The industrial versatility of *Gluconobacter oxydans*: current applications and future perspectives

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Abstract

*Gluconobacter oxydans* is a well-known acetic acid bacterium that has long been applied in the biotechnological industry. Its extraordinary capacity to oxidize a variety of sugars, polyols, and alcohols into acids, aldehydes, and ketones is advantageous for the production of valuable compounds. Relevant *G. oxydans* industrial applications are in the manufacture of L-ascorbic acid (vitamin C), miglitol, gluconic acid and its derivatives, and dihydroxyacetone. Increasing efforts on improving these processes have been made in the last few years, especially by applying metabolic engineering. Thereby, a series of genes have been targeted to construct powerful recombinant strains to be used in optimized fermentation. Furthermore, low-cost feedstocks, mostly agro-industrial wastes or byproducts, have been investigated, to reduce processing costs and improve the sustainability of *G. oxydans* bioprocess. Nonetheless, further research is required mainly to make these raw materials feasible at the industrial scale. The current shortage of suitable genetic tools for metabolic engineering modifications in *G. oxydans* is another challenge to be overcome. This paper aims to give an overview of the most relevant industrial *G. oxydans* processes and the current strategies developed for their improvement.

Keywords Bioprocess · Dihydroxyacetone · Gluconates · Metabolic engineering · Miglitol · Vitamin C

Abbreviations

2-KGA  2-Keto-D-gluconic acid
2KGADH  2-Keto-D-gluconate dehydrogenase
2KGR  2-Keto-gluconate-reductase
2-KLG  2-Keto-L-gulonic acid
2,5-KGA  2,5-Diketogluconic acid
5-KGA  5-Keto-D-gluconic acid
5KGR  5-Keto-gluconate-reductase
6NSL  6-(N-hydroxyethyl)-amino-6-deoxy-α-L-sorbofuranose
DHA  1,3 Dihydroxyacetone
DHA-P  Dihydroxyacetone phosphate
DNJ  1-Deoxynojirimycin
FAD  Flavin adenine dinucleotide
FDA  Food and Drug Administration
GA  D-gluconic acid
GA2DH  D-gluconate-2-dehydrogenase
GDH  D-glucose dehydrogenase
GLDH  Glycerol dehydrogenase
GNK  Gluconokinase
GPDH  Glycerol-3-phosphate-dehydrogenase
mDHs  Membrane-bound dehydrogenases
NHEG  N-2-hydroxyethyl glucamine
PPP  Pentose phosphate pathway
PQQ  Pyrroloquinoline quinone
SDH  Sorbose dehydrogenase
SLDH  D-sorbitol dehydrogenase

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SNDH  Sorbosone dehydrogenase
SSDH  Sorbose/sorbosone dehydrogenase

**Introduction**

Biotechnology plays an important role in generating a wide range of valuable compounds, providing novel opportunities for sustainable processes. Biocatalyst-based processes have been highlighted since their use allows an increased in selectivity of chemical reactions, which lowers byproduct formation and enhances product yield compared to chemical processes (Gavrilèscau and Chisti 2005). Among many microorganisms that can be applied in the biotechnological process, *Gluconobacter oxydans*, an obligatory aerobic and Gram-negative bacterium, has been recognized for its extraordinary capacity of incomplete regio- and stereoselective oxidation of sugars, polyols, and alcohols (De Ley et al. 1984; Gupta et al. 2001). This capacity made *G. oxydans* a convenient choice for synthesis of valuable compounds via a combination of biocatalysis and chemical methods.

*G. oxydans* oxidation is catalyzed by membrane-bound dehydrogenases (mDHs), located on the outer surface of the cytoplasmic membrane and does not involve phosphorylated reactions (Fig. 1). These dehydrogenases are quinoproteins or flavoproteins that have pyrroloquinoline quinone (PQQ) and flavin adenine dinucleotide (FAD) as prosthetic groups, respectively. Furthermore, since the active site of these enzymes is oriented towards the periplasm, both substrate and oxidized products can easily cross the outer membrane. Accumulation of oxidized products occurs in the culture medium, which favors their recovery. Membrane-bound dehydrogenases are linked to the respiratory chain and the electrons are transferred to oxygen as the final electron acceptor (Matsushita et al. 1994; Deppenmeier et al. 2002).

The direct oxidative pathways described above is related to the efficient and fast oxidation industrial reaction. There is a second oxidation pathway, in which a minor part of sugars or their oxidation products are catabolized. The substrate is taken up into the cytoplasm where the reactions occur (Fig. 1). In this case the oxidative reactions are catalyzed by NAD(P)+ dependent dehydrogenases. Phosphorylated intermediates are formed and metabolized via the pentose phosphate pathway (PPP) (Matsushita et al. 1994; Deppenmeier et al. 2002).

*Gluconobacter oxydans* has been used in biotechnological processes, namely in the production of: vitamin C (5) (Reichstein and Grüssner 1934), miglitol (9) (Ke et al. 2018), D-glucouronic acid (13) (GA) (Kulka et al. 1951; Ramachandran et al. 2006), 2-keto-D-glucuronic acid (14) (2-KGA) (Wang et al. 2019), 5-keto-D-glucuronic acid (16) (5-KGA) (Shinagawa et al. 1999) and 1,3 dihydroxyacetone (18) (DHA) (Claret et al. 1994). Each process requires different membrane-bound dehydrogenases to oxidize their respective substrates (Fig. 1).

The aim of this paper is overview biotechnological processes that have the versatile bacterium, *Gluconobacter oxydans*, as the biocatalyst. The emphasis is on current production strategies and new approaches that have been developed in the last few years to improve efficiency and profitability of these processes, focusing mostly on publications since 2014.

Recent developments rely on comprehension of metabolic features and genetic manipulation of *G. oxydans*. Research has been undertaken (Mientus et al. 2017; Kranz et al. 2018; Zhou et al. 2019a; Qin et al. 2021) to elucidate *G. oxydans* metabolism, which could pave the way for the improved performance of strains. For example, the genome of a strain with high oxidative power and low growth yield (*G. oxydans* ATCC 621H) was compared with that of a strain with good growth yield (*G. oxydans* DSM 3504) in order to understand how to boost growth and increase productivity (Kostner et al. 2015). It was found that the presence of an additional NADH dehydrogenase gene is important for increased growth yields but negatively impacts the activity of the membrane-bound polyol dehydrogenases responsible for substrate oxidation (Kostner et al. 2015).

