New perspectives into *Gluconobacter* catalyzed biotransformations

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Abstract

Differently from other aerobic microorganisms that oxidizes carbon sources to water and carbon dioxide, *Gluconobacter* catalyzes the incomplete oxidation of a variety of substrates with regio- and stereoselectivity. This ability as well as its capacity to release the resulting products into the reaction media locate *Gluconobacter* as a privileged member of a class of non-model microorganisms that may boost industrial biotechnology. Knowledge of new technologies applied to *Gluconobacter* has been pilling up in recent years. Advancements in its genetic modification, application of immobilization tools and careful designs of the catalyzed transformations have improved productivities and stabilities of *Gluconobacter* strains or enable new bioconversions for the production of valuable marketable chemicals. In this work we have summarized the latest advancements applied to *Gluconobacter* catalyzed biotransformations which we consider timely and needed regarding the increasing importance this class of bacteria for biotechnological applications.

Keywords

Gluconobacter; biotransformations; whole cell immobilization; genetic engineering; industrial biotechnology; process intensification; green chemistry

1. Introduction

Microbial biotechnology sustains a major part of industrial biotechnology and constitute the fundamental force driving the development and implementation of bio-based industries. Within the family *Acetobacteraceae*. acetic acid bacteria are important players in biotransformations. As such, *Gluconobacter* specie has been frequently used in the last 20 years exploiting its unique ability to incompletely oxidize sugars and alcohols. *Gluconobacter* is a distinct group of microorganisms as biocatalysts, as they lead to quantitative yields of oxidized products in reactions as industrially relevant as the preparation of vinegar, the vitamin C precursor l-sorbose, the tanning lotion additive dihydroxyacetone and 6-amino-l-sorbose used for the production of the antidiabetic drug miglitol. *Gluconobacter* catalysed oxidation reactions often have the added advantage of exquisite selectivity [1] which attracts more interest to its study and exploration of further applications.

The development of bio-based manufacture industry directly depends on the availability of suitable biocatalysts able to provide a wide spectrum of products at large scale. Microbial bioproduction has proven to be key to this development and harness enormous potential. However, there is a yet unexplored vast diversity of non-model organisms with unique features that may facilitate new or improved bioprocesses. One such microorganism is *Gluconobacter* that unlike other aerobic microoganisms that usually oxidize carbon sources to water and carbon dioxide it has the beneficial ability of regio- and stereoselective incomplete oxidation of a variety of substrates in the periplasm by membrane-bound dehydrogenases. As an added advantage this class of acetic bacteria releases the resulting products into the cultivation medium transform it into an excellent candidate for further studies. These advantages have recently prompted investigations on advancing the knowledge on its genetic manipulation and stabilization in order to leverage its phenotype on its full extent.

The last 5 years have seen a significant increase in the wealth of products generated through *Gluconobacter* biotransformations (Figure 1). Although dihydroxyacetone remains as the prevalent product judging from the work generated, it probably responds to the fact that the biotransformation serves as a case study for improvements as it is a very well-established bioconversion.

