where the SDS-PAGE is soaked in acetic acid after running a sample and the FAD-fluorescence is detected under UV-light. No difference was found between the two forms of the enzyme suggesting that both contained only covalently bound FAD. This conclusion was confirmed by the fact that no activation of GLO was observed when additional FAD (0.1–1 mM) was added to the assay mixture for GLO.

5.1.7 Production of D-EA (I)

Enzymatic conversion of glucose to D-EA was demonstrated using purified recombinant GLO in a reaction mixture containing GOD and catalase. In reactions, which were performed without pH control, 33% of glucose and 70% of D-gluconolactone were converted to D-EA. One of the most likely limiting factors for the reaction yield is the decrease in pH below 4 during the reaction caused by the hydrolysis of D-gluconolactone to gluconic acid and by the accumulation of D-EA (lactonase activity was measured from commercial

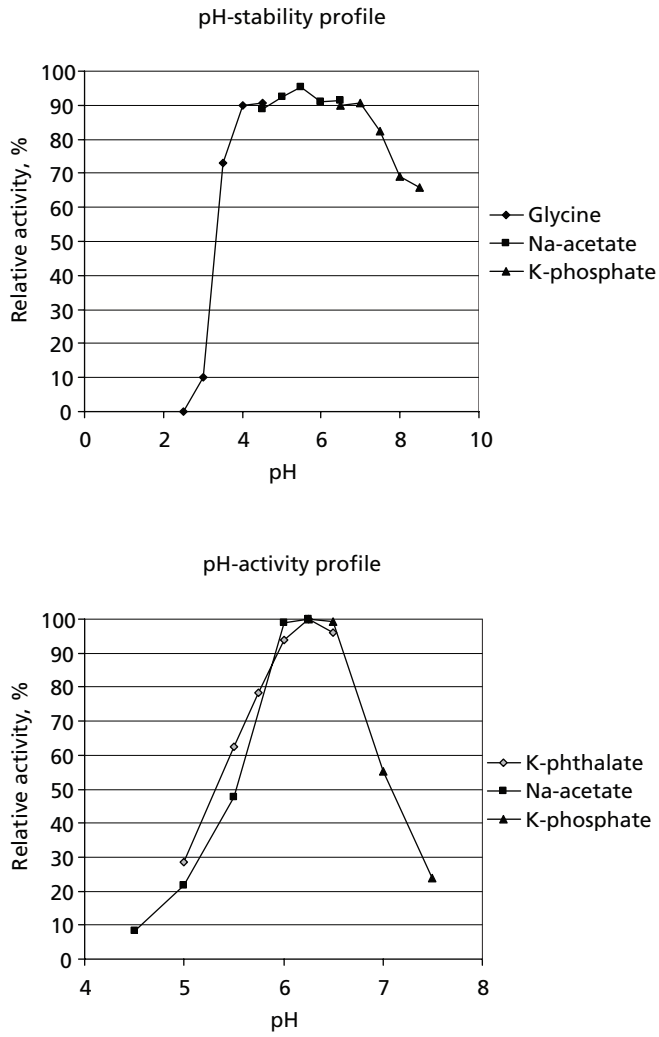

Figure 9 pH-stability and -activity profiles of recombinant GLO. The stability profile was obtained by incubating the sample over night at room temperature. The use of different buffers in 100 mM concentration is marked in separate panels on the graphs.

preparations of glucose oxidase and catalase). At such pH GLO is nearly inactive (Fig. 9). With suitable pH-controlling device the effect of pH on reaction would be straightforward to explore and the optimisation of the reaction pH together with substrate feeding should increase D-EA yields in this reaction even further.

One option for D-EA production is to immobilise GLO together with GOD and catalase in an enzymatic process to catalyse the conversion of glucose to D-EA. We found that GLO retains nearly 100% of its enzymatic activity when coupled to N-hydroxysuccinimide-activated sepharose. However, inactivation of immobilised GLO turned out to be consider-

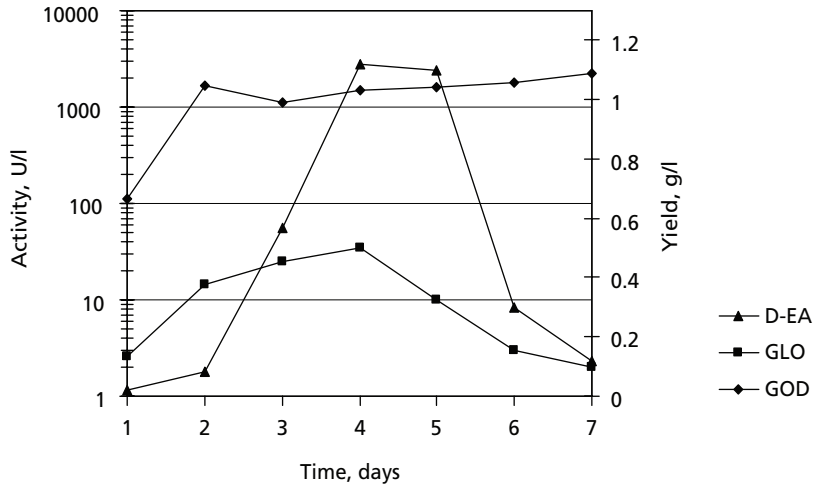

Figure 10 Production of D-EA in YPD medium containing 5% glucose by recombinant *P. pastoris* expressing both GLO and GOD. The cultivation was performed in shake flasks at 30°C and 200 rpm.

able during the conversion reaction. This is most probably due to the H_2O_2 formed during the reaction. Hydrogen peroxide has previously been shown to cause the inactivation of immobilised GOD, and the extent of this inactivation was decreased by removal of H_2O_2 from the microenvironment of GOD by co-immobilised catalase (Greenfield *et al.* 1975 and Prenosil 1979). More extensive studies of the factors affecting the enzymatic reaction are needed to increase yields and productivity. These remain to be done in the future since the effort in this study was mostly concentrated on novel fermentation based technology.

Gluconolactonase, which is produced at high levels by filamentous fungi, is also found in yeast (Brodie and Lipman 1955), but the enzyme is located only in the yeast cytosol and is present at much lower levels than in *A. niger*. No gluconolactonase activity was detected in the culture medium of *P. pastoris* and therefore *P. pastoris* was chosen as a model host for GLO production and to study the feasibility of direct fermentation of glucose into D-EA.