Overexpression of genes directly related to the reaction, gene knockout and heterologous expression have been applied to *G. oxydans* strains to improve the synthesis of 2-keto-L-gulonic acid (2-KLG = 4), 6-(2-hydroxyethyl)-amino-6-deoxy-α-L-sorburosanose (6NSL = 11), 2-keto-D-glucuronic acid (2-KGA = 14), 5 keto-D-glucuronic acid (5-KGA = 16) and dihydroxyacetone (DHA = 18) (Shi et al. 2015; Chen et al. 2016; Yuan et al. 2016a; Tan et al. 2019;
that improved the strain growth ability and substrate repression and the control of central carbon metabolism flux system in *G. oxydans*. An endogenous CRISPR/Cas system in *G. oxydans* WSH-003 allowed a multiplex gene regulation tools in the CRISPR/Cas system is also enriching the gene other strong promoters (Chen et al. 2021). The development of the CRISPR/Cas system is currently used for vitamin C production and comprises six steps (Wang et al. 2018) (Fig. 2). First, glucose is reduced to D-sorbitol (2), using nickel as catalyst, followed by its oxidation to L-sorbose (3) by *G. oxydans*. After a series of reactions to protect the hydroxy groups against oxidation, L-sorbose (3) is oxidized to 2-keto-L-gulonic acid (4). Enolization and lacticonization of 2-KLG (4) finally leads to L-ascorbic acid (5) (Reichstein and Grüssner 1934; Boudranton 1990).

Considered as an improvement of Reichstein’s process, a two-step fermentation is currently used for vitamin C (5) production and comprises six steps (Wang et al. 2018) (Fig. 2). Firstly, glucose is converted into D-sorbitol (2) by catalytic hydrogenation, which is efficiently transformed into L-sorbose (3) by *G. oxydans* through sorbitol dehydrogenase (SLDH) (Gao et al. 2014). This stage is industrially carried out in an airlift bioreactor and D-sorbitol (2) is applied as substrate (20–25%) in a batch fermentation, for 18–24 h at 32 °C and at natural pH. The oxygen transfer rate is maintained at 300–500 mmol L⁻¹ h⁻¹ and a productivity of 12–13 g L⁻¹ h⁻¹ is reached (Yang and Xu 2016). Sorbitol dehydrogenase is a major polyol dehydrogenase also known as a glycerol dehydrogenase (GDLH), which converts glycerol (17) to dihydroxyacetone (18), gluconic acid (13) to 5-keto-D-gluconic acid (16) and D-sorbitol (2) to L-sorbose (3) (Prust et al. 2005; Qin et al. 2022). This enzyme has PQQ as a prosthetic group, however a FAD dependent membrane bound SLDH also converts D-sorbitol (2) to L-sorbose (3) (Qin et al. 2022).

In the second fermentation, a mix-culture of *Ketogulonicigenium vulgare* and *Bacillus megaterium* or *Bacillus cereus* produces 2-KLG (4) (Fig. 2), the precursor of
| Original strain       | Mutant strain                      | Genetic modification                                                                 | Product titer (g L\(^{-1}\)) | References                  |
|----------------------|-----------------------------------|----------------------------------------------------------------------------------------|-------------------------------|----------------------------|
| **G. oxydans DSM 2003** | G. oxydans\_pBBR1PgHp0169-ga2dh | Overexpression of ga2dh gene (gluconate-2-dehydrogenase)                               | 2-KGA (14) 36.5              | (Shi et al. 2014)         |
| **G. oxydans WSH 003** | G. oxydans-sldhAB6               | Overexpression of slhdAB gene (sorbitol dehydrogenase)                                 | L-sorbos (3) 135.0            | (Xu et al. 2014a)         |
| **G. oxydans WSH 003** | G. oxydans/ pGUC-k0203-GS-k0095-pqqABCDE | Overexpression of slhd gene (L-sorbose dehydrogenase), sndh gene (L-sorbosone dehydro-
|                       |                                   | nase) and gene pqqABCDE (pyrroloquinoline quinone)                                     | 2-KLG (4) 39.2               | (Gao et al. 2014)        |
| **G. oxydans WSH 003** | G. oxydans pBBR1-PB392_2000-sldh | Overexpression of slhd gene (sorbitol dehydrogenase)                                 | L-sorbos (3) 144.5            | (Hu et al. 2015)          |
| **G. oxydans DSM 2003** | G. oxydans pBBR-3510-ga2dh       | Overexpression of ga2dh gene (gluconate-2-dehydrogenase)                               | 2-KGA (14) 486.0              | (Shi et al. 2015)         |
| **Ketogulonicigenium vulgare HKv604** | SyBE_Kv000116012 | Overexpression of ssda1-l gene (L-sorbose dehydrogenase)                               | 2-KLG (4) 79.97               | (Chen et al. 2016)        |
| **G. oxydans DSM 2003** | G. oxydans-tufB-ga2dh            | Overexpression of ga2dh gene (gluconate-2-dehydrogenase)                               | 2-KGA (14) 453.3              | (Li et al. 2016)          |
| **G. oxydans DSM 2343** | G. oxydans ZJU2/ pBB5-P0169-gcd  | Knocking out of ga2dh gene (GOX 1231—gluconate-2-dehydrogenase) and pdc gene (GOX 1081—pyruvate decarboxylase) / G. oxydans ZJU2 | 5-KGA (16) 117.75            | (Yuan et al. 2016a)      |
|                       |                                   | Heterologous expression of gcd gene (secondary alcohol dehydrogenase)                 |                               |                           |
| **G. oxydans ZJU2**   | G. oxydans ZJU7                  | Overexpression of sldAB gene (sorbitol dehydrogenase), and pqqABCDE and slD genes (pyrroloquinoline quinone-PQQ). Fusion of cyoBACD genes (cytochrome b03 oxidase) into the PQQ overexpression plasmid | 5-KGA (16) 162.0              | (Yuan et al. 2016b)      |
| **G. oxydans**        | G. oxydans H6 (SyE_Go00010313)   | Knocking out of B932-1330 gene (NADPH dependent L-sorbose reductase) and B932_1370  | 2-KLG (4) 76.6               | (Wang et al. 2016a)      |
|                       |                                   | gene (PT system transporter subunit IIA)                                               |                               |                           |
| **G. oxydans**        | G. oxydans gosldh-lrenox         | Overexpression of slhd gene (sorbitol dehydrogenase), Heterologous expression of lrenox gene (NAD(P)H oxidase) | L-sorbos (3) 145.0            | (Kim et al. 2019)        |
| **G. oxydans ZJB-605** | G. oxydans-gHp0169-sldAB         | Overexpression of sldAB gene (sorbitol dehydrogenase)                                 | 6NSL (11) 184.28             | (Ke et al. 2019b)        |
| **G. oxydans 621H**   | G. oxydans (G6) / PufB-gdh       | Overexpression of gdh gene (glycerol dehydrogenase)                                   | DHA (18) ~70*                | (Tan et al. 2019)        |
|                       |                                   |                                                                                       |                               |                           |
| **G. oxydans-gHp0169-sldAB** | G. oxydans-pGA004 | Overexpression of pqqABCDE and tld/D as a cluster (pyrroloquinoline quinone)           | 6NSL (11) 300.2              | (Liu et al. 2020b)       |
L-ascorbic acid (5), from L-sorbose (3). *K. vulgare* produces 2-KLG (4) by L-sorbose / L-sorbosone dehydrogenase (Asakura and Hoshino 1999) or by another sorbosone dehydrogenase that only catalyzes the conversion of L-sorbose to 2-KLG (4) (Miyazaki et al. 2006). *B. megaterium* cannot catalyze L-sorbose (3) transformation, and act as a companion strain to enrich 2-KLG (4) production by favoring the growth of *K. vulgare* (Ye et al. 2014). The industrial production of 2-KLG (4) is also a batch fermentation and is carried out on an airlift bioreactor. A temperature of 29 °C and a neutral pH (adjusted by sodium hydroxide) is maintained during the process, which lasts 40–65 h. L-sorbose (3) is added as the pasteurized broth obtained from the first fermentation initially at a concentration of 10 mg ml⁻¹ and then regularly added to the fermentation medium between 10–30 h. An oxygenation of 100 mmol L⁻¹ h⁻¹ is kept during the process to maintain the cells growth and a satisfactory L-sorbose (3) conversion. The process reaches a final concentration of 90–110 g L⁻¹ of 2-KLG (4) (Yang and Xu 2016). This co-cultivation of *K. vulgare* and *Bacillus* spp is an example of a successful consortia applied industrially.