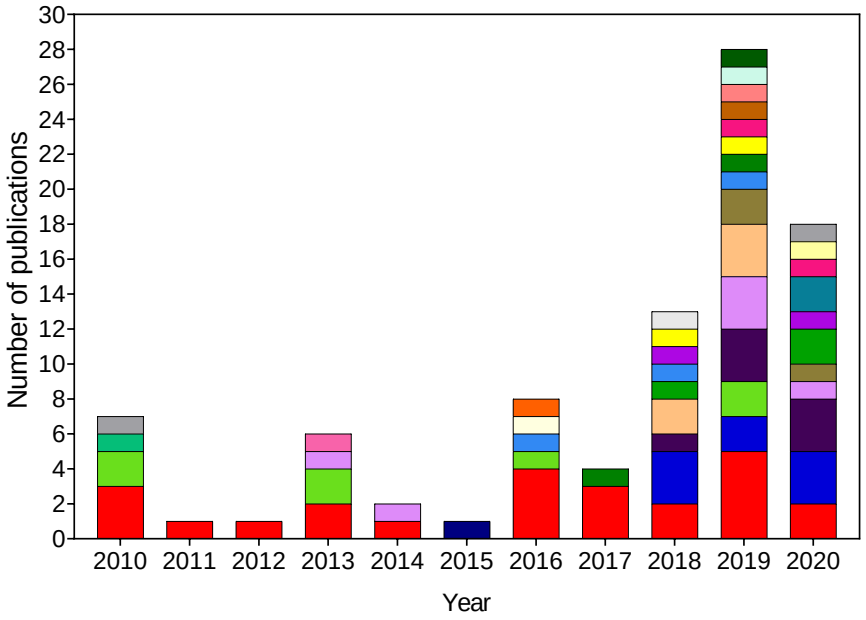


Figure 1: Products obtained through biotransformation using *Gluconobacter* strains as catalysts in the last 10 years.

Several previous reviews have focused on the physiology and application of *Gluconobacter* in specific biotransformations, often included in larger studies involving other acetic acid bacteria. However, the knowledge on this particular bacterium has significatively advanced and matured in the last decade opening up opportunities for examination and knowledge integration. In this work, we aim to contribute with a comprehensive analysis of the different approaches reported to improve the performance of *Gluconobacter* as a biocatalyst. We will review what we consider the most currently relevant examples of process intensification in *Gluconobacter* catalysed biotransformations. Particular attention will be given to the reaction design, including immobilization strategies and bioreactor configuration, both emerging as key players impacting the productivities in *Gluconobacter* catalysed reactions. Moreover, a special focus will also be put on recent molecular biology tools that promise to be game changers in the future application of this bacteria in new biotransformations. This review is not intended as an exhaustive summary of all *Gluconobacter* catalysed reactions reports but instead aims to demonstrate the wealth of enabling strategies that benefit this particular type of biotransformations highlighting the pertinent examples that illustrate significant improvements in *Gluconobacter* technology.

2. Improvements in fermentation

Successful bioprocess development requires addressing existing challenges for improved yields of target products. Process control, poor reaction engineering or long term operational stability are usual bottlenecks that impairs the applicability of otherwise interesting bioconversions at laboratory scale. In this section we summarize recent methods that aimed towards more efficient processes that involve *Gluconobacter* strains.

2.1. Improving catalyst endurance and reusability: Bacterial immobilization

Performing biocatalytic processes with whole cells at an industrial level presents a number of weaknesses [18]. Generally, biocatalyst separation from the reaction media is difficult, limiting the possibilities of reuse and increasing operational costs. Through immobilization, these difficulties can be circumvented, as the biocatalyst is confined in a region of space, with retention of its catalytic activity and enabling a continuous and repeated use [19].

Bacterial immobilization presents several advantages, allowing for increasements in productivity, resulting from an enhanced biocatalyst stability [20]. It also allows for a simpler separation of the catalysts from the reaction medium, which not only enables their reuse, but also the development of continuous systems [21]. However, immobilization can sometimes lead to diffusional problems, inactivation, or even an increase in process costs [20]. Therefore, a careful selection of both, the immobilization matrix and immobilization strategy is essential for the development of an efficient biocatalyst. This selection must also take into consideration the bioprocess in which the catalyst will be used [22]. Moreover, bacteria tend to be susceptible to the immobilization matrix. Particularly, bacteria of de genus *Gluconobacter* have been observed to modify its metabolism due to selection of the immobilization matrix [23]. Hence, the study of new strategies for *Gluconobacter* immobilization aiming for more efficient bioconversions continues to be necessary.

Ideally, the immobilization matrix should be physically resistant, stable, hydrophilic, inert, easily functionalized, biocompatible, resistant to microbial attack and cost-effective [24]. A wide variety of inorganic and organic matrixes are available for bacterial immobilization. Inorganic matrices include, for example, sintered glass, ceramic, activated carbon or cross-linked foam. Examples of natural matrixes are collagen, agar,

agarose, cellulose, κ-carrageenan and alginate, while synthetic matrixes are constituted by polymers such as acrylamide, polyurethane and polyvinyl [21,22].