Production of D-EA from glucose by direct fermentation using *P. pastoris* strains co-expressing both GLO and GOD genes was carried out in shake flasks using several different glucose containing media. The highest conversion yield obtained was 12% (yield 5.9 g/l) and productivity of 0.04 $gl^{-1}h^{-1}$ after 144 hours cultivation. The most likely reason for the inefficient productivity was the imbalance in the activity levels of GLO and GOD (Fig. 10) and as a result, accumulation of D-gluconolactone to the culture medium. Because D-gluconolactone is spontaneously hydrolysed to gluconate and this reaction is irreversible in these reaction conditions, most of the glucose is converted to gluconate instead of D-EA. The difference in the activity levels of GLO and GOD in a typical conversion experiment was nearly 40-fold in favour of GOD. The conversion efficiency could probably be increased by co-expression of catalase with GLO and GOD, since both GLO and GOD are inactivated by H_2O_2 formed in conversion.

5.2 Feasibility of TA production using recombinant GA 5-DH

5.2.1 Purification of *G. suboxydans* GA 5-DH (II)

GA 5-DH from *G. suboxydans* IFO 12528 was extracted from the membrane fraction of cell lysate with 10% *n*-octyl-β-D-glucoside and purified by a combination of cation-exchange chromatography on Mono-S and preparative PAGE under non-denaturing conditions (Table 7). Interestingly, the use of *n*-octyl-β-D-maltoside instead of *n*-octyl-β-D-glucoside as a detergent during the preparative PAGE purification step resulted in a dramatically improved separation of GA 5-DH and alcohol dehydrogenase, the major contaminant during GA 5-DH purification (Fig. 11).

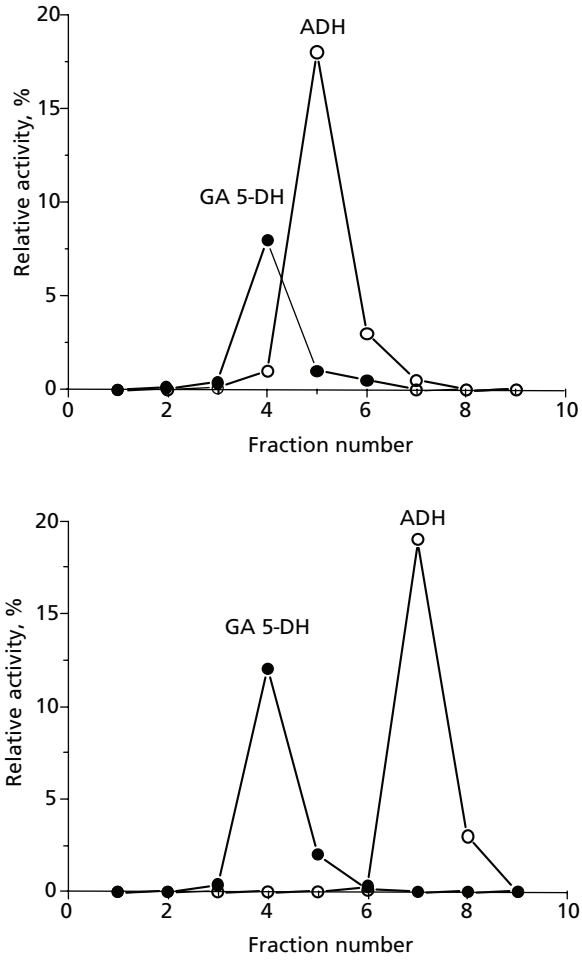

Figure 11 Purification of GA 5-DH by preparative electrophoresis under non-denaturing conditions. The separation of GA 5-DH and alcohol hydrogenase (ADH) was significantly improved with the change of detergent. Upper panel: separation in the presence of *n*-octyl β-D-glucoside. Lower panel: separation in the presence of *n*-octyl β-D-maltoside.

Table 7 Summary of GA-5 DH purification

Purification step	Total activity, U	Total protein, mg	Yield, %	Specific activity, U/mg
Membrane fraction	1005	1280	100	1.2
n-octyl β-D-glucoside	805	218	80	3.6
Mono-S chromatography	201	20	20	10
Non-denaturing PAGE	60	0.3	6	200

5.2.2 Cloning of GA 5-DH genes of *G. suboxydans* and *X. campestris* (II, III)

For *G. suboxydans* GA 5-DH, the N-terminus could not be sequenced by Edman degradation. This could be due to a blockage of the N-terminus of the enzyme. The partial amino acid sequences were obtained from the purified protein preparations after a preparative SDS-PAGE and in gel digestion with trypsin. Five of the obtained peptides were subjected to mass mapping by MALDI-TOF mass spectrometry and *de novo* sequencing by liquid chromatography/electrospray tandem mass spectrometry (LC / ESI-MS/MS).

A 0.8 kb PCR product was obtained by using oligonucleotides planned from the sequenced peptides and by using *G. suboxydans* genomic DNA as template. The PCR product displayed clear homology to the PQQ-dependent glucose dehydrogenase genes. It was used as a hybridisation probe to isolate a clone carrying a 5 kb hybridising DNA fragment from a *G. suboxydans* genomic library. The isolated fragment was sequenced (GenBank accession number AJ577472) and found to contain two open reading frames, 126 and 743 codons long (Fig. 12). There is no non-coding DNA between the two presumptive coding areas since the stop codon of the smaller ORF (TGA) partially overlaps the initiator codon of the larger ORF (ATG). The smaller of the two open reading frames encodes a polypep-

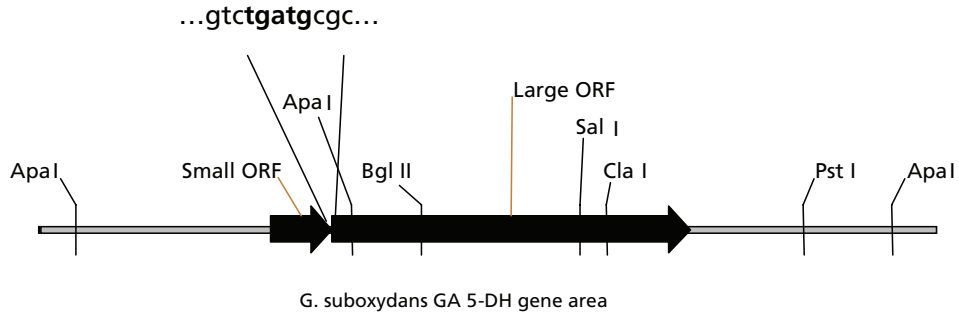

Figure 12 Restriction and functional map of the *G. suboxydans* chromosomal area containing the GA 5-DH operon. The overlapping sequence between the two adjacent open reading frames is shown. The stop-codon (tga) of the small ORF and the start-codon (atg) of the large ORF are marked in bold.