The knowledge on the type of interaction between the microorganisms is required for co-cultivation, since it affects metabolic efficiency. Due to advances in synthetic biology, metabolic and genetic engineering, researchers are working on developing efficient microbial consortia to increase productivity (Bhatia et al. 2018; Kyllidis et al. 2018). *Bacillus megaterium* and *K. vulgare* form a synergistically mutualistic co-culture (Zou et al. 2013; Ghosh et al. 2016) and their interaction is crucial for increased 2-KLG (4) yield (Yang et al. 2013). Other examples of biotechnological process being devised with defined consortia of microorganisms are in the production of lignocellulolytic enzymes (Hu et al. 2017a), butanol (Wu et al. 2016), lactic acid (Sun et al. 2021) and ethanol (Beri et al. 2020).

Despite effective production of vitamin C (5) by a two-step fermentation process, there are limitations such as the long incubation period and the need for additional sterilization (Wang et al. 2016a). In order to overcome these drawbacks, efforts to improve 2-KLG (4) production and develop a feasible one-step fermentation route are the major challenges of industrial vitamin C (5) production nowadays, which are discussed in greater detail by Wang and co-works (Wang et al. 2018). The design of a suitable microbial consortium and the genetic engineering of strains seem to be the paths to develop a successful one-step vitamin C (5) fermentation. Moreover, -omics technologies are important tools to understand changes occurring in microorganisms submitted for co-culture, providing insights for advancement of one-step fermentation.

Long-term adaptation of *K. vulgare* and *B. thuringiensis* strengthens their interaction due to both enhancement of amino acids metabolism and their exchange between

| Table 1 | (continued) |
|---------|-------------|
| Original strain | Mutant strain |
| *K. vulgare* WSH 001 and *Klebsiella pneumoniae* HS11286 | *Escherichia coli* pRSF-sal3-pET-ssda |
| *G. oxydans* AB-PQQ-VHb | Heterologous expression of *Vitreoscilla* hemoglobin-VHb |
| *G. oxydans* AB-PQQ-VHb | G. *oxydans* AB-PQQ-VHb |
| *G. oxydans* AB-PQQ-VHb | Heterologous expression of *Vitreoscilla* hemoglobin-VHb |

*Titer obtained from figures*
the strains. Furthermore, the adaptation mechanism of *B. thuringiensis* weakened its sporulation and increased its growth rate, which positively affected *K. vulgare* growth and productivity towards 2-KLG (4) (Ma et al. 2014; Jia et al. 2017). In addition, when *G. oxydans* are in co-culture with *B. endophyticus* and *K. vulgare*, a negative impact on its carbon metabolism (PPP–pentose phosphate pathway) occurred, which affected cells growth and protein synthesis. In contrast, the presence of *G. oxydans* in the three microbial consortium promoted an enhancement in fatty acids and purine nucleotide synthesis (Ma et al. 2019).

Towards genetic engineering, most efforts are concentrated on construct strains able to convert D-sorbitol (2) into 2-KLG (4) in mono or co-culture. Although, *Gluconobacter* synthesizes the necessary enzymes to produce 2-KLG (4) from D-sorbitol (2) (Saito et al. 1997), production is very low and only a few strains are able to form 2-KLG (4) (Deppenmeier et al. 2002). Meanwhile bioconversion from D-sorbitol (2) by *K. vulgare* is poorly efficient compared to that using L-sorbosone (3) as a substrate (Sugisawa et al. 2005). Furthermore, a high 2-KLG (4) production by *K. vulgare* is only achieved if a helper strain is present (Zhang et al. 2010) or by adding additional nutrients (Leduc et al. 2004). Thus, a construction of a recombinant strain of *G. oxydans* seems more likely.

The overexpression of *K. vulgare* genes in *G. oxydans* is also a target of research. Gao et al. (2014) expressed five genes encoding for SDH (sorbose dehydrogenase) and two genes for SNDH (sorbosone dehydrogenase) from *K. vulgare* WSH-01 in *G. oxydans* WSH-003. Ten distinct combination of SDH and SNDH genes were expressed generating ten new strains. Although all strains were able to produce 2-KLG (4) from D-sorbitol (2), the yield was low due to the accumulation of L-sorbosone. To overcome this shortcoming, linker peptides were used to fuse SDH and SNDH this led to higher yield of 2-KLG (4) (Gao et al. 2014).