As for bacterial immobilization strategies, coupling, encapsulation, aggregation and entrapment are the most frequently used [25,26]. Coupling, either by adhesion or adsorption, is the attachment of cells to a surface naturally or induced by the addition of binding agents [20]. Encapsulation consists of bacterial confinement, in which the bacteria are separated from the reaction medium by a barrier, which can simply be the boundary between two immiscible fluids in an emulsion [22,27]. Aggregation involves the formation of cell aggregates, linked together naturally or by the addition of flocculating or binding agents [22].

There are numerous examples in literature of *Gluconobacter* immobilization [28–32], mainly of strains from the *G. oxydans* species used in biotransformations [33–36]. However, *Gluconobacter* immobilization has not only been used for bioconversions, but also for the preparation of biosensors and microbial fuel cells [13,17,37–39]. In this section of the review, recent efforts concerning immobilization techniques for *Gluconobacter* are summarized, focusing on biotransformations.

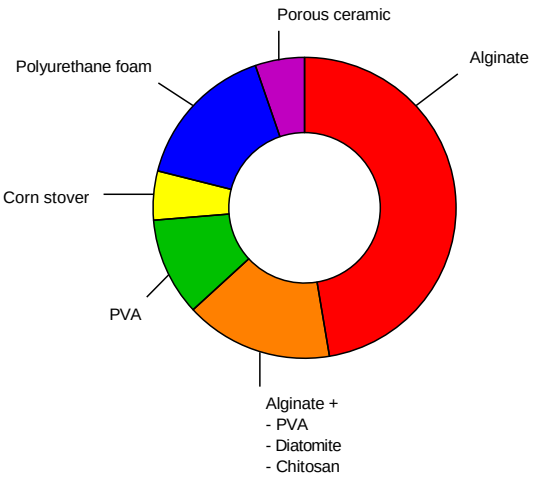


Figure 2. Immobilization matrixes used for *Gluconobacter* immobilization in the last ten years.

2.1.1. Alginate

Alginate is by far the preferred matrix for *Gluconobacter* immobilization. It is affordable, biocompatible, chemically inert and easy to operate, mostly in mild conditions [34,38].

Usually, the chosen format for bacterial immobilization in alginate is beads, as their preparation is simple, scalable and cost-effective [39–41]. It mainly involves the mixture of the biomass with an aqueous alginate solution, with a subsequent dropwise addition of the resulting mixture into a solution of $CaCl_2$. After incubation, the resulting beads can be easily separated with a strainer from the cross-linking solution.

With this immobilization technology, several products were recently obtained through *Gluconobacter* based preparations: dihydroxyacetone (DHA) [31,38,42,43], L-sorbose [44,45], benzaldehyde [34], 3-dehydroshikimate [46], vinager [41], xylonic acid [39,40], 3-hydroxypropionic acid and erythrulose [40].

The properties of alginate immobilized preparations depend on a series of parameters that when optimized can allow for high productivities and robust biocatalysts. Wu et al optimized some of these parameters using response surface methodology for the production of benzaldehyde with *G. oxydans* M5/ALDH, but their findings are transversal to most alginate immobilized systems [34]. Concerning the concentration of alginate used for immobilization, activity generally diminish with an increase in concentration. Typically, concentrations above 4% tend to hinder substrate and product diffusion, being concentrations of 2-3% optimal. Bead diameter also plays an important role in both expressed activity and mechanical stability of the biocatalyst, as smaller beads were shown to be more resistant to mechanical stress and allow a better mass transfer rate.

Despite the aforementioned diffusional problems usually associated with immobilization, an optimal design of the alginate biocatalyst may allow activities comparable with those of free cells [34].

