

# Tracking the right strain with the aid of a parallel bioreactor

Many companies have expressed great interest in the fermentative production of vitamin C. Researchers at the Institute of Biotechnology I in Juelich are therefore working on recombinant *Gluconobacter oxydans* strains for the microbial conversion of sorbitol to vitamin C, and on the characterisation of sorbitol/sorbose metabolism. A parallel bioreactor is supporting the Research Centre of Juelich in its efforts to find a bacterial strain for the fermentative production of vitamin C.

Vitamin C (L-ascorbic acid) is an essential nutrient component for human beings and a number of other mammals. Because of its high reductive power, it serves as a free radical scavenger, an antioxidant in the cell and as a redox buffer in blood. Besides its significance for the immune system and for signal transduction, vitamin C is also an essential co-factor in collagen synthesis and therefore necessary for the genesis and repair of various tissues and bone

material. Vitamin C deficiency results in diseases such as scurvy. About 50 percent of commercially produced L-ascorbic acid is used in pharmaceutical products. These include lotions for skin care and the treatment of burns as well as drugs to prevent degenerative diseases, infections and cancer. Commercially produced Vitamin C is also used as an antioxidant, a preserving and acidifying agent in the food and beverage industries (25 percent and 15

percent respectively) and in the animal feed industry (10 percent). The result is a steadily growing market with 110,000 tonnes of Vitamin C produced per year and an annual turnover of over US\$ 600 million.

While in China most commercially produced vitamin C is extracted from citrus fruits, European companies, such as Roche, BASF and Takeda manufacture a large part of commercially produced vitamin C from D-glucose using the Reichstein process. Because of the high temperature and pressure demanded by some of the chemical steps involved, there is great interest in a biotechnological process. In the past 20 years numerous attempts to produce vitamin C or the vitamin C precursor 2-keto-L-gulonic acid (2-KGLA) microbially, have been initiated. Besides *Acetobacter*, *Ketogulonigenium*, *Pseudomonas*, *Erwinia* and *Corynebacterium*, it is *Gluconobacter oxydans*, above all, that appears to be the most promising organism to use for the fermentative production of 2-KGLA.

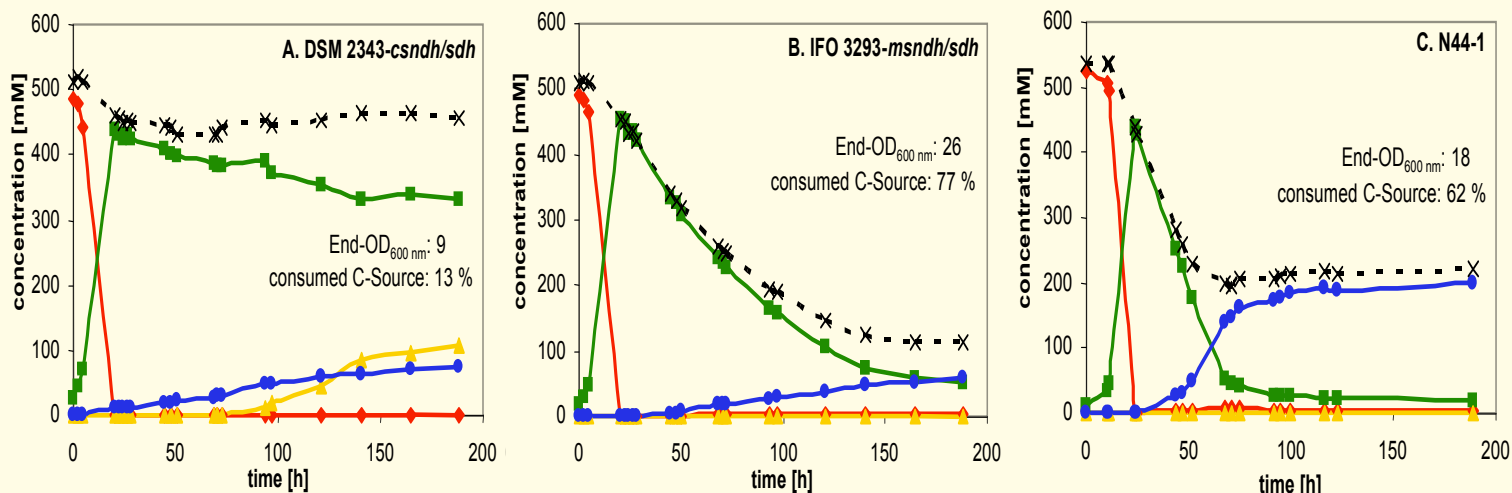


Figure 1. Substrate conversion, End-OD and C-source assimilation of various strains within a cultivation using 100 g/L (550 mM) Sorbitol in DASGIP Parallel Bioreactor System. Sorbitol-, Sorbose-, Sorbosone- and 2-KGLA concentrations were summed up to the overall C-Source concentration ( $\Sigma$  C-Source), because no other products could be detected via HPLC.