Furthermore, as SLDH, SDH and SNDH are PQQ dependent, an imbalance of enzymes and their cofactor
Miglitol

Miglitol (9) (N-hydroxyethyl-1-deoxynojirimycin) is an iminosugar developed by Bayer and has been used in the control of type 2 diabetes due to its competitive inhibition of α-glucosidase (Schedel 2000; Zhang et al. 2011a; Khobare et al. 2016). In 1996, miglitol (9) was approved by the Food and Drug Administration (FDA) as an antidiabetic drug (FDA 1996) and has been sold in many countries, such as France, USA, Japan, Spain and Australia (Sugimoto et al. 2015). Some of miglitol (9) manufacturers include Lunan Pharmaceutical Group Co., Ltd. (Linyi City, Shandong, China), Zhejiang Medicine (Shaoxing, Zhejiang, China) (Market Watch 2021) and Bayer HealthCare Pharmaceuticals Inc. (Berlin, Germany). Bayer has licensed to Pfizer the marketing rights of Glyset®, a trade name of miglitol (9) also registered by Bayer (Pfizer 2016).

Miglitol (9) can be obtained from DNJ (8), (1-Deoxynojirimycin) an iminosugar first isolated from plants in 1976 (Yagi et al. 1976). DNJ (8) can be extracted from plants or obtained by chemical synthesis or fermentation using Streptomyces and Bacillus. However, none of these methods are economically feasible on a large scale (Schedel 2000). Hence, the adopted industrial approach for miglitol (9) production is a combination of biocatalytic and chemical methods (Fig. 3). Biotransformation is applied to produce the key intermediate of miglitol (9) synthesis and then chemical steps are performed to obtain miglitol (9). Two routes with two different key intermediates (6-amino-6-deoxy-L-sorbose (7) or 6-(2-hydroxyethyl)-amino-6-deoxy-α-L-sorbofuranose (11)) are possible (Kinast and Schedel 1981; Grabner et al. 2003). Both use resting cells of G. oxydans grown in an appropriate culture medium with D-sorbitol (2) as the main carbon source (Schedel 2000).

In the first approach, D-glucose is chemically transformed through reductive amination to 1-amino-1-deoxy-D-sorbitol (6), which is regioselectively oxidated to 6-amino-6-deoxy-L-sorbose (7) by resting cells of G. oxydans. Stereoselective reductive ring closure of 6-amino-6-deoxy-L-sorbose (7) leads to DNJ (8) formation (Kinast and Schedel 1981; Schröder and Stubbe 1989; Schedel 2000). The addition of protection groups, (e.g., benzylloxycarbonyl group, and small hydrophilic groups like formyl- and dichloroacetyl-) prior the biotransformation of 1-amino-1-deoxy-D-sorbitol (6) is necessary (Kinast and Schedel 1981; Schröder and Stubbe 1989) to avoid the spontaneous intramolecular ring closure reaction. These protective groups have to be remove before the final reduction (Schedel 2000). DNJ (8) is chemically transformed into miglitol (9) (Fig. 3a).

The latest approach, also starts by a reductive amination reaction of D-glucose, which is converted into N-2-hydroxyethyl glucamine (10) (NHEG). Resting cells of G. oxydans are applied at 15 °C in sterile conditions and under aeration to the regioselective oxidation of NHEG (10) to 6-(2-hydroxyethyl)-amino-6-deoxy-α-L-sorbofuranose (11). The cell mass is removed and a catalytic hydrogenation reaction proceeds directly from the supernatant liquor to form miglitol (9) (Fig. 3b) (Grabner et al. 1999; Liu et al. 2020a). This process is more advantageous than the described above because no isolation and no protective group is needed. The conversion of NHEG (10) to 6NSL (11) requires a membrane-bound D-sorbitol dehydrogenase (SLDH) with activity and stability at the low process temperature of 15 °C. The selection or development of better strains that meet this latter requirement is until now a challenge (Ke et al. 2018; Liu et al. 2020a).

Despite advances in recombinant DNA and protein expression technologies, classical mutation is still a strategy used to improve industrial strains. High-throughput methods are needed for efficient and rapid screening of strains with high SLDH activity among the large amount of mutants generated by this method. New strategies based on chromogenic reactions have been reported. More than 1400 G. oxydans ultraviolet irradiation mutant strains were screened for 6NSL (11) production using a chromogenic reaction between 6NSL (11) and 2,4 dinitrophenylhydrazine. The reaction products formed under alkaline conditions present color intensities proportional to the amount of 6NSL (11), which is readily recognized (Ke et al. 2018). Another approach to high-throughput screening for 6NSL (11) producer strains was developed based on the reaction of L-sorbose (3) with Fehling’s reagent, which forms a yellow to red precipitate easily identified. This approach was used for the screening of 60Co-γ irradiation and microwave mutants, resulting in more than 1000 positive strains for 6NSL (11) (Liu et al. 2020a).

Regarding metabolic engineering, the increase in 6NSL (11) production was obtained by the overexpression of genes related to the SLDH enzyme. A G. oxydans strain overexpressing the sldAB gene cluster was constructed resulting in an accelerated catalysis towards substrate, which is directly related to an increase on SLDH activity (Ke et al. 2019b). The transcriptional level of SLDH was 27.3-fold higher compared to wild strain, and relative enzymatic activity.
towards NHEG (10) increased 2.05 fold in the recombinant strain.

As previously discussed for vitamin C (5), the small amount of PQQ synthesized by *G. oxydans* (Xiong et al. 2011) may represent a bottleneck for the action of dehydrogenase enzymes dependent on this coenzyme and, consequently, for the formation of miglitol (9). To overcome this shortage, the genes responsible for PQQ synthesis (gene cluster *pqqABCDE*) can be overexpressed (Liu et al. 2020b) or a medium fortified with amino acids can...
be applied as a simpler strategy (Ke et al. 2019a, b). In fact, supplement the medium with four exogenous amino acids (Glu, Ile, Thr, Arg), which are rapidly consumed by *G. oxydans* for biosynthesis of PQQ precursor (PqqA), enhances PQQ levels by 48% and favors SLDH activity, which showed an increase of 19.8% (Ke et al. 2019b).