tide of 13.7 kDa, and the large open reading frame encodes a polypeptide of 79.6 kDa. A BLAST search against protein sequences deposited in the GenBank revealed that sorbitol dehydrogenase from *G. suboxydans* strain IFO3255 (GenBank accession number BAC02909) is the closest homologue of GA 5-DH. Sorbitol dehydrogenase has a similar subunit structure and both large and small subunits show comparable levels of homology (around 90%) to GA 5-DH (Miyazaki *et al.* 2002). Lately it was shown by sequencing the *Gluconobacter* genome that these two separately isolated enzymes are actually encoded by only one gene in *G. oxydans* strain 621H (Prust *et al.* 2005). The enzyme sequence obtained by genomic sequencing is 100% identical on both amino acid and nucleotide levels to the GA 5-DH of *G. oxydans* strain IFO12528 used for the cloning of GA 5-DH in this study. No other highly homologous counterpart to sorbitol dehydrogenase was found from this strain. The difference in the homology between the sequences of sorbitol dehydrogenase from *G. oxydans* strain IFO3255 and GA 5-DH from strains IFO12528 and 621H is probably due to divergence between the different *Gluconobacter* strains and actually only one gene encoding the GA 5-DH/sorbitol dehydrogenase exists in *Gluconobacter* genome. Genomic sequence of the whole genome of *G. oxydans* 621H did not reveal any other highly homologous sequences to this enzyme.

Miyazaki *et al.* (2002) proposed, that the smaller subunit of sorbitol dehydrogenase was preceded by a putative promoter region of approximately 120 bp. Genes of both subunits were believed to be under this promoter and transcribed polycistronically. Similarly the Sequence Alignment Kernel Calculator (http://mendel.cs.rhul.ac.uk/mendel.php, Gordon *et al.* 2003) predicted location of a promoter region for GA 5-DH gene between the nucleotides -104 and -22 covering a region of 82 nucleotides upstream of the initiation codon. It is therefore possible that the small and large subunits of the GA 5-DH gene are located in an operon and the transcription of them occurs also polycistronically creating only one transcript that is spliced differently either for translation of the small or the large subunit of GA 5-DH.

All other homologues of GA 5-DH, except sorbitol dehydrogenase, are either glucose dehydrogenases from various sources or presumptive proteins identified in genomic projects and annotated as a putative glucose dehydrogenases. Particularly interesting are several dehydrogenases from *Xanthomonas campestris* and *Xanthomonas citri.* These enzymes appear to have approximately as close an evolutionary relationship with the GA 5-DH and sorbitol dehydrogenase as with glucose dehydrogenases. Unlike GA 5-DH and sorbitol dehydrogenase, these presumptive quinoproteins are encoded by a single polypeptide chain that is homologous to both large and small subunits of GA 5-DH. The one-subunit structure could make the expression of these enzymes more straightforward, which is important in large-scale production of enzymes.

The closest uncharacterised homologue (48% sequence identity) of GA 5-DH is found in the genomic DNA sequence of *X. campestris* ATCC 31339, a putative glucose dehydrogenase (GenBank accession NP_636946, Fig 13). This *Xanthomonas* enzyme consists of 823 amino acids with a calculated molecular weight of 88.4 kDa. In terms of sequence homology, NP_636946 is slightly closer to the gluconate dehydrogenases from *Gluconobacter* than the known glucose dehydrogenases but its apparent single-chain architecture is typical for a glucose dehydrogenase. Furthermore, *Xanthomonas* is not known to convert glucose to 5-KGA. Alignment of both *G. suboxydans* GA 5-DH and *X. campestris* NP_636946 with that of the amino acid sequence of methanol dehydrogenase from *Methylobacterium extorquens* and glucose dehydrogenase of *E. coli* suggests that D_{402} and K_{430} in *Gluconobacter* GA 5-DH and D_{480} and K_{515} in the *Xanthomonas* enzyme correspond the catalytically

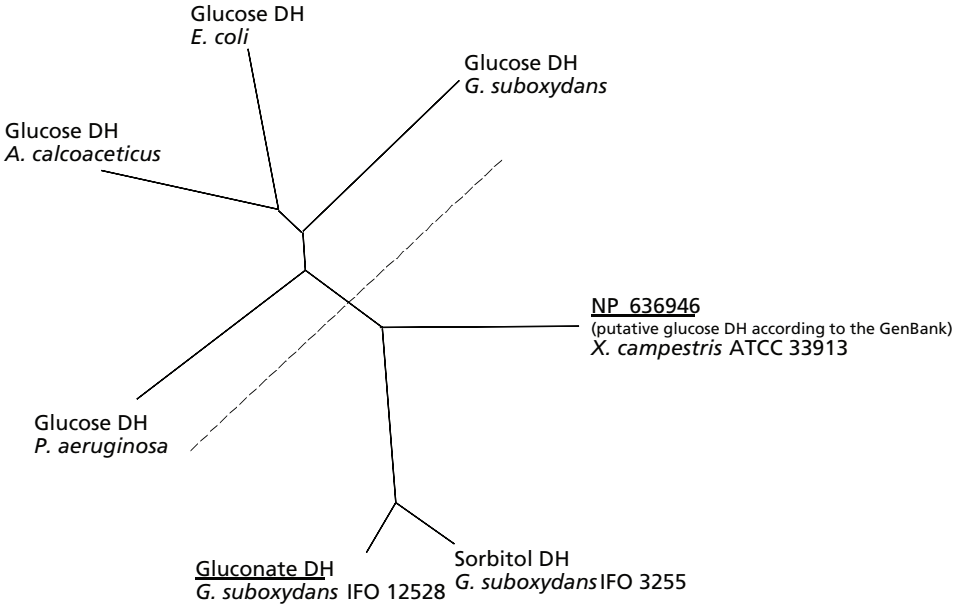

Figure 13 Graphic representation of the homology between the sequence of *G. suboxydans* GA 5-DH and sequences of several other PQQ-dependent enzymes. Note that the deduced amino acid sequence of dehydrogenase from *X. campestris* clusters with gluconate/sorbitol dehydrogenases from *G. suboxydans* rather than with glucose dehydrogenase from various species including *G. suboxydans* (clusters are separated with a dashed line). NCBI accession numbers for the sequences used in phylogenetic comparison: P05465 *A. calcoaceticus* glucose DH, AAC73235 *E. coli* glucose DH, CAA44594 *G. suboxydans* glucose DH, BAC02909 *G. suboxydans* sorbitol DH, NP_250980 *P. aeruginosa* glucose DH.

