Recent Patents on Vitamin C: Opportunities for Crop Improvement and Single-Step Biological Manufacture

Robert D. Hancock*

Plant Products and Food Quality, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA. United Kingdom

Received: August 1, 2008; Accepted: September 9, 2008; Revised: September 9, 2008

Abstract: Vitamin C is an essential human nutrient obtained primarily from plant foods. Despite the necessity of the vitamin for human health and the essential role that it plays in plant stress resistance, it is only in the last decade that an understanding of its biosynthesis in plants has emerged. These fundamental discoveries present for the first time the opportunity to manipulate crop plants for improved human nutrition and enhanced agronomic performance and are discussed in relation to recent patents protecting aspects of vitamin C synthesis and degradation in plants.

Vitamin C has a wide range of medicinal and industrial uses resulting in an annual market exceeding US$600 million. In recent years, there has been strong competition in vitamin C markets as a result of emerging Chinese producers and one response has been increased investment in research and development for the biological manufacture of the vitamin. Knowledge of the plant biosynthetic pathway has provided novel opportunities for the synthesis of vitamin C in yeasts, although successes have been limited to date. A more promising route is the direct synthesis of vitamin C in acetic acid bacteria and significant research and development has gone into the genetic improvement of appropriate strains.

Keywords: Ascorbic acid, fermentation, Reichstein process, *Gluconobacter*, sorbitol, sorbosone dehydrogenase.

INTRODUCTION

Vitamin C (L-ascorbic acid; AsA) is a powerful anti-oxidant and essential human nutrient that has extensive industrial and medicinal applications resulting in global requirements estimated at 154 thousand tons in 2007 [1]. Approximately 50% is used in the food industry where it is added during processing to prevent pigment discolouration and enzymatic browning, prevent loss of flavour and aroma, protect or enhance nutrient content and extend shelf-life [2]. A further 30% is used in the pharmaceutical industry for both medical applications and the production of vitamin supplements. The remainder is used in animal feeds (13%), the cosmetic industry (5%) and other industrial processes (2%) [1].

The commercial value of AsA manufacture was recognised in the early part of the 20th century and industrial processes have been heavily patented since the 1930's. Tadeus Reichstein first patented the synthesis of AsA from L-xylosone in 1935 [3] however difficulties in obtaining the starting material prevented the full commercial application of this process. In the same year a second patent was published demonstrating a method for the manufacture of AsA from 2-keto-L-gulonic acid [4] for which a facile method of production from the readily available L-sorbose had already been developed [5]. Further process improvements were introduced by Walter Haworth and colleagues who proposed the synthesis of L-sorbose from sorbitol by microbial fermentation [6]. This series of reactions formed the basis of the Reichstein process which dominated global AsA manufacture for over 60 years [7] Fig. (**1**).

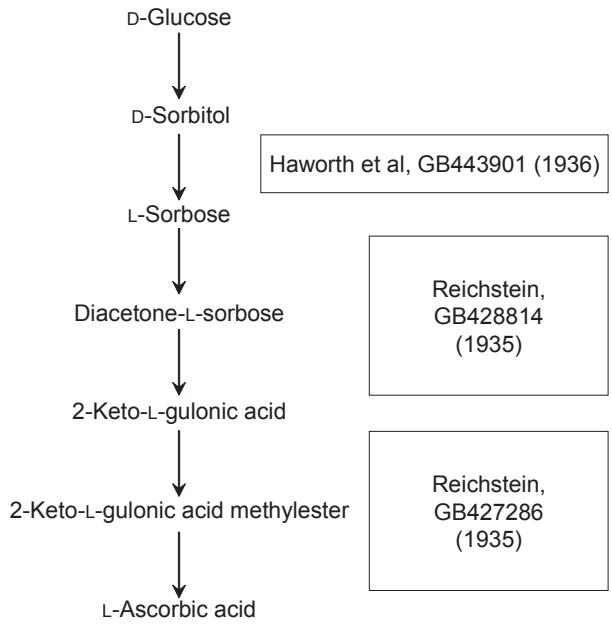

Fig. (1). Schematic of the Reichstein process for the industrial synthesis of vitamin C.
Intermediates of the Reichstein process are shown [1, 7, 10, 11]. Key patents protecting various stages are highlighted in orange boxes [4-6].

When commercial AsA production began the value of one kilo was over $1000 making the process highly profitable [1]. Since then prices have shown a steady fall as production efficiencies have improved. Following entry into the market by state subsidised Chinese producers AsA prices fell to an all time low of approximately $3 per kilo in the early 2000's but have since rallied and stood at $11 per kilo

*Address correspondence to this author at the Plant Products and Food Quality, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA. United Kingdom; Tel: +44 1382 562 731; Fax: +44 1382 568 575;
E-mail: Rob.Hancock@scri.ac.uk

1876-1429/09 $100.00+.00

in 2007 [8]. In part to overcome the threat represented by Chinese competition, there has been significant recent investment into research and development by European, American and Asian manufacturers resulting in a resurgence in AsA patent activity in recent years. Additional drivers for research have included scientific advances made in the areas of plant AsA biosynthesis and an appreciation of the metabolic flexibility of the yeast D-erythroascorbic acid pathway.

Vitamin C is an essential part of the diet, with the vast majority coming from plant foods and efforts to understand its biosynthesis in plants stretch back to the 1950's. Despite the pioneering efforts of Frank Loewus and colleagues, it wasn't until 1998 that a pathway compatible with all of the available scientific knowledge was finally published [9] and since then various other pathways have been proposed. Manipulation of expression of genes encoding biosynthetic enzymes from all of the proposed pathways have been demonstrated to impact on plant AsA levels. These novel discoveries have inevitably led to significant new patent activity.

The present review summarises the latest scientific research regarding the improvement of crop quality with respect to its AsA content and the latest developments in microbial fermentations for the industrial synthesis of AsA. The application of this work is highlighted by reference to recent patents in the field. Despite the fact that China now supplies more than 80% of vitamin C worldwide [8], very little information is available regarding the latest scientific and industrial developments hence the review focuses on work undertaken in the USA and Europe. Previous reviews have covered the scientific literature regarding the industrial and biological manufacture of vitamin C [7, 10, 11] and recent advances in understanding and manipulating plant vitamin C biosynthesis [12-14].

SYNTHESIS OF VITAMIN C IN PLANTS - EMERGING OPPORTUNITIES FOR THE IMPROVEMENT OF CROP AGRONOMY AND HUMAN NUTRITION

The last decade has seen rapid advances in understanding AsA biosynthesis and metabolism in plants and the commercial potential of these discoveries are reflected in the flurry of patent activity since the turn of the millennium. Claims primarily focus on the development of crops with improved agronomic and nutritional qualities although the significance of the discoveries for the biological manufacture of vitamin C has not been overlooked.

Despite the importance of vitamin C in human nutrition and plant physiology, it wasn't until 1998 that a widely accepted biosynthetic pathway that reconciled apparently conflicting previous data was presented [9]. A series of alternative pathways were subsequently proposed based on novel biochemical and molecular data and the engineering of pathways in transgenic plants [15-17]. As a result four potential routes to vitamin C have been described in plants within the last decade Fig. (**2**). Significant debate continues regarding the physiological significance of the different pathways, recently enlivened by the observation that mutation of the two genes encoding an enzyme specific to the L-galactose (L-Gal) biosynthetic pathway Fig. (**2**) is lethal in *Arabidopsis* seedlings [18]. Despite these discussions, the biotechnological potential of the alternative pathways has clearly been demonstrated and upregulation of genes encoding biosynthetic enzymes within the postulated pathways has resulted in enhanced plant AsA content. Scientific activity has also been strong regarding the metabolism and degradation of AsA [19, 20] and the manipulation of catabolic pathways represents an alternative prospect for engineering plant AsA content [21].