Moreover, in order to improve the mechanical stability of the beads, alginate was successfully mixed with chitosan [42], PVA [39], diatomite or SiO_2 [44] for the production of DHA, xylonic acid and L-sorbose, respectively.

As strict aerobes, the immobilization of *Gluconobacter* species in alginate is challenging, due to oxygen diffusion problems. However, strategies have been developed to improve oxygen transfer to the cells from reaction media. Black and Nair studied different aeration rates during DHA production by *G. xylinus* immobilized in chitosan-coated alginate beads [42]. An increase in the aeration rate from 0.3 to 1 vvm was accompanied by an increase in both DHA production and glycerol consumption. The use of different oxygen vectors, such as cetane, n-hexane and oleic acid, was assayed by Wang et al in the production of

L-sorbose [44]. In shake flask reactions, 3% oleic acid proved to increase the L-sorbose yield. However, the addition of oxygen vectors can make downstream processing and may lead to a longer fermentation [45].

As mentioned before, reusability is a desirable feature when developing an immobilized preparation. In literature, there are many examples of alginate immobilized *Gluconobacter* that are capable to be reused with high yields. Wu et al were able to reuse their immobilized preparations for ten cycles in the aforementioned synthesis of benzaldehyde, maintaining a residual activity of the 53.2%, in contrast to a residual activity of 15.7% presented by free cells [34]. Wang et al report the repeated use of *Gluconobacter oxydans* WSH-003 immobilized in a combination of alginate and diatomite [44]. In this work, the beads colud be reused up to ten times, achieving an L-sorobose yield of nearly 81%.

2.1.2. Polyurethane foam

Polyurethane foam is a novel immobilization matrix that has been recently studied by Dikshit et al for the immobilization of *G. oxydans* MTCC 904 aimed at the synthesis of dihydroxyacetone [32,47–50]. This immobilization matrix has high porosity and a large surface area that enables to obtain a high cell density in a relatively small volume [50]. Apart from including a low-cost matrix, the immobilization protocol is fairly simple [49].

Additionally, Dikshit et al describes an improvement in DHA production combining the immobilization of *G. oxydans* in polyurethane foam and the sonication of the fermentation media [47]. In the aforementioned work, *G. oxydans* was again immobilized in polyurethane foam and was sonicated at a 20% duty cycle together with the fermentation medium during the biotransformation. An increase in glycerol consumption of between 61.06 and 71.70% was obtained at different initial crude glycerol concentrations (20 – 50 g/L). The authors attribute this enhancement of DHA production with conformational changes in GlyDH induced by ultrasound, as they proved that while sonication had no effects in cell morphology, it may have affected the secondary structure of the enzyme.

Using this immobilization strategy Dikshit et al also carried out the biotransformation of crude glycerol to DHA in Fed-batch mode. In this work, various pulse-feeding strategies in shake flasks were tested [32]. In comparison with resting and free growing cells, the cells that were immobilized in polyurethane foam showed a better DHA yield, achieving a glycerol conversion of 87.52% equivalent to 65.64 g/L of glycerol after 4 glycerol feeding pulses (15

g/L). Nonetheless, DHA production diminished with each glycerol pulse. The kinetic constants for each segment between pulses was determined by fitting the data to a pseudo 1st order rate equation, with results that were consistent with product inhibition.

2.1.3. Polyvinyl alcohol (PVA)

Polyvinyl alcohol (PVA) is an interesting synthetic polymer that can be used for bacterial immobilization as it is a cheap, non-toxic and durable hydrogel [51,52]. It constitutes an alternative to natural polymers such as carrageenan, pectate and alginate that generally present poor mechanical stability, as it is scarcely biodegradable and resists temperatures up to 55 ºC and pH values between 3.1 y 8.5 [53]. This matrix has been recently used for biotransformations with a variety of microorganisms, alone or combined with other materials such as alginate and polyethylene glycol [54–56].