The growth of cells for biotransformation usually results in low yield and this step is responsible for 40% of the cost of producing 6NSL (11) (Hu et al. 2020). In addition, there is a loss of catalytic activity during biotransformation. Thus, cell reuse strategies help to lower the cost of the process and increase production (Hu et al. 2019, 2020). Hu et al. (2019) were able to reuse cells immobilized by adsorption four times, applying a repeated biotransformation strategy, using NH$_4$Cl as a nitrogen source and for pH control in a bubble column bioreactor with an aeration rate of 2.5vvm. They were also able to increase the number of reuse cycles to nine by regenerating the cells in situ using fresh medium. The NHEG (10) concentration was maintained at 50 g L$^{-1}$ at each repetition of the biotransformation, after verifying that above 60 g L$^{-1}$ there is a drop in the reaction rate, reflecting an inhibition of enzyme activity by the substrate (Hu et al. 2020).

**Gluconic acid and ketogluconic acids**

Organic acids represent one of the major building blocks obtained through biotechnology processes in the global market of high-volume bulk chemicals (Anastassiadis and Morgunov 2007). The market for gluconic acid (13), also known as pentahydroxycaproic acid (C$_6$H$_{12}$O$_7$), is expected to reach USD 1.9 billion in 2028 (Fior Markets 2021). Currently, annual production of GA (13) and its derivatives (Fig. 4) is approximately 80,000 tonnes (Karaffa and Kubicek 2021). Due to versatile chemical features, this organic acid and its derivatives have found wide applications in chemical, pharmaceutical, food, dairy, beverage and construction industries (Pal et al. 2016; Golikova et al. 2017).

Even though GA (13) can be obtained through chemical or enzymatic approaches, its industrial production is based on a dehydrogenation reaction of glucose by microbial fermentation (Fig. 1c). *G. oxydans* and *Aspergillus niger* are widely employed microorganisms and starch or sucrose are used as the main feedstock (Attwood et al. 1991; Vroemen and Béverini 1999; Sainz et al. 2016; Zhang et al. 2016b). Main manufacturers of gluconic acid (13) and/or its salts include Ruibang Laboratories (Whenzou, Zhejiang, China) (Ruibang Laboratories 2021) and Roquette Frères (France) (Roquette 2021).

![Fig. 4 Gluconic acid and ketogluconic acids from glucose oxidation by *Gluconobacter oxydans*; GDH glucose dehydrogenase, GL glucono-δ-lactonase, GA2DH gluconate-2-dehydrogenase, 2KGADH 2-keto-gluconate-dehydrogenase, GLDH glycerol dehydrogenase (Kataoka et al. 2015). *The reaction can be catalyzed by GL or occurs spontaneously](image)
The main pathway for gluconic acid (13) production is via membrane-bound D-glucose dehydrogenase that oxidizes D-glucose (1) to D-gluco-δ-lactone (12), which is subsequently converted to D-gluconate (13) spontaneously or by membrane-bound glucono-δ-lactonase (GL) (Fig. 4) (Kataoka et al. 2015). Some G. oxydans strains are able of carry out reactions transforming GA (13) into 2-keto-D-gluconate (14) or 5-keto-D-gluconate (16). Additionally, 2-keto-D-gluconate (14) may be further oxidized into 2,5-diketo-D-gluconate (15) (2,5-KGA). GA (13) can also be formed through a second pathway, that occurs inside cells by NADP+-dependent DH, but membrane-bound activity is 30-fold higher (Matsushita et al. 1994; Prust et al. 2005; Cañete-Rodríguez et al. 2016; Sainz et al. 2016).

Although some papers and patents report industrial production of GA (13) by Gluconobacter oxydans (Attwood et al. 1991; Vroemen and Bévérini 1999), the literature is mainly based on the process using A. niger and information on aspects of the G. oxydans process is limited. The fermentation yield in relation to glucose (1) (substrate) is higher with G. oxydans than with A. niger because the fungus uses part of the glucose (1) for its growth. The yield using G. oxydans can be close to 100%, although important nutrients are required for the growth of the microorganism (Vroemen and Bévérini 1999; Anastassiadis and Morgunov 2007). The process is carried out by submerged batch in aerated fermenters at controlled temperature (Anastassiadis and Morgunov 2007).

During growth on glucose (1), G. oxydans converts most of the sugar to GA (13) and ketogluconates (14, 16) (2-KGA and 5-KGA) (Fig. 4) at the expense of growth (Krajewski et al. 2010). The pH of the culture medium is the most influential factor in regulating the formation of GA (13) and its ketoacids (14, 16) (Ano et al. 2011; Cañete-Rodríguez et al. 2016). At pH between 3 and 5 the formation of 5-KGA (16) is privileged and above pH 5 this production decreases and favors the joint formation of 2-KGA (14). Gluconic acid (13) production is favored at pH below 3, but below pH 2 production is almost totally repressed (Velizarov and Beschkov 1994; Ano et al. 2011).

Glucose (1) up to a concentration of 90 g L⁻¹ is almost completely oxidized to GA (13) (Velizarov and Beschkov 1994). Acid accumulation due glucose transformation leads to a rapid decrease of pH to about 2.5. At this pH, the enzymes of the pentose phosphate cycle are almost totally inactivated and the energy needs of cells depend on the rapid oxidation by membrane dehydrogenases. Thus, almost quantitative conversion of glucose (1) to gluconate (13) is obtained and pH control is not required for the fermentation process at this glucose concentration (Velizarov and Beschkov 1998; Zhou and Xu 2019). At elevated glucose (1) concentrations the pH of the culture medium drops to around 2, a value where gluconate (13) production is almost totally repressed (Velizarov and Beschkov 1994).

The process continues to be studied, particularly for the use of glucose (1) derived from lignocellulosic biomass as a raw material (Zhang et al. 2016a; Zhou et al. 2017). Lignocellulosic biomasses contain a large amount of sugars, in the form of cellulose and hemicelluloses, and after treatment, fermentable sugars (mainly glucose and xylose) are released and can be oxidized to GA (Fujii et al. 2009). This is especially interesting as G. oxydans, unlike A. niger, is able to oxidize hexose and pentose sugars to their corresponding acids and is tolerant to inhibitors usually present in hydrolysates (Zhang et al. 2016a; Zhou et al. 2019a). The production of GA (13) simultaneously with other products arising from the sugar content of the lignocellulosic material has been seen as a good approach to the biorefinery concept.