It has been known for many years that the final step of AsA biosynthesis in plants is the oxidation of L-galactonolactone (L-GalL) by the mitochondrial enzyme L-GalL dehydrogenase (E.C. 1.3.2.3) [22]. Early work also showed that the vitamin C content of cress seedlings was enhanced following supply of D-galacturonic acid (D-GalUA) methyl ester and so a biosynthetic pathway was proposed in which D-galactose (D-Gal) was oxidised to D-GalUA then further metabolised to AsA via the intermediate L-GalL [23]. The implication of such a pathway was that the carbon skeleton of D-Gal would be inverted during the synthesis of AsA so that C1 of D-Gal would become C6 of AsA. This hypothesis was challenged following the supply of plant tissues with specifically labelled precursors which suggested that in fact, there was no inversion of the carbon skeleton when AsA was synthesised from sugars [24]. A later molecular analysis suggested that AsA could be synthesised from D-GalUA in strawberry fruit but that the source of D-GalUA was not D-Gal directly but uronic acids released from pectin depolymerisation [15] (D-GalUA pathway, Fig. (**2**)). A number of potential pathways were proposed that would be compatible with the data showing a lack of carbon chain inversion from sugars including one using the intermediates D-glucosone and L-sorbosone [25], the latter compound being an important precursor in industrial microbial fermentations today. Despite these efforts, it took over 40 years' investigation until a pathway consistent with all of the available biochemical evidence was proposed [9] [L-Gal pathway, Fig. (**2**)]. The principal advances leading to the discovery of the pathway were the observation that L-Gal was as effective an AsA precursor as L-GalL and the demonstration of enzymatic synthesis of free L-Gal from GDP-L-Gal in plant extracts. GDP-L-Gal itself was synthesised from GDP-D-mannose (GDP-D-Man) by the previously identified enzyme GDP-D-Man 3,5-epimerase (E.C. 5.1.3.18). The novel enzyme L-Gal dehydrogenase, responsible for the synthesis of L-GalL, was purified and N-terminal sequenced by Edman degradation and the identification of this sequence formed the basis of a patent protecting the biotechnological use of the pathway [26]. Subsequently, the gene encoding L-Gal dehydrogenase was cloned [27] and the utilisation of the full length protein or homologs and recombinant DNA encoding the protein were protected [28]. Several claims were made regarding both the utilisation of L-Gal dehydrogenase and the utilisation of knowledge regarding the proposed biosynthetic pathway. In particular the patents sought to protect organisms engineered to express L-Gal dehydrogenase with particular reference to those organisms that contained enhanced levels of AsA as a result of expressing the enzyme. A subsequent patent, outlined below, in which yeast were engineered to express plant GDP-D-mannose 3,5-epimerase, *myo*-inositol-1-phosphate phosphatase

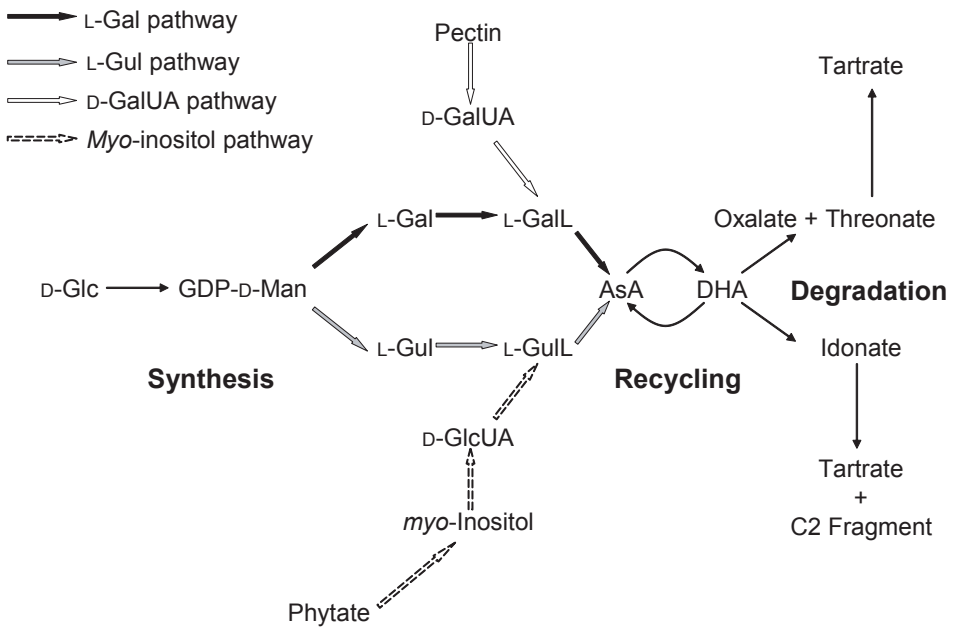

Fig. (2). Proposed pathways of synthesis and metabolism of ascorbic acid in plants.
Four pathways have been proposed for AsA biosynthesis in plants. The *de novo* route from photosynthetic sugars via the rare sugar L-galactose was proposed by Nick Smirnoff and colleagues in 1998 [9]. Subsequent kinetic analysis of the enzyme GDP-D-mannose 3,5-epimerase revealed that the reaction products were not limited to GDP-L-galactose but that GDP-L-gulose could also be formed. This data combined with other biochemical analyses led to the proposal that the true AsA biosynthetic pathway was via L-gulose [17]. In addition to the *de novo* pathways, a salvage pathway utilising galacturonic acid units recovered from pectin degradation was proposed in ripening strawberry fruit [15] and a further pathway utilising *myo*-inositol (released from phytate hydrolysis) has been described in Arabidopsis [16, 44]. Oxidation of AsA leads to the formation of dehydroascorbic acid which may either be recycled back to AsA via the ascorbate-glutathione cycle [12] or other reductive mechanisms. Alternatively dehydroascorbate can undergo C2/C3 cleavage to form oxalate and threonate [20] or C4/C5 cleavage to form tartrate and a C2 fragment [19]. Solid arrows represent single steps, dashed arrows represent multiple steps.
Abbreviations: D-Glc, D-glucose; GDP-D-Man, GDP-D-mannose, L-Gal, L-galactose; L-Gul, L-gulose; L-GalL, L-galactonolactone; L-GulL, L-gulonolactone; D-GalUA, D-galacturonic acid; D-GlcUA, D-glucuronic acid; AsA, L-ascorbic acid; DHA, dehydroascorbic acid.

and L-Gal dehydrogenase permitting the synthesis of AsA in yeasts [29] could therefore be considered an infringement. Another novel aspect that was protected under the L-Gal dehydrogenase patent was the development of herbicides targeting this enzyme [28]. Given the fact that AsA is essential for plants as well as animals and the divergence in AsA biosynthetic pathways between plant and animal kingdoms it was predicted that such herbicides would be lethal to plants but harmless to animals.

Following identification of the primary AsA biosynthetic pathway in plants, several groups set to work purifying proposed biosynthetic enzymes. A key enzyme was GDP-D-Man 3,5-epimerase responsible for the channelling of sugars into AsA biosynthesis. Although the enzyme activity had previously been identified in plant and algal extracts [30], it had not been purified beyond 18-fold [31] until Beata Wolucka and colleagues purified the *Arabidopsis* enzyme sufficiently to allow peptide fingerprinting by mass spectrometry in 2001 [32]. This allowed identification of the full amino acid sequence in bioinformatic databases and subsequently a full length functional gene was cloned and expressed in *Escherichia coli* [17]. The utility of the isolated enzyme and associated gene sequence was recognised and the generation of any of the downstream AsA pathway metabolites (GDP-L-Gal, L-Gal-1-phosphate, L-Gal, L-GalL) or AsA itself was protected either *in vitro* or in prokaryotic or eukaryotic cells [33]. In addition, the use of the recombinant enzyme for the development of antibodies was protected. Finally, high external mannose concentrations are highly toxic to plant cells. Mannose is taken up and rapidly phosphorylated, however most plant tissues have only a limited capacity for further metabolism of mannose phosphates resulting in sequestration of the inorganic phosphate pool [34]. It was proposed that one of the rate limiting enzymes for mannose phosphate metabolism was GDP-D-Man 3,5-epimerase and therefore the use of the enzyme as a selectable marker in transgenic cells was also protected [33].

Subsequent kinetic analysis of both the recombinant and native forms of GDP-D-Man 3,5-epimerase revealed that in addition to performing the double epimerisation of mannose to produce GDP-L-Gal, the enzyme was also capable of releasing the substrate following epimerisation at only the 5-position to form GDP-L-gulose (GDP-L-Gul). Furthermore, *Arabidopsis* suspension cells were capable of synthesising AsA when supplied with either L-Gul or L-GulL at rates similar to when supplied with the equivalent L-Gal derivatives [17]. It was therefore proposed that GDP-D-Man 3,5-epimerase was a dual function enzyme producing activated

L-galactosyl units for cell wall biosynthesis or alternatively producing GDP-L-Gul to be channelled into AsA biosynthesis L-Gul pathway, Fig. (**2**). The use of the L-Gul pathway for synthesising vitamin C was therefore subsequently protected in the USA [35].