Concerning *Gluconobacter* strains, there are recent reports that use PVA as an immobilization matrix. As an example, Mihal' et al report the immobilization of *Gluconobacter oxydans* NCIMB 8035 for the production of phenylacetic acid (PAA) from 2-phenyletanol (PEA) [57]. In this work, the LentiKats® technology is used, which consists on the formation of lenticular shaped hydrogels that present lesser diffusional problems and allow an easy separation from the reaction media due to their diameter [53]. The biotransformation was carried out in non-growing condition in an airlift bioreactor. Immobilization in PVA proved beneficial for the cells, as it protected them from death induced by high concentrations of PEA. However, an expected decrease in the rate of oxygen transfer as well as in the exchange rate of substrate and product to and from the biocatalyst was observed. Using the immobilized biocatalyst 7,17 g/L of PAA were obtained, which represents an 89.8% yield. The immobilized preparations could also be reused up to three times maintaining a 44% residual activity. In addition, it was demonstrated that the LentiKats® could be stored at 6°C in a 1 M phosphate buffer solution for 28 days, with minor effects in their activity.

When immobilizing in PVA, it is not uncommon to experience bead agglomeration. To avoid this phenomenon, alginate is frequently mixed with PVA, further improving the beads surface [56,58,59]. Hua et al recently used this strategy to immobilize *Gluconobacter oxydans* NL71 for the production of glycolic acid from ethylene glycol [33]. In this work, the immobilization was carried out by preparing an aqueous solution of 9% PVA with 1-2% alginate and mixing it with the dried biomass. The mixture was

then pumped into a crosslinking solution containing 5% boric acid and 1% $CaCl_2$, resulting in beads with a 2-3 mm diameter. The biotransformation experiments were carried out using free and immobilized cells, in an air-open bioreactor, an oxygen-open bioreactor and an oxygen-compressed bioreactor. After immobilization, a decrease in mass transfer was observed, that could be improved by oxygen supplementation, improving glycolic acid production for both free and immobilized cells.

With the same immobilization approach, Zhou et al prepared PVA-alginate beads containing *G. oxydans* NL71 for the production of xylonic acid from xylose [39]. In this work, a pure pressurized oxygen aerated and sealed stirred bioreactor (POA-SSB) was used to overcome this problem, obtaining 276.5 ± 5.4 g/L of xylonic acid after 48 hours in fed-batch mode. Repeated use of this immobilized preparations in 28 hours cycles starting from 200 g/L of xylose was tested, obtaining approximately 1,6 kg of xylonic acid after 8 repeated uses. Additionally, the beads could be used in a continuous system that incorporated bipolar membrane electrodialysis for the obtention of xylonic acid instead of its sodium salt. With this approach, 329.2 ± 7.2 g/L of xylonic acid was accumulated in the electrodialysis acid chamber after 48 hours.

2.1.4. Corn stover

In a recent report, Hu et al immobilized *G. oxydans* ZJB16009 on corn stover particles for the production of miglitol precursor 6-(N-Hydroxyethyl)-Amino-6-Deoxy-α-L-Sorbofuranose (6NSL) from N-2-hydroxyethyl glucamine [60]. This immobilization strategy involves cell adsorption to the lignocellulosic material. As corn stover bagasse is a residue of corn production, it is a cheap feedstock to use as an immobilization matrix, that also contributes to the biorefinery concept [61]. In these optimal conditions, the immobilized preparations could be reused up to 4 times, maintaining a residual activity above 70%. After 4 rounds, a production of 44.2 ± 1.5 g/L of 6NSL was achieved, corresponding to a conversion rate of 88.4 ± 2.0%.