Glucose (1) and xylose obtained from the hydrolysis of corn stover pre-treated with diluted acid and biodetoxified were converted simultaneously into GA (13) and xylonic acid. Yields obtained were 119.10 g L⁻¹ of GA (13) (97.12% yield) and 14.04 g L⁻¹ of xylonic acid. The conversion of xylose to xylonic acid was not complete, as the fermentation was stopped at 32 h to avoid the possible conversion of GA (13) to keto-gluconic acid (14, 16) (Zhang et al. 2016a). This difficulty was overcome by the addition of 10 g L⁻¹ of ZnCl₂ to the reaction medium, which inhibited the further transformation of GA into the by-product 2-keto-gluconic acid. Thus, the yield of xylonic acid increased to 93.36%, keeping that of GA (13) high (93.91%) (Zhou et al. 2017). Direct improvement in the conversion efficiency of non-glucose sugar acids was also achieved by adaptive evolution of G. oxydans. The conversion of xylose to xylonic acid improved significantly and the total fermentation time was reduced to 36 h from 72 h (Jin et al. 2019).

The pH value for the fermentation of glucose (1) to GA (13) by G. oxydans is in the scope of the pH of the enzymatic hydrolysis of lignocellulosic materials, thus allowing the conduction of the process as a simultaneous saccharification and fermentation (SSF). Hou et al. (2018) proposed a cascade hydrolysis of pretreated and biodetoxified corn stover and direct fermentation of glucose hydrolysate to GA (13) and xylonic acid. The approach avoided sugar loss and energy consumption by eliminating the solid/liquid separation step. A yield of 106.0 g L⁻¹ of GA (13) and 58.5 g L⁻¹ of xylonic acid were obtained in a 50 L bioreactor with a 30% (w/w) solids loading (Hou et al. 2018).

Sugarcane bagasse, a residue of sugar and ethanol industries, was used as a starting material for the simultaneous production of xylooligosaccharides (XOS) and GA (13). To obtain high yield of XOS, sugarcane bagasse was pretreated with acetic acid. The treated material was enzymatically hydrolyzed to glucose and used for the production of GA (13), leading to a good yield of 96.3%. In this process,
approximately 105 g of XOS and 340 g of GA (13) were obtained from 1 kg of sugarcane bagasse (Zhou and Xu 2019).

In another report, GA (13) replaced acetic acid and proved to be effective in the treatment of bagasse for the preparation of XOS in an eco-friendly biorefinery strategy (Zhou et al. 2019b). The maximum yield of XOS was achieved with 5% gluconic acid after 60 min of treatment, without consumption of gluconic acid (13). After removing the XOS solution, the solid was enzymatically hydrolyzed to glucose and subjected to fermentation by \textit{G. oxydans}, with 93.1% GA (13) yield in 14 h. It was also possible to use the newly prepared cellulose self-derived GA (13) for the production of XOS, with a maximum yield of 50.3% after 60 min at 150 °C.

### 2-keto-D-gluconic acid

2-keto-D-gluconic acid (14) (2-KGA) is an organic acid used in cosmetics, pharmaceutical and environmental industries. Moreover, it is a key intermediate in the synthesis of several products, including erythorbic acid (isoascorbic acid), which is an important food additive, preservative and mild antioxidant approved by the FDA (Chia et al. 2008; Sun et al. 2012b). There is an annual worldwide production of 2-keto-D-gluconic acid (14) of 50,000–60,000 tons (Sun et al. 2013), and it can be obtained by a chemical or biotechnological route (enzymatic conversion and microbial fermentation). In the chemical synthesis D-glucose (1) is oxidized to 2-KGA (14) with oxygen, in the presence of a metal catalyst (e.g. platinum) (Elseviers et al. 1998), but byproducts formed lower 2-KGA (14) yield. For enzymatic conversion expensive enzymes are required (Sun et al. 2012b). Microbial fermentation presents a high selectivity and yield conversion and is the main industrial 2-KGA (14) production method (Sun et al. 2020).

Fermentation can be performed by \textit{Glucobacter oxydans} (Kulka et al. 1951; Li et al. 2016), \textit{Klebsiella pneumoniae} (Wei et al. 2013; Sun et al. 2014), \textit{Pseudomonas} spp. (Lockwood et al. 1941; Hou et al. 2020) and \textit{Serratia marcescens} (Misenheimer et al. 1965; Zhang et al. 2011b). On the industrial scale, 2-KGA (14) has been produced mostly by \textit{Pseudomonas} spp. and \textit{G. oxydans} (Sun et al. 2012a). A major handicap in 2-KGA (14) fermentation is byproduct formation [GA (13), 5-KGA (16)] and inhibition caused by substrate or product, lowering its yield (Shi et al. 2015).

In an attempt to overcome the formation of by-products, \textit{H}_2\text{O}_2 was added to the culture medium for an intensive supply of oxygen, since the bio-oxidation reaction for 2-KGA (14) is highly dependent on the supply of oxygen. When \textit{H}_2\text{O}_2 was added to a synthetic media after 16 h of fermentation, glucose was converted to 2-KGA (14) with a rate of 63.9% compared to 43.5% conversion rate without adding \textit{H}_2\text{O}_2. Approximately the same conversion rate was observed in a corn stover enzymatic hydrolysate medium without \textit{H}_2\text{O}_2 addition. Further, \textit{H}_2\text{O}_2 addition did not stimulate the production of 2-KGA (14) as it did in the synthetic medium. The researchers found that \textit{H}_2\text{O}_2 and enzymatic hydrolysate degradation compounds up-regulated eight genes that were responsible for the production of membrane-bound enzymes, reflecting on the improved yield of 2-KGA (14) (Zhou et al. 2020).

Metabolic engineering of strains is another approach to increasing 2-KGA (14) yield. Overexpression of the \textit{ga2dh} gene encoding the membrane bound gluconate-2-dehydrogenase efficiently improved the production yield of 2-KGA (14) from GA (13) (Li et al. 2016; Shi et al 2015). Research also shows the use of new and robust promoters (Shi et al. 2014) and vector modification (Shi et al 2015) to overexpress the enzyme and enhance its level of 2-KGA (14) production. Genetic modifications of other species have also been developed to increase the production of 2-KGA (14) and may contribute with a framework for the improvement of \textit{G. oxydans} strains (Zeng et al. 2019; Sun et al. 2020).