Early in the new millennium, molecular analyses of strawberry fruit ripening reignited interest in the AsA biosynthetic pathway utilising D-GalUA as an intermediate. Although labelling studies carried out in the 1950's and 60's had provided evidence that the major AsA biosynthetic pathway in plants did not require inversion of the carbon skeleton from sugars, it was known that there was inversion of the carbon skeleton when AsA was synthesised from D-[1-^{14}C]GalUA in strawberry fruit [24]. This suggested the presence of an alternative biosynthetic route in which D-GalUA, salvaged from pectin breakdown, was reduced to L-GalL prior to conversion to AsA [D-GalUA pathway, Fig. (**2**)]. Furthermore, evidence for a soluble enzyme activity in pea that was capable of the conversion of esters of D-GalUA to L-GalL had previously been presented [36]. Although the pathway remained a scientific curiosity, no further progress was made until 2003 when the gene encoding D-GalUA reductase was identified following an analysis of gene expression during strawberry fruit development. A transcript homologous to aldo-keto reductases was correlated with AsA accumulation and positive identification of the gene product as D-GalUA reductase was achieved following heterologous expression in *Arabidopsis thaliana* and enzymatic assay of crude protein extracts [15]. In the same lines where the enzyme was overexpressed there was also up to threefold increases in plant AsA concentration demonstrating the utility of the enzyme for improving plant nutritional quality [37]. The use of the protein and/or DNA construct to produce L-GalL from D-GalUA, to produce plants with high levels of AsA for human consumption or for producing plants with increased oxidative stress resistance was protected. It should however be recognised that recent research has suggested the relationship between plant vitamin C levels and agronomic performance is not simply positively correlated as was once expected. For example, while the *Arabidopsis thaliana* vtc mutants which contain reduced levels of AsA are more susceptible to ozone damage [38], salt stress [39] and high light [40] they are more resistant to disease [41]. Conversely, work in our laboratory has shown that potato leaves containing high AsA levels allow more rapid aphid reproduction than those containing lower AsA levels (Vivera *et al.* unpublished). One mechanism for overcoming the potentially adverse physiological effects of manipulating plant AsA might be to limit AsA accumulation to specific organs such as tubers or fruits. This could be achieved via tissue specific gene expression or alternatively by manipulating the transport of AsA from source to sink tissues. The recent finding that symplastic phloem loaders appear to specifically tag AsA for transport may represent a mechanism by which such manipulation could be achieved [42].

A third alternative pathway was proposed that had similarities to the animal biosynthetic pathway. Where the pathway diverges from the animal pathway is in the synthesis of D-glucuronic acid (D-GlcUA) which is via *myo*-inositol in plants [*myo*-inositol pathway, Fig. (**2**)] but from phosphorylated intermediates in animals. It had been known for some time that L-gulonolactone (L-GulL) was a suitable AsA precursor when supplied to plant tissues [23] and a route to L-GulL had been proposed from *myo*-inositol via D-glucuronic acid (D-GlcUA) [43]. Evidence to suggest that such a pathway may operate *in vivo* was obtained following the analysis of homozygous *Arabidopsis* lines overexpressing an endogenous *myo*-inositol oxygenase (E.C. 1.13.99.1) responsible for oxidising *myo*-inositol to D-GlcUA. In the overexpressing lines foliar AsA levels were 2-3 fold higher than wild-type plants [16]. Further evidence to support the pathway came from the observation that increased conversion of phytate (inositol hexakisphosphate) to free *myo*-inositol could also enhance foliar AsA concentrations [44]. *Arabidopsis* mutants in which a phytase (inositol phosphate phosphatase) was upregulated by activation tagging showed two fold increased foliar AsA levels compared to wild-type plants. Similarly, plants expressing the gene under the control of the 35S constitutive promoter showed a 2-fold increase in foliar AsA. On the contrary insertion mutants in which the gene was deactivated had a 30% decrease in foliar AsA levels.

Unexpectedly, enhancing plant AsA levels via overexpression of *myo*-inositol oxygenase also improved the rate of accumulation of biomass in both *Arabidopsis* and lettuce [45]. Similarly, the overexpression of other genes encoding enzymes from the same pathway (gulonolactone oxidase and D-GlcUA reductase) enhanced biomass accumulation and AsA content. Those plants that had increased AsA levels also showed improved performance under salt stress compared to wild-type plants. These findings were included in a patent protecting the utility of upregulation of plant AsA content for increasing plant growth, biomass and stress tolerance [45]. It was envisaged that such applications would be useful for increasing food production for a growing population and because both aerial and below ground biomass was enhanced for improving carbon sequestration.

An alternative approach to the manipulation of plant AsA levels is modulation of recycling and degradation as illustrated by patents taken out by groups from the USA [46] and Australia [21]. The former approach was taken by Daniel Gallie and colleagues who manipulated the expression of the key recycling gene dehydroascorbate reductase (E.C. 1.8.5.1) in both tobacco and maize [47]. Gene expression levels were enhanced up to 100-fold with a similar increase in soluble enzyme activity resulting in up to 4-fold increases in AsA content of aerial tissues. The utility of manipulating dehydroascorbate reductase activity to control levels of AsA in plants was patented [46]. Further work in the authors' laboratory demonstrated a clear relationship between guard cell hydrogen peroxide levels, AsA redox state and the control of guard cell aperture [48]. It was therefore proposed that manipulation of foliar AsA content and redox state could be used to i) protect the plant from drought and aerosolised toxins by reducing the AsA content and inducing stomatal closure or ii) improve the carbon fixation activity of the plant by enhancing foliar AsA resulting in stomatal opening facilitating gas exchange with the atmosphere.

A second method for manipulating plant AsA content by altering AsA degradation focussed on the C4/C5 cleavage

pathway Fig. (**2**). Pioneering work in the 1970's and 80's had demonstrated the pathway intermediates as 2-keto-L-gulonate, L-idonate and 5-keto-D-gluconate [49] however the enzymes responsible for pathway catalysis had remained elusive, in part due to the difficulty in extracting and purifying active enzymes from fruit tissues. The problems were overcome following the application of transcriptional analysis to grape tissues actively accumulating tartrate. A candidate list of 87 genes showing transcriptional profiles correlating with tartrate accumulation was interrogated for those showing homology to oxido-reductases [19]. This was further refined by analysis of gene expression in Vitaceae fruit that accumulated different levels of tartrate. In particular one species (*Ampelopsis aconitifolia*) where tartrate could not be detected one of the candidate genes was not expressed and it was confirmed as encoding an L-idonate dehydrogenase following kinetic analysis of the recombinant protein. One important observation was that ripe fruit of *A. aconitifolia* contained three times the AsA concentration of fruit from tartrate accumulating species. Although not demonstrated through examples, the utility of the gene for manipulating AsA and tartrate levels in plants was protected through an international patent [21].

The capacity for plant AsA manipulation has now been clearly demonstrated under laboratory conditions however, a number of challenges remain before these findings can be fully commercially exploited. AsA as the main contributor to cellular redox state in plants plays a key role in the perception and transduction of environmental signals [50] and it has yet to be determined how plants with manipulated AsA levels would perform under field conditions. In addition, while GM crops continue to increase in acreage considerable consumer resistance remains in many parts of the World such as Europe and Australasia; therefore either consumers must be convinced of the clear benefits of GM crops with enhanced AsA levels or alternative breeding technologies need to be found.