active aspartic acid and lysine residues (Anthony 1996 and Elias *et al.* 2000). The *E. coli* glucose dehydrogenase residues (D_{466} and K_{493}) have been proposed to function in the initiation of glucose oxidation and in electron transfer from the reduced form of PQQ. These amino acids are conserved also in *G. suboxydans* sorbitol dehydrogenase (D_{398} and K_{426}) (Miyazaki *et al.* 2002). The same alignment demonstrates that many of the amino acids implicated in binding of Ca^{2+} and PQQ in methanol dehydrogenase (E_{177}, S_{174}, R_{109}, R_{331}, N_{394}) are not conserved in GA 5-DH and NP_636946. However, according to a search of the NCBI conserved domain database (Marchler-Bauer *et al.* 2003), amino acids 96–722 of the deduced amino acid sequence of the large subunit of GA 5-DH show homology to dehydrogenases with PQQ as cofactor (NCBI conserved domain database accession number cd00216).

5.2.3 Expression of both GA 5-DH genes in *E. coli* (II, III)

The pTAC expression vector used for expression of GA 5-DH in *E. coli* included the strong and inducible *tac* promoter and a high-copy number replicon from pUC19. However, the

protein content in the expression experiments varied greatly and reproducible production of recombinant GA 5-DH in *E. coli* under standard *tac* promoter induction conditions (Sambrook *et al.* 1989) turned out to be difficult. One of the reasons for this was quite apparent, expression of GA 5-DH gene was noxious to the *E. coli* cells. Induction of expression of GA 5-DH gene led to cessation of growth and extensive lysis of the *E. coli* transformants and therefore the cultivation temperature and IPTG concentration needed to be carefully optimised for the production of recombinant GA 5-DH in *E. coli*.

Under optimised cultivation conditions (see Materials and Methods) it was possible to obtain reproducibly, about 0.03 mU/ml × OD_{600} of GA 5-DH activity in cell lysate of *E. coli* strains transformed with pTAC(GA 5-DH). This expression level is rather modest, since gluconate dehydrogenase activity in cell suspensions of *G. suboxydans* IFO12528 is about 0.06 mU/ml × OD_{600} (Table 8). This value is a sum of two activities since *G. suboxydans* is known to produce comparable levels of GA 5-DH and gluconate 2-dehydrogenase. Therefore, it was concluded that GA 5-DH activity in recombinant *E. coli* strains was approximately equal to the activity in native *G. suboxydans* strain. Such a level of expression is sufficient for characterisation of the recombinant enzyme and experiments at the "proof-of-concept" level to demonstrate the feasibility of a bioprocess based on expression of GA 5-DH gene in heterologous host.

One possible avenue towards improvement of expression levels of recombinant GA 5-DH was to try to find a homologue of GA 5-DH with better expressability. The preliminary analysis of *X. campestris* culture broth grown on glucose containing medium showed on TLC-plates that one of the fermentation products was actually 5-KGA. However, the 5-KGA levels on these cultivations were very low and not easily detectable. The results of measurement of enzymatic activity in *E. coli* carrying pTAC(GADX) were more encouraging, although pTAC(GADX) was found to be similarly toxic to the host cells of *E. coli* as pTAC(GAD) if induction was carried out in standard conditions. The GA 5-DH homologue in *X. campestris* turned out to have gluconate dehydrogenase activity, and not glucose dehydrogenase activity, as was expected from the sequence homology data (Fig. 13).

Moreover, expression levels, typically about 0.8 mU/ml × A_{600}, were around 30 times higher than those obtained with the *G. suboxydans* enzyme (Table 8) in otherwise similar expression experiments. It would be interesting to understand how much of this improvement in expression level is a consequence of the monomeric tertiary structure of the *X. campestris* GA 5-DH compared with the dimeric tertiary structure of *G. suboxydans* GA 5-DH. This question could be addressed, for example, by constructing and expressing a

Table 8 Enzyme activities of GA 5-DHs obtained in cultivations of the wild type strains and with recombinant microbial strains over-expressing the enzymes

Strain	Construct	Promoter	Signal	Enzyme	Activity, mU/ml × OD_{600}
G. suboxydans	wt	wt	–	GA 5-DH and GA 2-DH	0.06[a]
E. coli	pTAC(GA 5-DH)	*TAC*	–	GA 5-DH	0.03
E. coli	pTAC(GADX)	*TAC*	–	GA 5-DH	0.8

[a] Contains both GA 5-DH and GA 2-DH activities.

single chain chimeric enzyme by combining the sequences of both small and large subunits of *G. suboxydans* GA 5-DH.

The recombinant *X. campestris* GA 5-DH was not purified to homogeneity. However, estimates based on SDS-PAGE analysis of the two *E. coli* strains expressing gluconate dehydrogenases from *G. suboxydans* and *X. campestris* suggested that most of the difference in expression levels was explained by a higher specific activity of the *Xanthomonas* enzyme.

5.2.4 Characterisation of GA 5-DHs (II, III)

The recombinant *E. coli* strains expressing GA 5-DHs provided a convenient source and allowed the characterisation of the enzymes. This could not have been done earlier because of the difficulty in purification of GA 5-DH and the presence of many membrane dehydrogenases (e.g. GA 2-DH) in *G. suboxydans*.