2.1.5. Porous ceramic

Hu et al also used porous ceramic for the immobilization of *G. oxydans* ZJB09113 for the production of dihydroxyacetone from glycerol [62]. three different porous ceramics were tested, with pore sizes of 30, 40 and 50 µm and a 10 mm diameter maximum DHA production was achieved with ceramics with 40 µm pores. With this strategy, an approximate 4.8 g/L of cells were immobilized. The optimum pH value selected was pH

4.5, as DHA production is favored at low pH and there is less probability of contamination with another bacteria. Several aeration rates were tested and 1.2 vvm proved to be the optimal for glycerol bioconversion. In comparison with a previous work from Hu et al [63], the authors discuss that optimal aeration rate for resting free cells was higher than that for immobilized cells. This is an advantage of bacterial immobilization in porous ceramic carriers, as gas dispersion is beneficiated, extending the residence time of air in the bubble column bioreactor. The production of DHA was studied in the optimized condition with different glycerol and urea feeding strategies. Continuous feeding of a glycerol and urea solution achieved the highest DHA concentration (177.2 ± 6.8 g/L).

2.1.6. Polyelectrolyte complex

Bertóková et al immobilized *G. oxydans* NCIMB 8035 in polyelectrolyte complex capsules (PEC) for the production of phenylacetic acid (PAA) [64]. The complex consisted of a mixture of sodium alginate, cellulose sulphate, poly(methylene-co-guanidine), $CaCl_2$ and NaCl.

the immobilized preparations experienced mass transfer restrictions in comparison with free cells. However, at high substrate concentrations, the specific activity of the capsules was comparable with that of free cells. Additionally, the reusability of the capsules was assessed, maintaining full catalytic efficiency for 12 cycles, while free cells lost the ability to produce PAA after the 7^{th} cycle. The immobilized preparations also could be stored for up to 64 days with no loss in activity. In a bubble column reactor, a concentration of 25 g/L of PAA was achieved with this immobilization technology after subsequent uses during a 7-day period.

2.2. Improving oxygen availability: Bioreactor selection

As obligate aerobes, bacteria of the genus *Gluconobacter* tend to present limitations when used as biocatalysts in large-scale fermentations. The design of the bioreactor must account for the elevated oxygen demand of the bacteria. Moreover, when working with immobilized bacteria it is frequent to encounter mass transfer limitations that hinder productivities. With a proper bioreactor design, these mass transfer limitations can be overcome. In recent years, several reactor configurations have been studied in order to obtain improved yields in a wide variety of *Gluconobacter* catalysed biotransformations, with both free and immobilized cells.

2.2.1. Open fermentation system

At fermenter scale, oxygen transfer can be easily benefited by replacing air with pure oxygen. With this strategy, recently Hua et al achieved high titers of xylonic acid (588.4 g/L), 3-hydroxypropionic acid (69.4 g/L), and erythrulose (364.7 g/L) in a sealed oxygen supplied bioreactor (SOS-BR) [40]. The strengthening of oxygen partial pressure inside the bioreactor not only had virtually no effect in the cell retention rate of the alginate beads, but also inhibited the formation of foam. With a similar approach, Zhou et al used a traditional open fermentation system with a pure oxygen supply in the production of xylonic acid by alginate-PVA immobilized *Gluconobacter oxydans* ATCC 621 [39]. In this case, the immobilized preparations could be reused effectively for 8 cycles, with a 1,6 Kg yield of xylonic acid.

2.2.2. Bubble column bioreactors

Bubble column bioreactors have a very simple structure and count with no mechanical agitation. Both aeration and agitation are attained by gas sparging [65]. They are presented as a low cost and low energy consuming option for bioconversions with obligate aerobes. Recently, this technology was used by Hu et al to produce DHA and 6-(N-Hydroxyethyl)-Amino-6-Deoxy-α-L-Sorbofuranose (6NSL) with immobilized *G. oxydans* ZJB09113 [60,62]. DHA production was carried out in a home-made bubble column bioreactor with a 2L working volume [62]. The height-to-diameter ratio of the fermenter was 6.0 and it included a sintered glass gas difusser with pores ranging from 80 to 120 μm. To maintain a temperature of 30ºC water was circulated within an outer jacket, pH was controlled manually. A water cooler condenser was added at the top of the reactor and sterile water was added as needed to avoid volume reduction due to evaporation. Polyethylene glycol was used as an antifoaming agent. With this reactor configuration several aeration rates were tested using porous ceramic immobilized *G. oxydans* as catalysts. An aeration rate of 1.2 vvm allowed for the maximum DHA yield. The authors proposed that the immobilization carrier could benefit gas dispersion, allowing for a larger air retention time, further contributing to achieve high productivities with less air expenditure.