The gram-negative and obligate aerobic *G. oxydans* is a member of the *Acetobacteriaceae*. The natural habitats of *G. oxydans* include fruit, blossom nectar, wine and beer. While most aerobic bacteria metabolise substrates entirely to form H<sub>2</sub>O and CO<sub>2</sub> and only accumulate intermediate products under limiting conditions, *G. oxydans* only oxidises Carbon sources (C-sources) partially, and accumulates intermediate products due to an incomplete citrate cycle. As a result, fewer reduction equivalents (NADH, FADH) are available for the energy production via oxidative phosphorylation. In *G. oxydans* this is compensated for by a huge number of dehydrogenases (>20 sugar/polyol dehydrogenases, >30 alcohol dehydrogenases and >80 dehydrogenases/reductases with unknown functions), since the electrons generated from the oxidation reactions flow directly into the respiratory chain via Pyrroloquinolin-Quinone (PQQ) or FAD and contribute to energy production. The large number of stereo- and regio-selective dehydrogenases, which effectively oxidise many sugars, sugar alcohols and alcohols, make *G. oxydans* a very interesting organism for the biotechnical production of, for example, vinegar, flavouring agents, sweeteners, dihydroxyacetone, gluconate, tartaric acid and the diabetes drug Miglitol.

The microbial synthesis of the vitamin C precursor 2-KGLA can be carried out in *G. oxydans* via the so-called "L-sorbosone pathway" [1]. Sorbitol is oxidised to

2-KGLA via the intermediates sorbose and sorbosone. These reactions are catalysed by a membrane-associated sorbitol DH (SLDH), a membrane-associated sorbose DH (SDH) and a cytosolic sorbosone DH (cSNDH).

## Development of a microbial 2-KGLA production system

The aim of the group around Professor Hermann Sahn, head of the Institute for Biotechnology I at the Research Centre of Juelich, was to set up a microbial 2-KGLA production system using *G. oxydans* in a one-step bio-transformation. That required the construction and characterisation of recombinant *G. oxydans* strains with sufficiently high enzyme activity in order to oxidise sorbitol efficiently to 2-KGLA.

First of all, the most important growth and production parameters (pH values, temperature, oxygen partial pressure, substrate concentration, agitation speed) were optimised with the help of a parallel bioreactor system (DASGIP AG, Juelich). Furthermore, the characterisation of numerous *G. oxydans* strains with regard to biomass and product formation allowed the selection of suitable wild type bacteria for the construction of 2-KGLA production strains. Two *G. oxydans* wild types, namely *G. oxydans* DSM 2343 and *G. oxydans* IFO 3293, proved to be especially suitable for the construction of recombinant 2-KGLA production strains, since they could already carry out the first 2-KGLA synthesis step (the oxidation of

sorbitol to sorbose) completely and achieved this with low biomass generation and a very high product formation rate.

For the subsequent conversion of sorbose to 2-KGLA, in both strains the genes of the membrane-bound SDH and the cytosolic cSNDH were over-expressed under the control of the constitutive *E. coli* *tufB* promoter. In addition to the gene for the cytosolic cSNDH, the gene of a membrane-bound mSNDH of *A. liquefaciens* was also functionally over-expressed, so that all reaction steps from sorbitol to 2-KGLA could take place in the periplasm and no membrane transport of intermediate products was required. Once the expression of the heterologous gene combinations was examined at the transcriptional level (Northern Blot), the translational level (SDS PAGE, mass spectroscopy) and the enzyme activity level, defined strains were available containing all necessary enzymes for 2-KGLA synthesis with suitable activity.

The SDH/cSNDH overproducers converted 100 g/L sorbitol with a specific 2-KGLA yield of 7 g 2-KGLA per g cell dry weight to 15 g/L 2-KGLA [Figure 1]. With the SDH/mSNDH overproducers, up to 12 g/L 2-KGLA could be produced from 100 g/L sorbitol. Comparison of the product formation from *G. oxydans* DSM 2343-*csndh/sdh*, *G. oxydans* IFO 3293-*msndh/sdh* and from an industrial strain (*G. oxydans* N44-1) showed that the development of the total C-sources concentration in the three strains was very different over time.