METABOLIC FLEXIBILITY OF THE YEAST D-ERYTHROASCORBATE PATHWAY – NEW OPPORTUNITIES FOR EUKARYOTIC MANUFACTURE OF VITAMIN C

Yeasts do not synthesise AsA but instead produce its 5-carbon analogue D-erythroascorbate (EAsA) through sequential oxidation of the pentose D-arabinose [51]. The enzymes catalysing these reactions lack specificity and are capable of oxidising a range of sugars and their corresponding lactones [52-54]. *In vitro* studies showed that the final pathway enzyme, D-aribono-1,4-lactone oxidase (E.C. 1.1.3.37) was capable of oxidising a range of substrates to generate a number of products including EAsA, L-erythroascorbic acid, D-isoascorbic acid, 6-deoxy-L-ascorbic acid and AsA [11]. *In vivo* studies have additionally shown that AsA can be synthesised by yeasts when provided with an external supply of L-Gal, L-GulL or L-GalL [55]. It therefore appears that the reason yeast cells are unable to synthesise AsA from common substrates is due to metabolic deficiencies with respect to the synthesis of L-Gal. This has led to the development of novel yeast strains in which various approaches have been taken to engineer the synthesis of L-Gal from cheap starting materials.

The discovery that plants synthesise AsA via GDP-D-Man and L-Gal represented an opportunity to extend AsA biosynthetic capacity to yeasts by engineering of the appropriate enzymes into suitable yeast strains. Yeasts are capable of the synthesis of GDP-D-Man which acts as a precursor for cell wall components and substrate for protein glycosylation reactions. Therefore, expression of only three enzymes, GDP-D-Man 3,5-epimerase, GDP-L-Gal phosphorylase and L-Gal-1-phosphate phosphatase should be sufficient for the extension of L-Gal synthesis to yeasts. In fact, expression of only GDP-D-Man 3,5-epimerase from *Arabidopsis thaliana* in *Saccharomyces cerevisiae* was sufficient for the engineered strain to produce small quantities of AsA [29]. This suggests that yeast cells contain enzyme activities capable of dephosphorylating GDP-L-Gal to free L-Gal. However, further improvements in yield were obtained when the *A. thaliana myo*-inositol-1-phosphate phosphatase (E.C. 3.1.3.25), catalysing the dephosphorylation of L-Gal-1-phosphate was also expressed; suggesting endogenous dephosphorylating activities were limited. Further yield enhancements were achieved following the expression of a rat GDP-L-fucose pyrophosphorylase (E.C. 2.7.7.30) capable of the conversion of GDP-L-galactose to L-galactose-1-phosphate [56]. The best strains were those in which heterologous genes encoding *A. thaliania* L-Gal dehydrogenase and *S. cerevisiae* D-arabinonolactone oxidase (E.C. 1.1.3.37) were also expressed however, AsA yields were still only 200 $\mu g\ L^{-1}$. Now that the sequences encoding GDP-L-Gal phosphorylase [57] and L-Gal-1-phosphate phosphatase [58] are available, further strain improvements may be expected.

Although AsA yields from the transgenic yeast strains were too low to represent a viable industrial process, the increased cellular soluble antioxidant content improved the stress resistance of the engineered strains. Strains expressing GDP-D-Man 3,5-epimerase, *myo*-inositol-1-phosphate phosphatase, L-Gal dehydrogenase and D-arabinonolactone oxidase were highly resistant to H_2O_2 reaching culture stationary phase in medium containing 2 mM H_2O_2 within 24 h, a period not significantly different from cells grown in medium without H_2O_2. In contrast, wild-type strains which had growth curves indistinguishable from engineered strains in medium lacking H_2O_2 were completely unable to grow in 2 mM H_2O_2 [29]. Engineered yeast cells were not only resistant to oxidative stress but also showed enhanced resistance to acid stress, a condition frequently encountered in industrial fermentations. Wild-type cells were almost completely inhibited for growth in medium at pH 2.2 and this inhibition was further enhanced by the addition of 0.5 M lactic acid to the medium. On the contrary, strains engineered to synthesise AsA continued to grow well in acid media supplemented with 0.5 M lactate [56]. Several claims were therefore made for the engineered yeast cells, firstly that they could be used for the industrial synthesis of AsA and secondly that they could be used to increase production, productivity or yield of any other product by virtue of their ability to withstand osmotic, pH, temperature or oxidative stress [29].

An alternative approach to induce L-Gal synthesis in yeast used bacterial enzymes to convert the readily available substrate L-sorbose. In this application, the broad substrate

specificity of bacterial sugar metabolising enzymes was exploited to synthesise L-Gal in a two step pathway. D-Tagatose epimerase isolated from *Pseudomonas* sp. is known to use not only D-tagatose but also D-fructose, L-psicose and L-sorbose as substrates [59]. A homologous sequence (AE008210) was cloned from *Agrobacterium tumefaciens* and the expressed protein was able to convert L-sorbose to L-tagatose *in vitro* [60]. L-Fucose isomerase (E.C. 5.3.1.25) from *Escherichia coli* also has broad substrate specificity being capable of the isomerisation of not only L-fucose and L-fuculose but also the structurally similar 5-carbon analogues D-arabinose and D-ribulose [61]. As L-fucose is 6-deoxy-L-Gal, it was postulated that the same enzyme would also be capable of the isomerisation of L-tagatose and L-Gal and a recombinant L-fucose isomerase from *E. coli* (NC000913) was observed to undertake this reaction *in vitro* [60]. Co-cultivation of the purified enzymes, *S. cerevisiae* cells and L-sorbose resulted in the synthesis of AsA via the pathway shown in Fig. (**3**) [60] however, attempts to synthesise AsA from L-sorbose in yeast cells engineered to express the two enzymes were unsuccessful. One possible explanation is that the low enzyme specificity prevented conversion of the desired substrates as a result of competition for active sites from endogenous metabolites. Future process improvements might include protein engineering to improve enzyme specificity or the development of yeast strains in which pathways producing competing metabolites are blocked.

PATHWAY ENGINEERING IN BACTERIA - EFFICIENT SINGLE STEP PROKARYOTIC MANUFACTURE OF VITAMIN C

Bacterial transformations have been integral to industrial AsA synthesis since the earliest days and a great number of bacterial strains have been used to produce AsA intermediates, frequently in mixed or sequential cultures [11]. Industrial prokaryotes were generally not considered capable of synthesising AsA directly and so strains were engineered for the rapid and efficient production of AsA precursors in single stage fermentations. Many of these developments have been discussed in recent reviews [7, 10, 11, 62] and here the focus will be on the latest developments for the direct single-stage fermentation of AsA in prokaryotic microorganisms.

The genus *Gluconobacter* and related organisms have been valued in the biotechnology industry for many years. One of the primary features pertaining to their utility is the wide array of both periplasmic and intracellular dehydrogenases which are used in their sugar-rich natural environments (flowers and fruit) for the partial oxidation of sugars and polyols which are then frequently returned to the environment inhibiting the growth of competitor organisms [63]. In industrial AsA synthesis, *G. oxydans* initially found use within the Reichstein process for the oxidation of D-sorbitol to L-sorbose Fig. (**1**) and further developments allowed the direct fermentation of 2-keto-L-gulonic acid (2-