Substrate specificity of the recombinant GA 5-DHs was investigated with a number of substrates. It was found, that gluconate is not a preferred substrate for either of the two enzymes (Table 9). The highest activity in both cases was measured with a mixture of different isomers of 2,3-butanediol. Many other polyols with carbon chain length from 3 to 7 were also good substrates. Significant activity was also measured with certain amino derivatives of polyols and aldonic acids. Among simple aliphatic alcohols, highest activity was measured with the two lower secondary alcohols – isopropanol and 2-pentanol. Also, a primary alcohol, isobutanol was accepted as a substrate but no activity was detected with ethanol. The relatively low activity measured with gluconic acid is mainly a consequence of the very high K_m, 161 mM for *G. suboxydans* GA 5-DH and 109 mM for *X. cambestris* GA 5-DH, compared to Kms 4.6 mM and 1.4 mM respectively for D-arabitol (Table 9). V_{max} with gluconate as a substrate was 36% (*G. suboxydans* GA 5-DH) and 51% (*X. cambestris* GA 5-DH) of that measured with D-arabitol. Also several aldoses and ketoses were tested and most of them were not accepted as substrates, presumably because the reactive hydroxyl was protected by cyclic hemiacetal formation in solution. The only exception was D-ribose that was oxidised efficiently, presumably because of its unusually high equilibrium concentration of linear (aldehyde) form in water solution (8.5%) (Pigman and Horton 1972). For example for glucose the equilibrium concentration of linear form in water solution is only 0.024%. The GenBank annotation of the enzyme of *X. campestris* encoded by locus NP_636946 as glucose dehydrogenase is obviously erroneous, as the data presented in Table 9 shows.

Many similarities in terms of substrate specificity were found between the enzymes from *G. suboxydans* and *X. campestris* as can be seen from Table 9. However, there were striking differences in the specificity of the two enzymes. Many poor substrates of *G. suboxydans* GA 5-DH were oxidized by the *X. campestris* dehydrogenase with high efficiency. Five to ten folds increase in relative reaction rates were observed with aliphatic alcohols 2-pentanol and isobutanol, amino-sugar N-methylglucosamine, and a seven-carbon polyol perseitol. It is not an easy task to give these enzymes a short but sufficiently descriptive name to cover all the substrates they accept. "Secondary alcohol dehydrogenase" could be the most suitable name.

Specificity profiles of GA 5-DH of *G. suboxydans* with respect to different polyols show clear correlation with the Bertrand–Hudson rule (Fig. 3) that summarises a set of empirical observations on the oxidation of polyols by acetic acid bacteria (Kulhánek, 1989). Polyols matching the Bertrand–Hudson rule (such as D-sorbitol, ribitol, D-arabitol, glycerol, etc)

Table 9 Substrate specificity of GA 5-DH of *G. suboxydans* and *X. campestris*

	Relative activity of GA 5-DH[a]	
Substrate	***G. suboxydans***	***X. campestris***
D-Arabitol	100	100
D-Sorbitol	77	91
Ribitol	51	91
Galactitol	0	0
D-Mannitol	57	89
meso-Erythritol	109	100
2,3-Butanediol	129	116
Glycerol	106	66
N-Methylglucosamine	3	49
2-Pentanol	7	34
Isobutanol	2	8
2-Propanol	5	5
L-Arabitol	0	0
Perseitol	2	13
L-Fucitol	3	14
Acetoin	0	0
D-Glucuronic acid	0	0
L-Gulonic acid	0	0
D-Galactonic acid	0	0
D-Gluconic acid	6	27
2-Ketogluconic acid	0	0
D-Glucosaminic acid	6	40
D-Fructose	0	0
D-Galactose	0	0
D-Ribose	2	28
D-Glucose	0	0

[a]The values in the table are expressed relative to the activity of the enzyme with 30 mM arabitol as substrate (= 100%). Substrate concentration used in the activity assays is 30 mM.

are all good substrates of GA 5-DH while those that do not match the rule (L-arabitol, xylitol, galactitol) are either poor substrates or not accepted at all. The products of GA 5-DH catalysed oxidation of D-sorbitol, D-arabitol, ribitol and D-mannitol were analysed by HPLC and found to be sorbose, xylulose, ribulose and fructose, respectively. Thus, in all tested

cases, positional specificity of oxidation of polyols by GA 5-DH also followed the Bertrand–Hudson rule. This correlation suggests that enzymes similar to *G. suboxydans* GA 5-DH may be responsible for the bulk of polyol dehydrogenase activity found in acetic acid bacteria.

The results of the studies of substrate specificity of both GA 5-DHs significantly broaden the scope of potential applications of these enzymes. This is particularly important with respect to the enzyme from *X. campestris,* which is the first single chain GA 5-DH/sorbitol dehydrogenase/secondary alcohol dehydrogenase discovered. Its good expressability properties mean that it may be a valuable tool not only for TA process development, but also for other bioprocesses e.g. oxidation of sorbitol into L-sorbose, a step in vitamin C manufacture, glycerol to dihydroxyacetone and meso-erythritol to L-erythrulose, both tanning substances used by cosmetics industry. In vitamin C synthesis by Reichstein process, the second step is a fermentation of D-sorbitol to L-sorbose. The fermentation is done with high yield of nearly 100% with *G. oxydans* (De Wulf *et al.* 2000). However, the efficiency of the reaction could possibly be improved by overexpression GA 5-DH, the enzyme responsible for this reaction, in the fermentation host. Alternatively, GA 5-DH could be used in formation of a more efficient strain combining the pathway from glucose to 2-keto-L-gulonic acid for conversion of glucose in one step fermentation directly to 2-keto-L-gulonic acid, the fifth intermediate of Reichstein process. (Fig 14. Saito Y. *et al.* 1997 and 1998, for rewiev see Hancock and Viola, 2002).