While the total C-source concentration with *G. oxydans* DSM 2343-*csndh/sdh* remains almost constant throughout the fermentation process and hardly any C-source was metabolised, the total C-source concentration, that was generated from the *G. oxydans* IFO 3293 wild type strain by mutagenesis [2], drops sharply with *G. oxydans* IFO 3293-*msndh/sdh* and *G. oxydans* N44-1. In *G. oxydans* IFO 3293-*msndh/sdh* and *G. oxydans* N44-1, more than half of the C-source was metabolised.

### CO<sub>2</sub>: a competitor of 2-KGLA-formation

In order to obtain high yields of 2-KGLA, it is necessary to prevent the cells' internal catabolism of intermediates of the 2-KGLA synthesis (mainly sorbose), since it is in competition with the periplasmic 2-KGLA production. Therefore further experiments were performed in order to discover the metabolic pathway that is responsible for sorbose degradation in *G. oxydans*. Exhaust gas analysis and the comparison of various strains in a parallel bioreactor system (DASGIP AG, Juelich) showed correlation between the assimilation of sorbose and the formation of biomass

(7 g/L cell dry weight) in *G. oxydans* IFO3293 (not shown) and in the industrial strain *G. oxydans* N44-1, in which up to 60 percent of the sorbose is converted to CO<sub>2</sub> [Figure 2]. The *G. oxydans* strain DSM 2343-*csndh/sdh*, on the other hand, only formed 2 g/L cell dry weight and only converted 6 percent of sorbose to CO<sub>2</sub>.

Since the enzymes of the citrate cycle are not entirely represented in *G. oxydans* [3] and hence the pentose-phosphate pathway (PPW) is the only way to produce CO<sub>2</sub>, the PPW in *G. oxydans* must be the main metabolic pathway to metabolise 2-KGLA intermediates [Figure 3]. Beside the activities of the fructokinase and fructose-1,6-bisphosphatase, primarily the activities of the PPW enzymes transaldolase (TAL) and phosphoglucose-isomerase (PGI) are significantly higher in *G. oxydans* N44-1 and *G. oxydans* IFO 3293 than in *G. oxydans* DSM 2343.

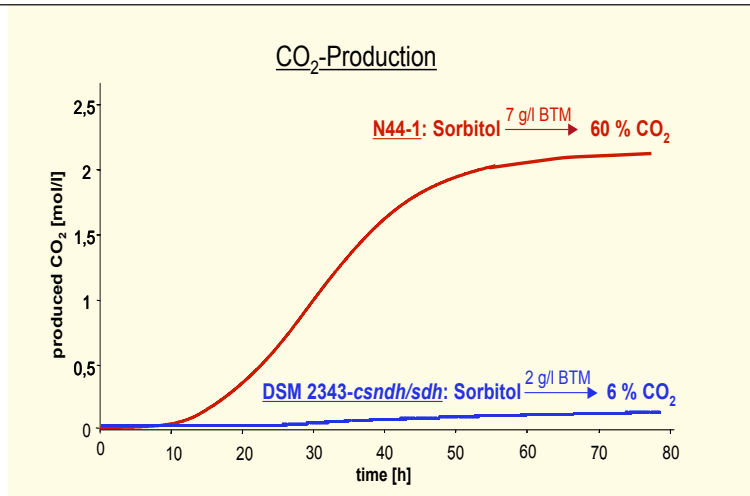


Figure 2. Carbon dioxide production within a fermentation using 100 g/L (550 mM) sorbitol in the DASGIP Parallel Bioreactor System.

In order to determine the exact influence of transaldolase and phosphoglucose-isomerase activity on PPW metabolism performance, both enzymes were constitutively overproduced in *G. oxydans*. Within a fermentation using 100 g/L sorbitol as C-source, a 3.3 times higher activity of both enzymes (PGI and TAL) resulted in 1.5 fold higher growth [Figure 4A] and 1.3 fold higher CO<sub>2</sub> production [Figure 4B]. This proves that the PPW is responsible for the degradation of sorbose leading to the