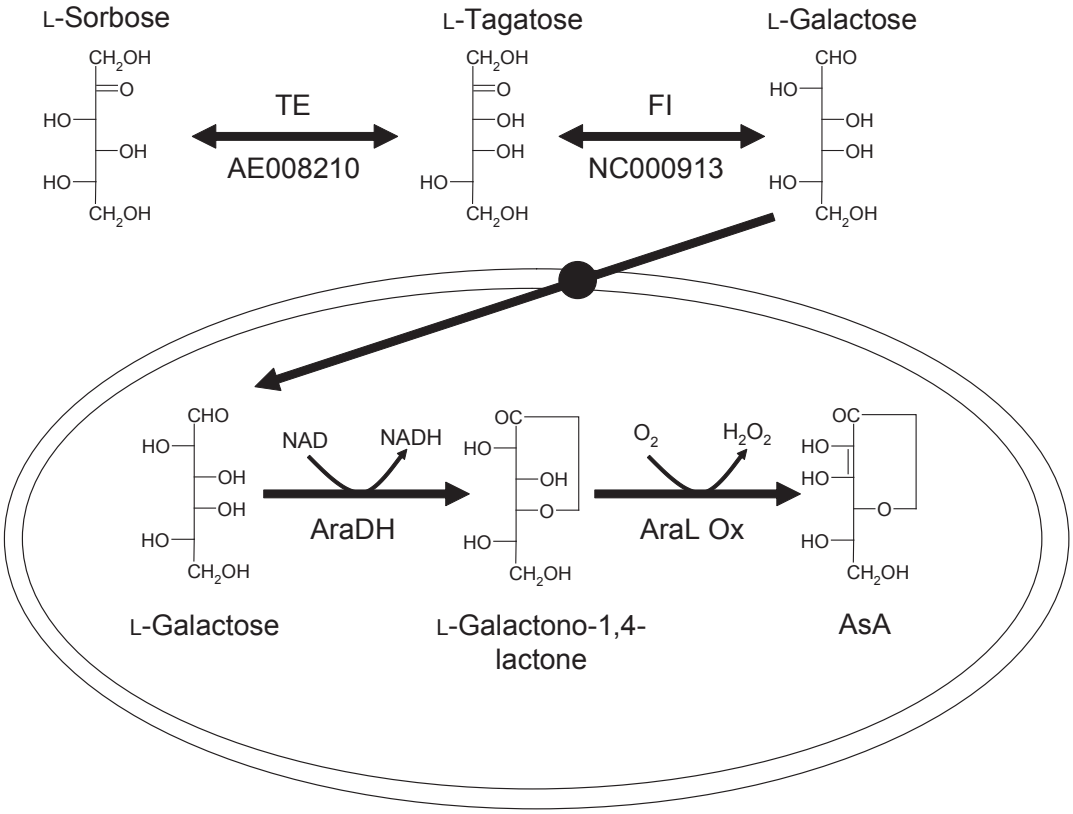

Fig. (3). Proposed mechanism of synthesis of AsA using partially purified enzymes and yeast cells.
L-Sorbose is sequentially isomerised to L-galactose by the action of recombinant tagatose epimerase (TE, NCBI accession number AE008210) and fucose isomerase (FI, NC000913). L-Galactose is then taken up by intact yeast cells via a sugar transporter (●) prior to conversion to AsA by the endogenous enzymes arabinose dehydrogenase (AraDH) and arabinonolactone oxidase (AraL Ox).

KLG); which is transformed to AsA in a single chemical step, from sorbitol [64]. Process efficiency can be reduced through the transport of partially oxidised substrates across the plasma membrane resulting in their channelling into central metabolism via the pentose phosphate pathway Fig. (**4**).

In 1995, it was observed that a particular strain of *G. oxydans* (later renamed *Ketogulonigenium vulgare* DSM 4025) [65] commonly used to produce 2-KLG carried an enzyme capable of oxidising L-GulL to AsA [66]. The organism produced AsA yields of approximately 15% from L-GulL in resting cell systems however as a method for the industrial synthesis of AsA was not cost effective due to difficulties in obtaining the reaction substrate. Subsequent analysis of the biosynthetic capacities of the same strain revealed that it was not only capable of AsA synthesis from L-GulL but also from D-sorbitol, L-sorbose and L-sorbosone the substrates usually supplied for the production of 2-KLG [67]. Yields varied with L-sorbosone providing 1.04 g L^{-1} AsA following 24 h incubation from a 0.5% feedstock, L-sorbose providing 0.87 g L^{-1} from an 8% feedstock and D-sorbitol providing 90 mg L^{-1} also from an 8% feedstock. Significant quantities of 2-KLG were also produced when sorbosone was supplied as a substrate however, 2-KLG was not converted to AsA as no AsA synthesis was observed when 2-KLG was supplied. *K. vulgare* and related genera such as *Gluconobacter* and *Acetobacter* contain sorbitol and sorbose dehydrogenases capable of sequentially oxidising D-sorbitol to L-sorbosone [7]. It is well established that they also contain sorbosone dehydrogenases capable of the conversion of L-sorbosone to 2-KLG. The sorbosone dehydrogenases from *K. vulgare* DSM 4025 were purified and three enzymes capable of the pyrroloquinoline quinine (PQQ) dependent conversion of L-sorbosone directly to AsA were identified following native polyacrylamide gel electrophoresis [68]. All three enzymes were made up of homo- or mixed oligomers of 75 or 55 kDa subunits encoded by full length or truncated expression of the same gene, *sndH*. The enzymes were also capable of the conversion of L-sorbosone to 2-KLG although the pH optimum for AsA production was lower than that for 2-KLG production at pH 7-8 and greater than pH 8.5, respectively. The use of the nucleotide sequence, purified proteins or recombinant organisms for the production of AsA were protected [69].

The finding that periplasmic PQQ-dependent L-sorbose dehydrogenases are capable of producing not only 2-KLG but also AsA has led to a great deal of additional research and patent activity primarily by the Dutch company DSM [70, 71]. The main focus has been gene discovery and the improvement of *Gluconobacter*, *Acetobacter* and *Ketogulonicigenium* strains via up- or down-regulation of genes involved in sorbitol/sorbose metabolism (*sms* genes) or sugar transport systems (*sts* genes). In addition, the impact of changing expression of genes involved in the synthesis of respiratory chain enzymes or cofactors (*rcs* genes) or those described as being vitamin C production system (*vcs* genes) was investigated. The latter included a diverse array of genes encoding enzymes (e.g. GDP-mannose pyrophosphorylase) and regulatory genes (e.g. transcriptional regulatory protein OmpR).

Untransformed bacterial strains were capable of synthesis of AsA from L-sorbosone in stationary culture over a period of 20 h with up to 1.74 g L^{-1} AsA accumulation from 10 g L^{-1} sorbosone in the strain *G. oxydans* IFO 3293 (Table **1**). Southern probes designed using the *G. oxydans* DSM 17078 *sndH* gene hybridised with all of the AsA producing strains

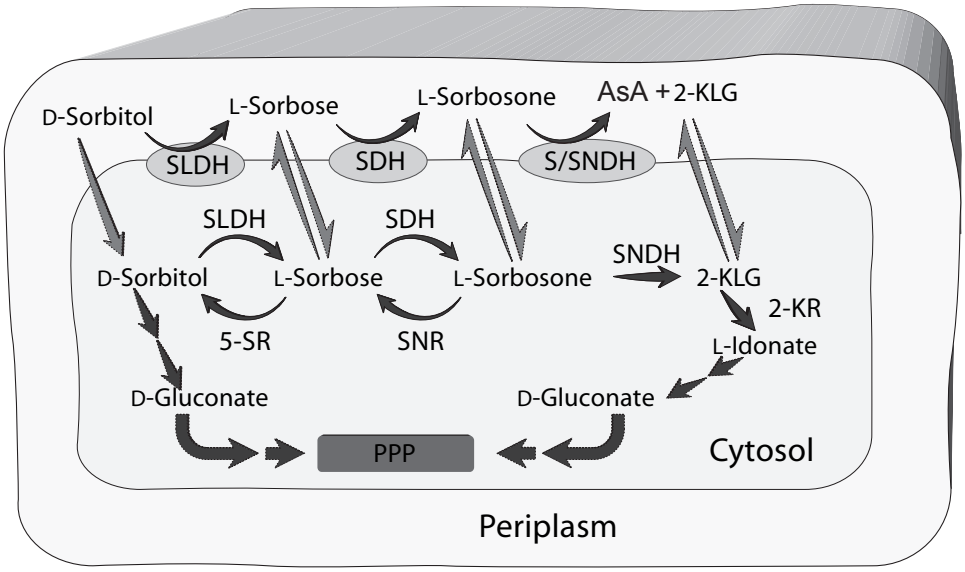

Fig. (4). Proposed pathways of sorbitol metabolism in *Gluconobacter oxydans.*
D-Sorbitol may be oxidised via a series of periplasmic dehydrogenases to yield AsA and 2-keto-L-gulonate (2-KLG). *G. oxydans* and other acetic acid bacteria also contain a series of cytoplasmic dehydrogenases and reductases capable of the interconversion of D-sorbitol, L-sorbose and L-sorbosone. Cytoplasmically localised L-sorbosone may enter central metabolism via a cytoplasmic sorbosone dehydrogenase that is not known to produce AsA. Alternatively, D-sorbitol may enter central metabolism via D-gluconate. SLDH, D-sorbitol dehydrogenase; SDH, L-sorbose dehydrogenase; SNDH, L-sorbosone dehydrogenase; SR, D-sorbitol reductase; SNR, L-sorbose reductase; KR, ketogulonate reductase; PPP, pentose phosphate pathway.

with the exception of the *A. aceti* strains which also failed to produce products in PCR reactions using primers designed to the DSM 17078 gene (Table **1**) suggesting broad diversity in AsA producing sorbosone dehydrogenase enzymes. In a further series of production experiments, resting cells of strain DSM 17078 synthesised 1.8, 2.0 and 5.1 g L^{-1} AsA from D-sorbitol, L-sorbose or L-sorbosone, respectively in 48 h starting from 20 g L^{-1} of the appropriate substrate.