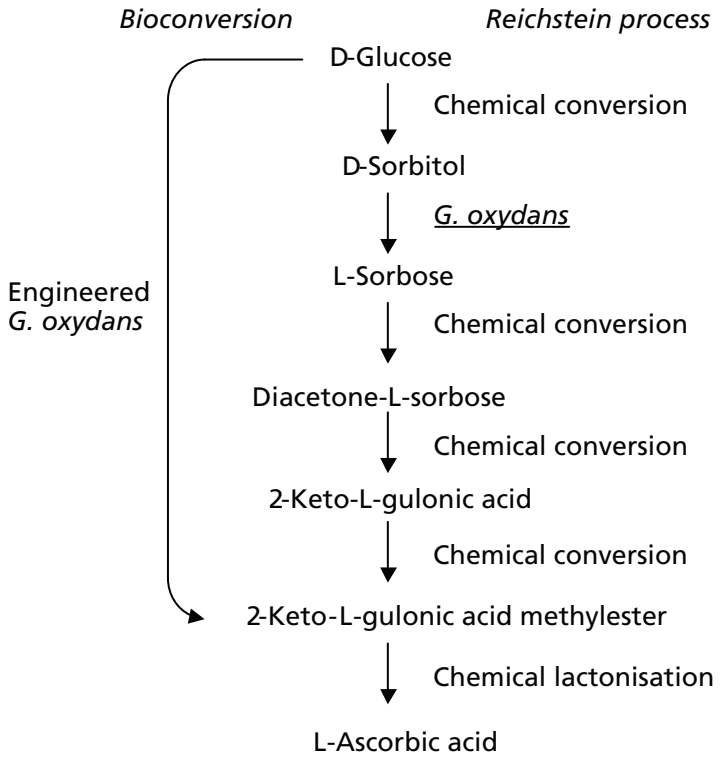

Figure 14 Reichstein process and modern biotechnology based process for L-AA production. The first five steps in Reichstein process have been replaced by on-step fermentation using metabolically engineered *G. oxydans* (Saito *et al.* 1998)

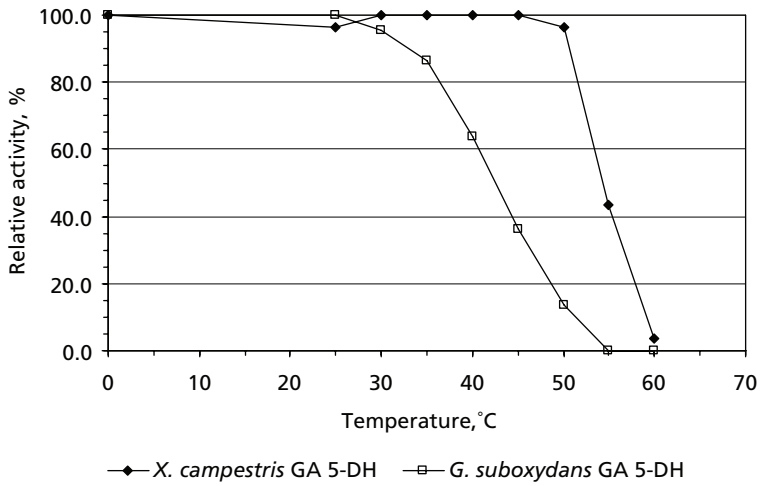

Figure 15 Temperature stability profiles for GA 5-DH of *G. suboxydans* and *X. campestris*.

The pH-activity profile of GA 5-DH from *X. campestris* was very similar to that of the GA 5-DH from *G. suboxydans*, with a maximum activity at around pH 6.0, but thermostability of the two enzymes was quite different (Fig. 15). The enzyme from *Xanthomonas* retained nearly full activity after 20 min at 50ºC while the *Gluconobacter* enzyme was almost completely inactivated under these conditions. This could be due to the more stable one-subunit structure of the *Xanthomonas* enzyme compared to the two-subunit structure of the *Gluconobacter* enzyme. Different thermostability was also reflected in different temperature optima (in a 20 min assay) of the two enzymes, 42.5 and 35ºC respectively.

It is well known that most PQQ-dependent enzymes require Ca^{2+} ions and are inactivated by EDTA (Anthony and Ghosh 1997). This was also the case with the cloned GA 5-DHs. Both enzymes were dependent on the presence of divalent cations for their activity and could be completely inactivated by treatment with EDTA. Full re-activation of the EDTA-treated enzyme required the presence of both Ca^{2+} and Mg^{2+} ions. The effect of Mg^{2+} and Ca^{2+} was synergistic since no increase in enzyme activity could be achieved by simply raising the concentration of either of the two ions alone. The only significant difference was that GA 5-DH from *G. suboxydans* was activated to a significant extent by Mg^{2+} alone, whereas the *X. campestris* GA 5-DH was almost inactive when supplemented with $MgCl_2$. These findings are in contrast with the results obtained by Sugisawa and Hoshino (2002) who reported that the sorbitol dehydrogenase of *G. suboxydans* IFO3255 is insensitive to EDTA and does not require divalent cations for activity.

Another enzyme apparently similar to GA 5-DH, arabitol dehydrogenase from *G. suboxydans* IFO3257, was reported to be inhibited by EDTA but restored to full activity in the presence of Ca^{2+} alone. Among other PQQ-dependent enzymes, divalent ion requirements vary significantly, in most cases Ca^{2+} is the preferred divalent metal. For some enzymes, particularly membrane-bound glucose dehydrogenases, Ca^{2+} can be substituted with Mg^{2+} (Goodwin and Anthony 1998) and Mg^{2+} can even be the preferred divalent ion for reconstitution of certain apoenzymes. However, it is not clear how common is the synergistic effect of the two divalent metal ions on activity of PQQ-dependent dehydrogenases.

The influence of several heavy metal ions on the activity of the two GA 5-DHs was

tested. The enzymes were completely inactivated by low concentrations of Cu^{2+} and strongly (about 80%) inhibited by 1.8 mM Zn^{2+}, similar to the inactivation of sorbitol dehydrogenase by the same ions. We did not, however, see any activation of GA 5-DHs holoenzyme by Co^{2+} as reported by Sugisawa and Hoshino (2002).

5.2.5 Production of 5-KGA (II, III)

As expected, *E. coli* strain expressing the GA 5-DH gene from *X. campestris* was more efficient in conversion of glucose and sodium gluconate to 5-KGA than the *E. coli* strain expressing the *G. suboxydans* gene. Two problems in using the cloned GA 5-DH gene from *G. suboxydans* as a basis for developing fermentation process for 5-ketogluconate were identified. Firstly, the enzyme had relatively low activity with D-gluconic acid (about 10% of the activity with D-sorbitol as substrate, Table 10). Secondly, expression of GA 5-DH gene was toxic to *E. coli* and only modest expression levels (about 0.06 mU/ml × OD_{600}, Table 8) were obtained using a *tac* promoter based expression vector. However, the approach of searching for the new enzymes with more suitable properties was successful.