### 5-keto-D-gluconic acid

5-keto-D-gluconic acid (16) (5-KGA) is formed by \textit{G. oxydans} from GA (13) originating from the oxidation of glucose (Fig. 4). Further, this organic acid can be converted into L-tartaric acid by a chemical reaction (Matzerath et al. 1995; Merfort et al. 2006). Tartaric acid has been applied in textile and food industries, as a chiral reagent, as an acidic reducing agent and as antioxidant (Matzerath et al. 1995). Although the microbial production of 5-KGA (16) using \textit{G. oxydans} is not yet industrialized, attempts are being made to increase this production, thus enable the further synthesis of tartaric acid (Yuan et al. 2016b). The main problem with the process is the accumulation of high concentrations of gluconate (13) during glucose oxidation and the production of 2-KGA (14) as a competing product. Thus, the strains used in the studies are generally genetically modified, in which the gene encoding the 2-KGA-forming enzyme has been disrupted (Merfort et al. 2006; Yuan et al. 2016a).

A recombined \textit{G. oxydans} was constructed by knocking out two genes (GOX 1231 and GOX 1081) and heterologous expression of quinoprotein glucose dehydrogenase from \textit{Xanthomonas campestris}. The first gene is responsible for gluconate-2-dehydrogenase (GA2DH) biosynthesis, which catalyses GA (13) to 2-KGA (14) (Fig. 4). The deletion of the gene enhanced 5-KGA (16) production by 140.33%. The second gene deleted led to the reduction of acetaldehyde and, consequently, acetic acid formation. These byproducts not only seriously affect cell growth but also may diminish 5-KGA (16) yield. In addition, \textit{X. campestris} quinoprotein glucose dehydrogenase is highly active in producing 5-KGA.
(16) from GA (13). The recombinant strain of G. oxydans was used in fermenters with pH control at 4.5 and dissolved oxygen above 20%, resulting in a yield of 81.85% of 5-KGA (16) after 55 h of cultivation (Yuan et al. 2016a).

5-KGA (16) accumulation was also enhanced using a combinatorial metabolic engineering technique in G. oxydans strain. The genes responsible for sorbitol dehydrogenase (sldAB), PQP (pqqABCDE and tldD) and bo3 oxidase (cvoBACD) were overexpressed with the strong promoter, P0169. After the optimization of culture conditions, the increase of 5-KGA (16) titer was tenfold higher (162 g L⁻¹) of that produced by wild strain (G. oxydans DSM2343) in a fed-batch process to maintain the glucose at 40—50 g L⁻¹. The dissolved oxygen was kept above 20% and the pH was controlled at 5.5 in the first stage (during glucose oxidation and cell growth) then shifted to 4.5, facilitating 5-KGA (16) formation (Yuan et al. 2016b).

**Dihydroxyacetone**

Dihydroxyacetone (18) (DHA) is a valuable product due to its importance in chemical, cosmetic, pharmaceutical and food industries (Rajatanavin et al. 2008; Carnali et al. 2012; Stasiak-Różańska and Błazejak 2012; Ciriminna et al. 2018). This compound is a building block in organic synthesis of a variety of fine chemicals, including pharmaceuticals and polymers (Hekmat et al. 2003; Enders et al. 2005; Weiser et al. 2011; Lux and Siebenhofer 2015; Korley et al. 2016).

DHA (18) has unique relevance in the cosmetic industry as a tanning agent, and is considered one of the most suitable self-tanning product due to its satisfactory and durable results (Ciriminna et al. 2018). The tan promoted by DHA (18) on the skin surface is a consequence of its reaction (Maillard reaction) with proteins, which form brown pigments known as melanoidin (Carnali et al. 2012).

In 2019, the global market for dihydroxyacetone (18) was USD 400 million and it is expected to reach USD 500 million by 2026 (Facts and Factors 2020). Manufactures of DHA (18) include Hisun Pharmaceuticals USA Inc. (Bridge-water, NJ, USA) (Hison Pharmaceuticals USA 2019) and Merck KGAa (Darmstadt, Germany) (Merck 2020).

The industrial production of DHA (18) is based on microbial biotransformation of glycerol (17) (Fig. 5) by high activity membrane-bound glycerol dehydrogenase present in some species of acetic acid bacteria (Stasiak-Różańska et al. 2018). Industrial process for DHA (18) production is performed in aerated stirred tank reactors with G. oxydans as a biocatalyst and glycerol (17) as a substrate. The process is conducted in a fed-batch operation mode and the reaction time occurs up to 70 h (Hekmat et al. 2003).

Regarding oxidative biotransformation of glycerol (17), two distinct pathways can be undertaken by G. oxydans (Fig. 1b). In the periplasmic space most glycerol (17) is oxidized, which is essential for providing the energy for cell growth (Claret et al. 1994). Glycerol (17) is directly converted into DHA (18) by glycerol dehydrogenase (GLDH). In the second pathway, the substrate enters the cell and glycerol (17) is phosphorylated to glycerol-3-phosphate, which is dehydrogenated into DHA-phosphate, that further enters the pentose phosphate pathway (Claret et al. 1994; Matsushita et al. 1994).

Despite that Gluconobacter is already used industrially to oxidize glycerol (17) into DHA (18), some hindrances still need to be overcome. The viability of Gluconobacter decreases exponentially with exposure time to DHA (18), and the decrease rate is determined by DHA concentration (Ohrem and Voß 1995). Furthermore, a high initial glycerol (17) content can affect both specific growth rate of G. oxydans and specific rate of DHA (18) production. However, DHA (18) shows more influence in causing an inhibitory effect on microbial growth than glycerol (17) (Claret et al. 1992; de la Morena et al. 2019a). Despite cell damage caused by DHA (18) accumulation in the medium, the formation of this product continues for a period, due to robustness of glycerol dehydrogenase activity (Bauer et al. 2005).