To further improve process efficiency strain DSM 17078 underwent a series of genetic modifications. Increasing the gene dosage of *sndH* through the introduction of *sndH* bearing plasmids into the parental strain almost doubled AsA production in a resting cell assay using 1% L-sorbosone where the parental strain produced 2.5 g L^{-1} while three independent transformants produced 4.2, 4.2 and 4.1 g L^{-1}, respectively. Strains transformed with the *sndH* gene were used to demonstrate the AsA production process at the laboratory scale. Cells were grown in a 1.25 L growth vessel in a medium containing 100 g L^{-1} sorbitol, yeast extract and salts to reach OD 600nm of 20 after which further medium was pumped into the growth vessel and cell culture transferred to a second vessel containing 5 L of production medium (100 g L^{-1} sorbitol, 0.3 g L^{-1} NaCl, 0.12 g L^{-1} $CaSO_4$) at a rate of 125 mL h^{-1}. Additional production medium was added to allow continuous harvesting from the production vessel at a rate of 500 mL h^{-1} which was fed through a crossflow ultrafiltration module with cells returned to the production vessel until an OD 600 nm of 100 was achieved. At this stage, AsA could be harvested from the production vessel at a rate of 2 g h^{-1}. Further downstream processing included electrodialysis, treatment with chelating, anion and cation exchange resins, removal of impurities with activated carbon and crystallisation producing 98% pure free AsA with an 80% yield in the purification steps [71].

For maximum conversion efficiency, an entirely periplasmic sorbitol oxidation pathway is desirable Fig. (**4**) and transfer of reaction intermediates (L-sorbose, L-sorbosone) to the cytoplasm has detrimental effects on product yield therefore efforts were made to isolate and clone genes encoding sugar transporters (*sts* genes). One of the isolated genes, named *sts24*, had sequence homology to the P-loop ATPase of phosphotransferase systems. Disruption of the gene in *G. oxydans* DSM 17078 resulted in improved AsA production from D-sorbitol, L-sorbose or L-sorbosone in resting cell systems with the parental strain producing 145, 212 and 1130 mg L^{-1} AsA from the respective substrates while the strain carrying the gene disruption produced 1070, 1470 and 3230 mg L^{-1} AsA, respectively. The relatively higher improvement in AsA production from the former substrates suggests that phosphotransferase systems may be involved in transport of all three substrates across the cytoplasmic membrane into the cell preventing their periplasmic conversion to AsA. Disruption of *sts24* was a more effective method for improvement of AsA production than increasing gene dosage of *sndH* however, combination of both mutations resulted in even higher AsA production Table **2**. A second disruption in the sugar transport system was shown to be effective for improving AsA production. In this case *sts01*, a D-arabinitol transporter was upregulated by engineering the endogenous gene with a strong constitutive promoter. Strains carrying this mutation in combination with enhanced *sndH* expression produced 20% more AsA than strains carrying the enhanced *sndH* expression alone when resting cells were incubated with sorbitol [71], presumably as a result of improved export of sorbitol or other downstream intermediates from the cytoplasm back to the periplasm.

An alternative to maximising substrate partitioning to the periplasmic space is to downregulate cytoplasmic pathways leading into central metabolism. One major entry point for L-sorbosone into the pentose phosphate pathway is via cytoplasmic sorbosone dehydrogenase Fig. (**4**). The gene for a NAD(P)-dependent sorbosone dehydrogenase (*sms05*) was disrupted in *G. oxydans* DSM 17078 and the capacity of the

Table 1. Characteristics of AsA Synthesising Bacterial Strains

Species	Strain	AsA in Medium (mg L^{-1})	Southern Hybridisation	PCR Product
Gluconobacter oxydans	IFO 3293	1740	Yes	Yes
	IFO 3244	1280	Yes	Yes
	DSM 17078	570	N/A	N/A
	IFO 3292	410	Yes	Yes
Acetobacter sp.	ATCC 15164	310	Yes	Yes
G. cerinus	IFO 3266	140	Weak	No
A. aceti subsp. *xylinum*	IFO 13693	120	No	No
G. oxydans	IFO 3287	60	Yes	Yes
G. frateurii	IFO 3260	50	Weak	No
A. aceti subsp. *orleanus*	IFO 3259	30	No	No

For the analysis of AsA biosynthesis, cells were suspended at a density of 10 absorbance units at 600 nm in 0.5 mL 1% L-sorbosone, 0.3% NaCl and 1% $CaCO_3$. Incubation was continued for 20 h in 5 mL tubes with shaking at 200 rpm and AsA quantified by HPLC. Southern hybridisation and PCR reactions were undertaken using Southern probes or PCR primers designed using the DSM 17078 *sndH* gene as template. All data obtained from Beuzelin-Ollivier et al. 2006 [71]. N/A - not applicable.

Table 2. Impact of Genetic Manipulation on AsA Production in *Gluconobacter oxydans*

			AsA in medium (mg L^{-1}) from		
Strain	**Culture**	**Incubation**	**D-Sorbitol**	**L-Sorbose**	**L-Sorbosone**
17078	Resting	20 h	180	360	2050
17078/*sndH*↑			750	760	2890
17078/*sts24*↓			1460	1330	5210
17078/*sndH*↑/*sts24*↓			2400	2080	6770
17078	Resting	20 h	270	670	
17078/*sms05*↓			1540	1990	
17078	Resting	20 h			1300
17078/*sms05*↓					1800
17078/*sndH*↑/*sms05*↓					6100
17078	Growing	48 h	60		
17078/*sndH*↑			120		
17078/*sms05*↓			260		
17078/*sndH*↑/*sms05*↓			320		
17078	Resting	20 h	145	212	1130
17078/*vcs01*↓			1490	2220	2830
17078	Resting	20 h	240		
17078/*sndH*↑			640		
17078/*sndH*↑/*vcs01*↓			1100		
17078	Resting	20 h	180	360	2050
17078/*sndH*↑			750	760	2890
17078/*vcs08*↓			650	1050	3810
17078/*sndH*↑/*vcs08*↓			1900	2440	6640

Cells of *G. oxydans* DSM 17078 or derivative strains following genetic manipulation were cultured for the time period shown in either resting or growing culture systems. Up or downregulation of gene expression is indicated by up or down arrows. Individual experiments are separated by horizontal lines. Gene notations were *sndH*, PQQ-dependent sorbosone dehydrogenase; *sts24*, P-loop ATPase protein family involved in phosphotransferase system; *sms05*, NAD(P)-dependent sorbosone dehydrogenase; *vcs01*, mannose-1-phosphate guanyltransferase/phosphomannose isomerase; *vcs08*, glutamine-hydrolysing asparagine synthase. All data from Beuzelin Ollivier et al. 2006 [71].

mutant strain to synthesise AsA from 2% D-sorbitol or L-sorbose was assessed following 20 h in resting culture. Under these conditions, the strain containing the disrupted *sms05* gene produced 1540 and 1990 mg L^{-1} AsA from the two substrates, respectively compared with only 270 and 670 mg L^{-1} AsA in strain DSM 17078 Table **2**. In a further series of experiments, gene disruption of *sms05* in a background of overexpressing *sndH* provided further increases in product recovery either from L-sorbosone in resting culture or from D-sorbitol in actively growing culture Table **2**.

As the sorbosone dehydrogenase responsible for AsA synthesis was PQQ-dependent, efforts were made to boost the availability of this cofactor through overexpression of the *rcs21* gene, annotated as PQQ biosynthesis protein A. This mutation was reported to increase AsA synthesis from D-sorbitol in a resting cell system by a further 20% when expressed in a background of upregulated *sndH* [71], perhaps suggesting cofactor limitation, at least in strains overexpressing *sndH*.