The biotechnological potential of GA 5-DH from *X. campestris* was illustrated by a model experiment on the preparative conversion of D-gluconate into 5-KGA using cells of recombinant *E. coli* transformed with either pTAC(GAD) or pTAC(GADX) (Table 11). To achieve 75% conversion with cells expressing the gluconate dehydrogenase gene from *G. suboxydans*, 100 h incubation with a concentrated cell suspension (OD_{600} = 20) was required. Using the *E. coli* cells expressing *X. campestris* enzyme, almost similar conversion yield was achieved in 16 h and at low cell density (OD_{600} = 2). No gluconic acid transformation products were detected in control reactions with the *E. coli* strain XL1-Blue MRF' cells transformed with empty pTAC-vector. Also, 2-KGA and or 2,5-diketogluconic acid, the common contaminants of 5-KGA produced in *G. suboxydans* were completely absent in these reaction mixtures as was expected.

Obviously, GA 5-DH could also be useful for the production of L-sorbose, dihydroxyacetone, D-erythrulose, rare pentuloses such as L-ribulose and amino-sugar derivatives, such as those used as intermediates in Miglitol production (Deppenmeier *et al.* 2002). It is highly probable that the closest uncharacterised homologues of this enzyme (e.g. NP_641965, NP_638430, NP_643520, encoded by other *Xanthomonas* chromosomal loci) would be similarly applicable.

The literature suggests that laboratory strains of *E. coli* K-12 (e.g. JM 109) express large amounts of membrane-bound glucose dehydrogenase apoenzyme that can be activated

Table 10 Michaelis–Menten constants of GA 5-DH from *G. suboxydans* and *X. campestris*

	***G. suboxydans* GA 5-DH**		***X. campestris* GA 5-DH**	
Substrate	**K_m, mM)**	**V_{max}, mM/min**	**Km, mM**	**V_{max}, mM/min**
D-Arabitol	4.6	1	1.4	1
D-Sorbitol	57	2.2	5.6	0.93
D-Gluconic acid	161	0.36	109	0.51

Table 11 Conversion of glucose and gluconic acid into 5-KGA by cells of recombinant *E. coli* expressing a GA 5-DH gene of *G. oxydans* or *X. campestris*

Strain	Biomass concentration	Substrate	Incubation time, h	5-KGA accumulation, %
E. coli pTAC(GA 5-DH)	10 x	Na-Gluconate	100	75
E. coli pTAC(GA 5-DH)	10 x	Glucose	100	+
E. coli pTAC(GADX)	1 x	Na-Gluconate	16	60
E. coli pTAC(GADX)	1 x	Glucose	16	1

Note: The activity of glucose dehydrogenase of *E. coli* was not confirmed. In the experiment with GA 5-DH of *G. suboxydans* the pH of the conversion reaction mixture was adjusted to pH 5.5. The conversion reaction with GA 5-DH from *X. campestris* was done in LB without pH adjustment.

by simply supplying the cells with PQQ (Yum *et al.* 1997). However, the conversion of glucose to gluconic acid by *E. coli* strain XL1-Blue MRF' supplied with PQQ was very inefficient. Therefore, it was not surprising that incubation of 1 or 5% glucose solutions with cell suspensions of induced cultures of *E. coli* XL1-Blue MRF' transformed with pTAC(GAD) yielded only low amounts of 5-KGA. The only other bioconversion product that was detected in these reactions was TA, presumably formed spontaneously through oxidation of 5-KGA by oxygen during prolonged incubations. Since the reaction broth did not contain any added vanadate, it raises an intriguing possibility that this conversion may somehow be enzyme-catalysed.

5.2.6 Feasibility of enzymatic conversion of 5-KGA into TA (II)

5-KGA has long been implicated as part of the tartaric acid biosynthetic route in plants (Saito and Kasai. 1984) but no enzymes catalysing this conversion have ever been isolated. The idea that bacteria may contain enzymes capable of catalysing conversion of 5-KGA into TA is interesting.

It was found that transketolases encoded by both *tkt*A and *tkt*B genes of *E. coli* and the yeast D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase can accept 5-KGA as substrate. Removal of keto-moiety from 5-KGA by the transketolase activity leads to tartaric acid semialdehyde (Fig. 16).

Two transketolase genes of *E. coli* encoding transketolase A (*tkt*A) and transketolase B (*tktB*) were cloned and expressed. Expression levels of both genes were quite high (4–6 U/mg i.e. about 500-fold higher than in wild-type *E.coli*). The K_m values of these enzymes and purified transketolase from *S. cerevisiae* for 5-KGA and xylulose 5-phosphate were determined (Table 12). Kinetic parameters of the reaction catalysed by the microbial transketolases were similar, with a K_m of about 20 mM and V_{max} of approximately 25% of the V_{max} for xylulose 5-phosphate, the preferred substrate of the enzymes.

Tartaric acid semialdehyde production was tested *in vitro* and the reaction products were analysed by HPLC. It was found that a product with a retention time similar to that of TA was formed in the reaction. The UV-spectrum of this product was different from that of TA, supporting the idea that the product formed was tartaric acid semialdehyde. A

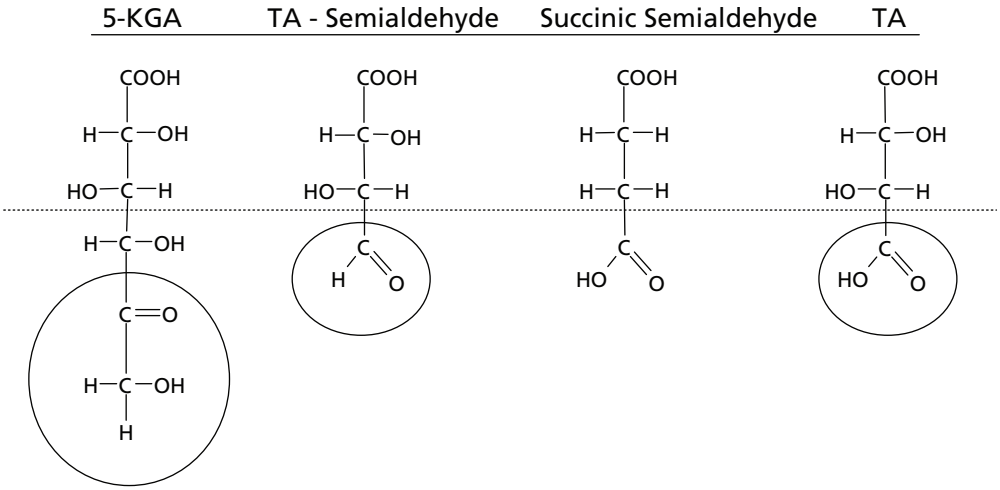

Figure 16 When the ketol moiety is removed from 5-KGA, a tartaric acid semialdehyde is formed. TA-semialdehyde could possibly be converted to TA by succinic semialdehyde dehydrogenase.

candidate for tartaric acid semialdehyde dehydrogenase was identified through literature searches as succinic semialdehyde dehydrogenase, an enzyme found in a number of bacterial species. TA semialdehyde and succinic semialdehyde are structurally similar (Fig. 16). However, cloning of the succinic semialdehyde dehydrogenase and testing of its activity towards tartaric acid semialdehyde remains still to be done in future.