The efforts to enhance industrial DHA (18) production mostly involve the search for new methods to prevent or decrease inhibitory effects of the substrate/product (Hu and Zheng 2011; Dikshit and Moholkar 2016a), strain improvement in bioconversion (Lin et al. 2016; Tan et al. 2019) and optimization of process conditions (Hu et al. 2017b; Sudarshan and Sanjay 2018; de la Morena et al. 2019a). Regarding reduction of costs, the use of biodiesel-derived crude glycerol (17) has been tested as a substrate for DHA (18) fermentation due to its low price on the market (Jun et al. 2010; Stasiak-Różańska et al. 2018; Dikshit and Moholkar 2019; Jittjlang et al. 2020). The environmental commitment for the use of a by-product can also be highlighted. In the last few years, different approaches have been proposed to produce DHA (18) from crude glycerol (17), without prior purification, as shown in Table 2.

Another way to reduce costs is to obtain a purer product already in the biocatalysis phase, in order to reduce the costs of the downstream process since large amounts of impurities and high DHA (18) solubility hinder its purification and crystallization (Xie 2018). Hu et al. (2017b) successfully...
obtained a near colorless DHA (18) solution with few impurities by applying inorganic nitrogen sources or amino acids in the fermentation medium, instead of a yeast extract that hinders DHA (18) recovery.

In order to optimize DHA (18) production the control of the factors that most influence the process is important. De la Morena et al. (2020) successfully established four kinetic model equations to correlate the oxygen transfer rate (OTR) and DHA (18) production, which represents a guide towards the influence of OTR on glycerol (17) to DHA (18) transformation by resting cells of *G. oxydans*. Other recent studies that analyzed oxygen interference on DHA (18) production have been published (Zheng et al. 2016; Zhou et al. 2016; Poljungreed and Boonyarattanakalin 2017; de la Morena et al. 2019b).

Regarding the use of genetic engineering, efficient strains can be constructed by overexpressing or suppressing the genes related to metabolic pathways of DHA (18) production. A 2014 patent (Xu et al. 2014b) claimed an invention that provides a method for constructing a DHA (18) high-producing strain. The inventors constructed a new strain excessively expressing the membrane system of glycerol dehydrogenase, which positively affect DHA (18) production. Tan et al. (2019) overexpressed the gene encoded glycerol transporter (*GlpF*) in *G. oxydans* ATCC 621H aiming to overcome the inhibition caused by high glycerol (17) concentration and to improve cell growth. The authors have

| Microorganism      | Mode of operation | Type of bioreactor | Glycerol concentration (g L⁻¹) | DHA concentration (g L⁻¹) | References                  |
|--------------------|-------------------|---------------------|-------------------------------|--------------------------|-----------------------------|
| *G. oxydans* ATCC 621 | Immobilized extract | Shake flasks      | 30                            | 9.0                      | (Stasiak-Różańska et al. 2017) |
| *G. frauterii* CGMCC 5397 | Free cells | 30 L bioreactor | Initial: 60 / Maintained: 5–25 | 175.7                    | (Zheng et al. 2016)          |
| *G. oxydans* MTCC 904 | Immobilized cells | Shake flasks      | 20                            | 12.5                     | (Dikshit et al. 2018)        |
| *G. oxydans* ATCC 621 | Free cells | Shake flasks      | 30                            | 20.3                     | (Stasiak-Różańska et al. 2018) |
| *G. oxydans* MTCC 904 | Free cells | Shake flasks      | Initial: 20 / Fed-batch: 15 + 15 | 35.0                     | (Dikshit and Moholkar 2019)  |
| *G. oxydans* MTCC 904 | Immobilized cells | Shake flasks      | 20                            | 14.1                     | (Dikshit and Moholkar 2016b) |
| *G. oxydans* MTCC 904 | Free cells | Shake flasks      | 20                            | 13.9                     | (Dikshit and Moholkar 2016a) |
| *G. oxydans* MTCC 904 | Immobilized cells | Shake flasks      | 30                            | 15.5                     | (Jackson et al. 2019)       |
| *G. oxydans* NBRC 14819 | Resting cells | Shake flasks      | Initial: 50 / Fed-batch: 50 + 50 | 52.7                     |                              |
| *G. frauterii* NBRC 103465 | Resting cells | Shake flasks      | Initial: 25 / Fed-batch: 25 + 25 + 25 | 30 (approximately) |                              |
| *G. oxydans* MTCC 904 | Immobilized cells | Shake flasks      | 20                            | 17.8                     | (Dikshit et al. 2017)       |
| *G. thailandicus* TBRC 3351 | Free cells | 7 L stirred-tank bioreactor | 60                            | 53.8                     | (Jittjang et al. 2020)      |
| *G. oxydans* spp. *suboxydans* DSM 50049 | Free cells | 20 L stirred-tank bioreactor | 30                            | 25.8                     | (Liebminger et al. 2014)    |
| *G. frauterii* NKC115 | Free cells | Shake flasks      | 200                           | 37.3                     | (Tanamool et al. 2018)      |
| *G. oxydans* NBRC 14819 | Immobilized cells | Shake flasks      | 50                            | 43.1                     | (Ripoll et al. 2021)        |
| *G. oxydans* MTCC 0904 | Free cells | Shake flasks      | 100                           | 92.6                     | (Sudarshan and Sanjay 2018) |
been able to efficiently construct strains tolerant to high glycerol (17) concentrations.

**Conclusion**

This review highlights the industrial versatility of *Gluconobacter oxydans*, an outstanding bacterium that has been widely used in biotechnological processes. All this versatility is linked to the extraordinary capacity of incomplete regional and stereoselective oxidation of sugars, polyols, and alcohols. All *G. oxydans* products reported here have diverse and important applications and comprise a high value market. The vitamin C production process is one of the oldest industrial applications of *G. oxydans*; the microbial production of 5-keto-D-glucuronic acid is still potential, but of great industrial interest. Strategies for process improvement mainly include optimization of fermentation medium and culture conditions, use of residues and by-products as raw materials, cell immobilization, microbial consortia and genetically improved strains.

The application of agro-industrial wastes or by-products as substrate have been explored to lower process costs and make processes more environmentally friendly. Lignocellulosic biomass and biodiesel-derived glycerol have shown potential to be applied to biotechnological processes and a future advance towards this area can be glimpsed. Regardless of the bioprocesses reviewed, all displayed efforts to genetically improve *G. oxydans* strains, despite the current shortage of suitable genetic tools. The construction of robust strains successfully enhanced bioproduction yields, whether by deleting genes that generate byproducts, expressing heterologous genes or using more potent promoters and vectors. Through -omics technologies, a better understanding of metabolic networks of microbial consortia has been reached. The great versatility already demonstrated by *G. oxydans* points to a potential yet to be explored in new future applications.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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