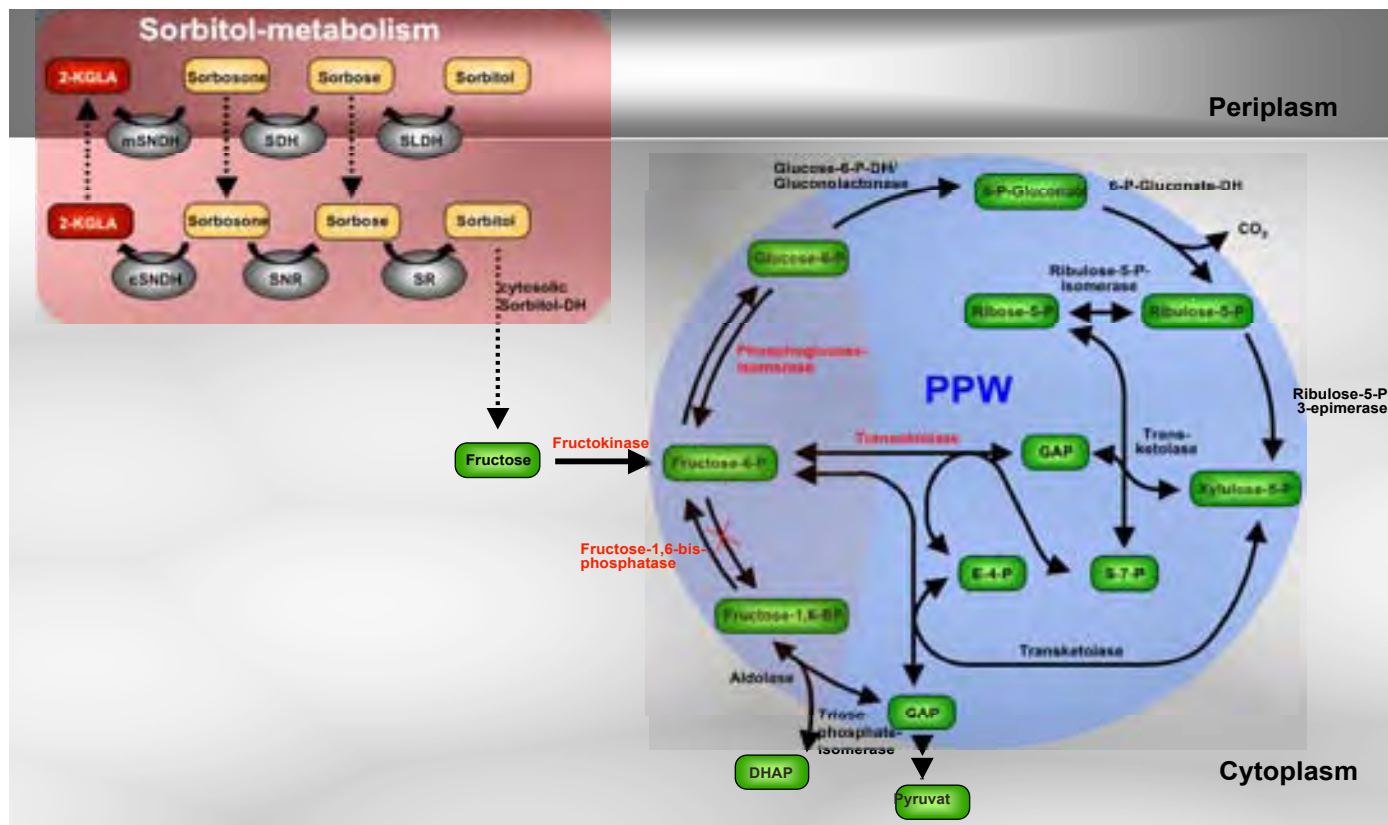


Figure 3. In *G. oxydans* the pentose-phosphate cycle is the main degradation pathway for intermediates of the sorbose metabolism.