While the mutations outlined above might perhaps be rationally chosen in a process improvement programme, the improvements conferred from altering expression of the *vcs* genes are more difficult to predict. Two examples were provided in which genes encoding metabolic enzymes were disrupted. In the first the impact of disrupting a gene encoding mannose-1-phosphate guanyltransferase/ phosphomannose isomerase (*vcs01*) was shown to have positive impacts on AsA synthesis in *G. oxydans* DSM 17078 with resting cells carrying the *vcs01* disruption producing at least twice as much AsA from D-sorbitol, L-sorbose or L-sorbosone Table **2**. Again, concurrent disruption of *vcs01* with overexpression of *sndH* resulted in even higher product

recoveries. Why the disruption of an enzyme involved in cell wall biosynthesis and protein glycosylation would enhance AsA synthesis is difficult to imagine. Similarly, disruption of *vcs08*, a gene encoding glutamine hydrolysing asparagine synthase had strong positive impacts on AsA synthesis from D-sorbitol, L-sorbose and L-sorbosone in resting cell cultures Table **2**. Again, why manipulation of a gene encoding an enzyme involved in primary metabolic pathways would impact on a periplasmic AsA biosynthetic pathway is difficult to imagine.

In the last few years, DSM have filed in excess of 30 patents relating to AsA synthesis in acetobacteria and perhaps the fact that they are the only remaining Western producer of industrial vitamin C is testament to the success of such an aggressive research and development strategy. Their patenting strategy has covered the fermentative production of vitamin C from the D-sorbitol metabolisation pathway, the use of the *sndH* gene or the protein that it encodes in addition to in excess of 50 genes in the SMS, STS, RCS and VCS systems. The company have also specifically covered the use of a broad range of bacterial genera including *Pseudomonas*, *Pantoea*, *Escherichia*, *Corynebacterium*, *Ketogulonicigenium*, *Gluconobacter*, *Acetobacter* and *Gluconacetobacter*.

CURRENT & FUTURE DEVELOPMENTS

Over the last decade several fundamental findings have translated into intellectual property with a very real possibility for their future exploitation. The discovery of the primary AsA biosynthetic pathway in plants as well as several other potential routes to its synthesis has created the opportunity for the development of second generation biotech crops with enhanced nutritional value and potentially improved agronomic performance. Given that several global agbiotech companies such as Syngenta, Monsanto and Dupont are already involved in the commercial development of crops with improved nutritional traits [72], it seems likely that the high consumer recognition of vitamin C will encourage the development of such crops in the near future.

Market conditions for the industrial synthesis of vitamin C have been difficult over the past decade with exceptionally low prices and flooding of global markets with vitamin C produced in China. Low employment costs and undoubted innovation within the manufacturing plants along with state subsidies have allowed the production of vitamin C within China at costs all but impossible for Western manufacturers to compete with and the result has been that only one vitamin C manufacturing plant remains in the West located in Dalry, Scotland. Perhaps in part as a result of overseas competition, the parent company of the manufacturing plant DSM, have invested heavily in process improvement primarily through single-step bacterial fermentations. Although much of the underlying development work is now in the public domain in the form of international patents, it is difficult to obtain information regarding how far these processes have gone into commercial production.

ACKNOWLEDGEMENTS

Ascorbic acid research in the author's laboratory has been supported by GlaxoSmithKline, the UK Biotechnology and Biological Sciences Research Council, the Scottish Government Rural and Environmental Research and Analysis Directorate, the UK Horticultural Development Council and the Scottish Enterprise Proof of Concept Fund. Figure **4** is a modified version of a figure that originally appeared in an article by the same author in Trends in Biotechnology, reprinted here with permission.

REFERENCES

[1] Marz U. World markets for citric, ascorbic, isoascorbic acids: Highlighting antioxidants in food. Business Communications Company Inc. Report number FOD031A 2002.
[2] Chauhan AS, Ramteke RS, Eipeson WE. Properties of ascorbic acid and its applications in food processing: A critical appraisal. J Food Sci Technol Mysore 1998; 35(5): 381-392.
[3] Reichstein, T.: GB425198 (**1935**).
[4] Reichstein, T.: GB428814 (**1935**).
[5] Reichstein, T.: GB427286 (**1935**).
[6] Haworth, W.N., Hirst, E.L., Jones, J.K.N., Smith, F.: GB443901 (**1936**).
[7] Hancock RD, Viola R. Biotechnological approaches for L-ascorbic acid production. Trends Biotechnol 2002; 20(7): 298-305.
[8] Scherer R, Ford P. China's grip on key food additive. Christian Science Monitor 2007, July 20. Accessed online, http: //www.csmonitor.com/2007/0720/p01s01-woap.html 12 August 2008.
[9] Wheeler GL, Jones MA, Smirnoff N. The biosynthetic pathway of vitamin C in higher plants. Nature 1998; 393(6683): 365-369.
[10] Bremus C, Herrmann U, Bringer-Meyer S, Sahm H. The use of microorganisms in L-ascorbic acid production. J Biotech 2006; 124(1): 196-205.
[11] Hancock RD, Viola R. The use of microorganisms for L-ascorbic acid production: Current status and future perspectives. Appl Microbiol Biotechnol 2001; 56(5-6): 567-576.
[12] Hancock RD, Viola R. Biosynthesis and catabolism of L-ascorbic acid in plants. Crit Rev Plant Sci 2005; 24(3): 167-188.
[13] Ishikawa T, Dowdle J, Smirnoff N. Progress in manipulating ascorbic acid biosynthesis and accumulation in plants. Physiol Plant 2006; 126(3): 343-355.
[14] Zhang L, Wang W, Xia Y, Kai G, Chen W, Tang K. Metabolic engineering of plant L-Ascorbic acid biosynthesis: recent trends and applications. Crit Rev Biotechnol 2007; 27(3): 173-182.
[15] Agius F, González-Limothe R, Caballero JL, Muñoz-Blanco J, Botella MA, Valpuesta V. Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nature Biotechnol 2003; 21(2): 177-181.
[16] Lorence A, Chevone BI, Mendes P, Nessler CL. Myo-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. Plant Physiol 2004; 134(3): 1200-1205.
[17] Wolucka BA, van Montagu M. GDP-Mannose 3',5'-Epimerase forms GDP-L-gulose, a putative intermediate for the *de novo* biosynthesis of vitamin C in plants. J Biol Chem 2003; 278(48): 47483-47490.
[18] Dowdle J, Ishikawa T, Gatzek S, Rolinski S, Smirnoff N. Two genes in *Arabidopsis thalina* encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. Plant J 2007; 52(4): 673-689.
[19] DeBolt S, Cook DR, Ford CM. L-Tartaric acid synthesis from vitamin C in higher plants. Proc Natl Acad Sci USA 2006; 103(14): 5608-5613.
[20] Green MA, Fry SC. Vitamin C degradation in plant cells via enzymatic hydrolysis of 4-O-oxalyl-L-threonate. Nature 2005; 433(7021): 83-87.
[21] DeBolt, S., Cook, D.R., Ford, C.M.: WO07006087 (**2007**).
[22] Mapson LW, Isherwood FA, Chen YT. Biological synthesis of ascorbic acid: The conversion of L-Galactono-γ-lactone into L-ascorbic acid by plant mitochondria. Biochem J 1954; 56(1): 21-28.
[23] Isherwood FA, Chen YT, Mapson LW. Synthesis of L-ascorbic acid in plants and animals. Biochem J 1954; 56(1): 1-15.
[24] Loewus FA. Tracer studies on ascorbic acid formation in plants. Phytochem 1963; 2(2): 109-128.
[25] Saito K, Nick JA, Loewus FA. D-glucosone and L-sorbosone, putative intermediates of L-ascorbic acid biosynthesis in detached bean and spinach leaves. Plant Physiol 1990; 94(3): 1496-1500.
[26] Smirnoff, N., Wheeler, G.L.: WO9933995 (**1999**).