Table 12 The determined K_m and relative V_{max} values of 5-KGA and xylulose 5-phosphate for the microbial transketolases

Enzyme	K_m (5-KGA), mM	K_m (xlu-5-P), mM	V_{max} (5-KGA):V_{max} (xlu-5-P)
E.coli tktA	20	2	1:4
E.coli tktB	25	1	1:5
S. cerevisiae tkt	22	2	1:4

Note: V_{max} was measured using crude preparations of *E. coli* transketolases. Therefore, relative rather than absolute V_{max} values are listed.

6 Conclusions

In the beginning of this work two goals were set. The first was to study on concept level the possibility to produce two different organic acids, 5-KGA and D-EA by fermentation. The second goal was the evaluation of these two studies to be further developed for a production process. To reach both goals, research routes for the production of these acids were taken based on literature search and analysis of the current production processes for 5-KGA and D-EA. The analyses pointed out that the economically most feasible production processes should be based on recombinant microorganism.

The central technical challenge in developing an efficient fermentation-based technology for the conversion of glucose into 5-KGA or D-EA was the lack of microbial strains capable of efficiently converting glucose, via D-glucono-1,5-lactone, into these acids. The mechanisms controlling formation and consumption of ketogluconates or D-EA in *G. suboxydans* and *P. cyaneo-fulvum*, respectively, are complex and incompletely understood. No literature documenting of production of these acids by microbial strains outside of the *Gluconobacter* or *Penicillium* family exists. Therefore the most direct and promising avenue towards efficient process for fermenting glucose into 5-KGA or D-EA was to clone and over-express the genes of gluconate 5-dehydrogenase and D-gluconolactone oxidase in suitable hosts. The possible production process would be built around these recombinant enzymes or production strains and therefore, the enzyme expression level and fermentation productivity were the key parameters defining the usability of these strains.

Both D-gluconolactone oxidase of *P. cyaneo fulvum* and gluconate 5-dehydrogenase of *G. suboxydans* were purified successfully. The genes encoding these enzymes were cloned, overexpressed and the recombinant enzymes characterised. Identification of a novel single chain gluconate 5-dehydrogenase encoded by the *X. campestris* chromosomal locus NP_636946 in the course of this study was a valuable discovery. This enzyme proved to be a superior tool not only for 5-KGA production, but also for production of other industrially interesting compounds such as L-sorbose, an intermediate of vitamin C production, and dihydroxy acetone, a functional compound in self tanning cosmetics, and many other compounds as well. Finally, feasibility of the production of both D-EA and 5-KGA by one-step fermentation was demonstrated. Based on the results of this study, an efficient process for high-yield fermentation of glucose into 5-KGA and D-EA using recombinant hosts seems to be a feasible route to novel fermentation-based production technology. Critical evaluation of these two subprojects indicated that the production of 5-KGA using *X. campestris* GA 5-DH would have the highest success probability and is recommended to be prioritised for further process development.

Major task in strain development still lies in over-expression of the enzymes. With both enzymes, the level of the overexpression was still insufficient for process purposes. In future, more focus should be aimed at testing the expression of GA 5-DH genes in several Gram-negative hosts, such as *Acetobacter*, *Gluconobacter, Serratia*, and *Pseudomonas* to obtain more viable and active strains for fermentation. The selection criteria for such expression hosts would include at least following aspects: endogenous PQQ-production (1) and glucose dehydrogenase activity (2), not a 2-KGA (3) producer or gluconate (4) or ketogluconate (5) consumer, and fast growing (6) to high cell densities (7). Exploring the other uncharacterised homologues of GA 5-DHs could offer other new enzymes with the same substrate specificity, and possibly with different expression character. This could be an alternative route aiming for more efficient 5-KGA production strains. An alternative ap-

proach could be using the random or rational protein engineering techniques to improve the properties of the enzymes of this study. Especially, improvement of specific activity of *X. campestris* GA 5-DH towards D-gluconic acid would make it more suitable for production purposes. The problems in the expressability of the enzyme could be overcome simultaneously using random mutagenesis and high throughput screening technique.

The ideal one-step process for production of TA from glucose has taken a long step towards reality. Microbial transketolases were shown to accept 5-KGA as substrate and to produce most probably TA semialdehyde. Earlier no microbes with this pathway have been reported. However, to prove the feasibility of such a process, even at a concept level, one would still need to demonstrate that an enzyme capable of oxidising TA semialdehyde into tartaric acid exists. The odds for this can be considered good.

The over expression level of GLO proved out to be modest even under the strong *AOX1* promoter of *P. pastoris*, and should be the major target in attempts to create a second generation D-EA production strain. However, cloning of the GLO gene and elucidating its novel character as a secreted sugar lactone oxidase pointed out some possible reasons for the earlier unsuccessful attempts to use the *Penicillium* sp. in production of D-EA by direct fermentation on glucose. The influence of lactonases present in the fungal glucose oxidation system must have a strong negative effect on the D-EA yields in the fermentations. A great deal of gluconolactones formed from glucose by glucose oxidase could have been ended up to gluconic acid instead of D-EA. Therefore, it would be very interesting to study how inactivation of the lactonase gene or probably genes from *Penicillium* sp. would affect on the fermentation yields of D-EA. A tempting and natural extension of this approach of second generation D-EA producing recombinant microbe would be the homologous overexpression of GLO in *Penicillium*-mutant lacking the lactonase activity

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