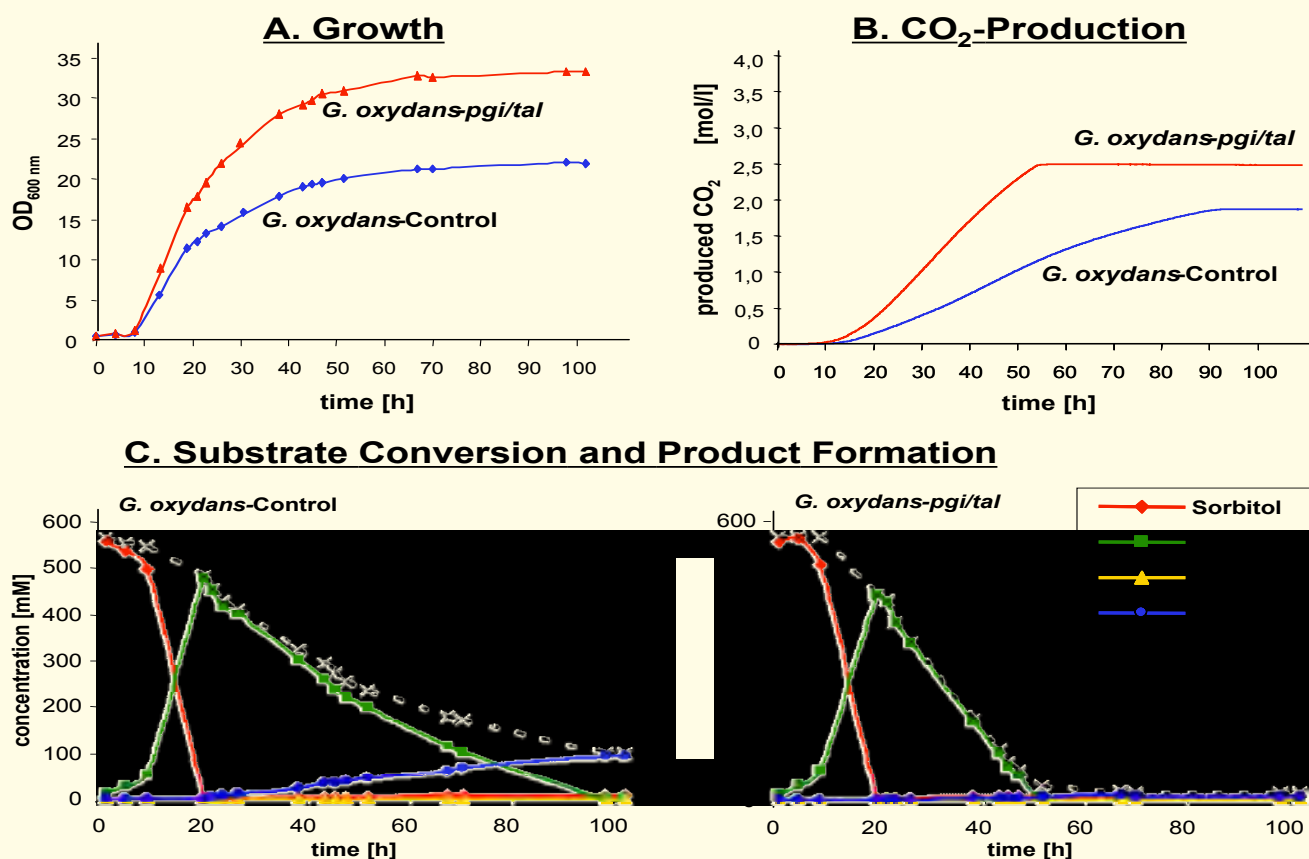


Figure 4. Growth (A.), Carbon Dioxide Production (B.) and temporal development of substrate conversion and product formation (C.) under PGI/TAL overproduction compared with control strain (*G. oxydans* without the gene of interest). Cultivation in medium with 100 g/L (550 mM Sorbitol) in a parallel bioreactor system (DASGIP AG, Juelich).

generation of energy for biomass formation. Within the PPW, PGI and TAL activities are responsible for the metabolic performance of the PPW. The development over time of the substrate conversion and product formation [Figure 4C] leads to the assumption that cytosolic sorbose degradation to carbon dioxide and periplasmatic production of 2-KGLA remain in competition with each other. With almost no detectable 2-KGLA, PGI/TAL overproduction leads to a much faster degradation of sorbose compared to the control.

### Summary: a suitable strain for the microbial 2-KGLA production has been found

In some *G. oxydans* strains, the CO<sub>2</sub> generated in the PPW represents the main competitor product for 2-KGLA synthesis. Since both PGI and TAL manifest far less activity in *G. oxydans* DSM 2343 than in those strains deriving from *G. oxydans* IFO 3293, less than 10 percent of the substrate is converted to CO<sub>2</sub> with the constructed *G. oxydans* DSM 2343 strains during sorbitol

biotransformation. In accordance with that there is only a small biomass formation. As a result, in *G. oxydans* DSM 2343, about 90 percent of the substrate is available for the production of 2-KGLA. In contrast to that, the 2-KGLA yield in the other analysed strains is limited by the fact that large amounts of substrate are used in the PPW to generate energy for the production of biomass. With the generated *G. oxydans* DSM 2343 strains there are now strains available in which sorbose metabolism and all the enzymes involved in 2-KGLA synthesis are well characterised. This is the basis for further improvement of 2-KGLA production using targeted modifications in sorbose metabolism [4].

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