[27] Gatzek S, Wheeler GL, Smirnoff N. Antisense suppression of L-Galactose dehydrogenase in *Arabidopsis thaliana* Provides Evidence for its role in ascorbate synthesis and reveals light modulated L-galactose synthesis. Plant J 2002; 30(5): 541-553.
[28] Smirnoff, N., Wheeler, G.L.: US20040053235 (**2004**).
[29] Branduardi, P., Porro, D., Sauer, M., Mattanovich, D.: WO06113147 (**2006**).
[30] Barber GA. The synthesis of L-glucose by plant enzyme systems. Arch Biochem Biophys 1971; 147(2): 619-623.
[31] Barber GA. Observations on the mechanism of reversible epimerization of GDP-D-mannose to GDP-L-galactose by an enzyme from *Chlorella pyrenoidosa*. J Biol Chem 1979; 254(16): 7600-7603.
[32] Wolucka BA, Persiau G, van Doorsselaere J, *et al*. Partial Purification and Identification of GDP-mannose 3,5-epimerase of *Arabidopsis thaliana*, a key enzyme of the plant vitamin C pathway. Proc Natl Acad Sci USA 2001; 98(26): 14843-14848.
[33] Wolucka, B.: WO02103001 (**2002**).
[34] Herold A, Lewis DH. Mannose and green plants: Occurrence, physiology and metabolism, and use as a tool to study the role of orthophosphate. New Phytol 1977; 79(1): 1-40.
[35] Wolucka, B.: US20060156431 (**2006**).
[36] Mapson LW, Isherwood FA. Biological synthesis of ascorbic acid: the conversion of derivatives of D-galacturonic acid into L-ascorbic acid by plant extracts. Biochem J 1956; 64(1): 13-22.
[37] Agius-Guadalupe, M.F., Botella-Mesa, M.A., Valpuesta-Fernandez, V.: US20050150001 (**2005**).
[38] Conklin PL, Barth C. Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ 2004; 27(8): 959-970.
[39] Huang C, He W, Guo J, Chang X, Su P, Zhang L. Increased sensitivity to salt stress in an ascorbate-deficient *Arabidopsis* mutant. J Exp Bot 2005; 56(422): 3041-3049.
[40] Müller-Moulé P, Golan T, Niyogi KK. Ascorbate-deficient mutants of arabidopsis grow in high light despite chronic photooxidative stress. Plant Physiol 2004; 134(3): 1163-1172.
[41] Barth C, Moeder W, Klessig DF, Conklin PL. The timing of senescence and response to pathogens is altered in the ascorbate-deficient Arabidopsis mutant *vitamin C-1*. Plant Physiol 2004; 134(4): 1784-1792.
[42] Hancock RD, Chudek JA, Walker PG, Pont SDA, Viola R. Ascorbic acid conjugates isolated from the phloem of *Cucurbitaceae*. Phytochem 2008; 69(9): 1850-1858.
[43] Davey MW, Gilot C, Persiau G *et al*. Ascorbate biosynthesis in arabidopsis cell suspension culture. Plant Physiol 1999; 121(2): 535-543.
[44] Zhang W, Gruszewski HA, Chevone BI, Nessler CL. An arabidopsis purple acid phosphatase with phytase activity increases foliar ascorbate. Plant Physiol 2008; 146(2): 431-440.
[45] Nessler, C.L., Lorence, A., Chevone, B., Mendes, P.: WO06104503 (**2006**).
[46] Gallie, D.R.: US20030215949 (**2003**).
[47] Chen Z, Young TE, Ling J, Chang S-C, Gallie DR. Increasing vitamin C content of plants through enhanced ascorbate recycling. Proc Natl Acad Sci USA 2003; 100(6): 3525-3530.
[48] Chen Z, Gallie DR. The ascorbic acid redox state controls guard cell signaling and stomatal movement. Plant Cell 2004; 16(5): 1143-1162.
[49] Saito K, Kasai Z. Synthesis of L-(+)-Tartaric acid from L-ascorbic acid via 5-keto-D-gluconic acid in grapes. Plant Physiol 1984; 76(1): 170-174.
[50] Foyer CH, Noctor G. Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. Plant Cell 2005; 17(7): 1866-1875.
[51] Murakawa S, Sano S, Yamashita H, Takahashi T. Biosynthesis of D-erythroascorbic acid by *Candida*. Agric Biol Chem 1977; 41(9): 1799-1800.
[52] Huh W-K, Kim S-T, Yang K-S, Seok Y-J, Hah Y-C, Kang S-O. Characterization of D-arabinono-1,4-lactone oxidase from *Candida albicans* ATCC 10231. Eur J Biochem 1994; 225(3): 1073-1079.
[53] Kim S-T, Huh W-K, Kim J-Y, Hwang S-W, Kang S-O. D-arabinose dehydrogenase and biosynthesis of erythroascorbic acid in *Candida albicans*. Biochim Biophys Acta 1996; 1297(1): 1-8.
[54] Kim S-T, Huh W-K, Lee B-H, Kang S-O. D-arabinose dehydrogenase and its gene from *Saccharomyces cerevisiae*. Biochim Biophys Acta 1998; 1429(1): 29-39.
[55] Hancock RD, Galpin JR, Viola R. Biosynthesis of L-ascorbic acid (vitamin C) by *Saccharomyces cerevisiae*. FEMS Microbiol Lett 2000; 186(2): 245-250.
[56] Branduardi P, Fossati T, Sauer M, Pagani R, Mattanovich D, Porro D. Biosynthesis of vitamin C by yeast leads to increased stress resistance. PLOS One 2007; 2(10): e1092.
[57] Laing WA, Wright MA, Cooney J, Bulley SM. The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-Galactose guanyltransferase, increases leaf ascorbate content. Proc Natl Acad Sci USA 2007; 104(22): 9534-9539.
[58] Laing WA, Bulley S, Wright M, *et al*. A Highly specific L-galactose-1-phosphate phosphatase on the path to ascorbate biosynthesis. Proc Natl Acad Sci USA 2004; 101(48): 16976-16981.
[59] Itoh H, Izumori K. Enzymatic production of L-tagatose and L-fructose from L-sorbose and L-psicose, respectively. J Ferm Bioeng 1996; 81(4): 351-353.
[60] Hancock, R.D., Hunter, G.D., Shaw, J., Viola, R.: WO06134374 (**2006**).
[61] Green M, Cohen SS. Enzymatic conversion of L-fucose to L-fuculose. J Biol Chem 1956; 219(2): 557-568.
[62] Survase SA, Bajaj IB, Singhal RS. Biotechnological production of vitamins. Food Technol Biotechnol 2006; 44(3): 381-396.
[63] Prust C, Hoffmeister M, Liesegang H, *et al*. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nature Biotechnol 2005; 23(2): 195-200.
[64] De Muynck C, Pereira CSS, Naessens M, Parmentier S, Soetaert W, Vandamme EJ. The Genus *Gluconobacter oxydans*: Comprehensive overview of biochemistry and biotechnological applications. Crit Rev Biotechnol 2007; 27(3): 147-171.
[65] Urbance JW, Bratina BJ, Stoddard SF, Schmidt TM. Taxonomic characterization of *Ketogulonigenium vulgare* gen. nov., sp. nov. and *Ketogulonigenium robustum* sp. nov., which oxidize L-sorbose to 2-keto-L-gulonic acid. Int J Systematic Evol Microbiol 2001; 51(3): 1059-1070.
[66] Sugisawa T, Ojima S, Matzinger PK, Hoshino T. Isolation and characterization of a new vitamin C producing enzyme (L-gulono-γ-lactone dehydrogenase) of bacterial origin. Biosci Biotechnol Biochem 1995; 59(2): 190-196.
[67] Sugisawa T, Miyazaki T, Hoshino T. Microbial production of L-ascorbic acid from D-sorbitol, L-Sorbose, L-gulose, and L-sorbosone by *Ketogulonicigenium vulgare* DSM 4025. Biosci Biotechnol Biochem 2005; 69(3): 659-662.
[68] Miyazaki T, Sugisawa T, Hoshino T. Pyrroloquinoline quinone-dependent dehydrogenases from *Ketogolonicigenium vulgare* catalyze the direct conversion of L-sorbosone to L-ascorbic acid. Appl Environ Micobiol 2006; 72(2): 1487-1495.
[69] Hoshino, T., Miyazaki, T., Sugisawa, T.: US20060127994 (**2006**).
[70] Berry, A., Lee, C., Mayer, A.F., Shinjoh, M.: US20070184534 (**2007**).
[71] Beuzelin Ollivier, M-G., Chevreux, B., Dalluege, M., Gelder, VM., Goese, M., Hauk, C., Koekman, B.P., Lee, C., Mayer, A.F., Meury, A., Mouncey, N.J., Schipper, D., Shinjoh, M., Toepfer, C., Vollebregt, A.W.: WO06084719 (**2006**).
[72] Yonekura-Sakakibara K, Saito K. Review: Genetically modified plants for the promotion of human health. Biotechnol Lett 2006; 28(12): 1983-1991.