For 6NSL production, a 1 L home-made bubble column reactor with a height-to-diameter ratio of 6.0 was used [60]. The gas difusser was made of sintered glass with pores ranging

from 80 to 120 µm. The system was completed with a cooling jacket and a pH controlling system to maintain 15ºC and pH 5.5. Polyethylene glycol was added as an antifoaming agent. Aeration proved to be a key element both for the production of the target compound and the maintenance of bacterial fitness during the reaction. At aeration rates below 1 vvm 6NSL production was hindered and cell damage was observed, as the immobilized preparations could not be reused more than two times. An increase in aeration rate was accompanied by an increase in 6NSL production, reaching a maximum at 2.5 vvm where the oxygen supply was sufficient for mixing and for the biotransformation. This technology proved to be a simple alternative to more complex reactors, allowing for high fluid circulation, proving good mass and heat transfer with lower shedding stress. This last characteristic makes this kind of reactors especially suitable for use with immobilized bacteria.

2.2.3. Airlift bioreactors

Like bubble column bioreactors, airlift bioreactors are pneumatically agitated, relying only in the aeration rate for both providing the oxygen needed for the biotransformation and for mixing. The main difference between these types of bioreactors is that liquid circulation is achieved in the airlift bioreactors in addition to that caused by the bubble flow [65]. As previously discussed in the immobilization section of this review, Mihal' et al used immobilized and free biomass of *Gluconobacter oxydans* NCIMB 8035 for the production of phenylacetic acid (PAA) from phenyl ethanol (PE) utilizing an internal loop airlift bioreactor [57]. In this case, results showed that for immobilized cells, the aeration rate had only a minor effect on the PAA production. However, for free cells, aeration in the reactor had a significant impact on PAA production depending on the biomass concentration. In the conditions of the experiment, the final PAA concentration was approximately 9.3 g/L, 30% higher than the one achieved with the immobilized preparations.

2.2.4. Oxygen-compressed bioreactors

Reactions with obligate aerobes require high oxygen environments that most of the times cannot be achieved simply by supplying air into the reactor. Replacing air with pure oxygen is the simplest way to achieve an oxygen enriched environment [40]. However, due to the elevated cost of oxygen, this strategy results non-viable when working with open systems. Alternatively, the use of oxygen-compressed bioreactors for these

biotransformations allows for oxygen enriched environments with high partial pressures that result in a high utilization rate of oxygen for a fraction of the cost [40]. Additionally, oxygen-compressed bioreactors eliminate the requirement for antifoaming agents due to the high pressures achieved inside the vessel, easing the obtention of more pure end products [39]. These bioreactors have been recently used for the production of DHA, sorbose, acetoin, erythrulose and 3-hydroxypropionic acid with *G. oxydans* NL71 free cells and glycolic acid and xylonic acid with immobilized preparations of this strain (Table X).

Table X: Production of various compounds using free and immobilized cells of *G. oxydans* NL71 in different types of stirred bioreactors.

Product	**Cells**	**Productivity (g/L.h)**			**Ref**
		Air-aerated open bioreactor	**Oxygen-aerated open bioreactor**	**Oxygen compressed bioreactor**	
Sorbose	Free	4.32 (72 h)	6.12 (72 h)	6.01 (72 h)	[66]
Xylonic acid	Free	10.7 (24 h)	25.4 (24 h)	24.5 (24 h)	[40]
	Immobilized	7.9 (24 h)	11.7 (24 h)	12.2 (24 h)	[40]
	Free	1.59 (48 h)	-	1.95 (48 h)	[39]
	Immobilized	0.86 (48 h)	-	1.39 (48 h)	[39]
3-Hydroxypropionic acid	Free	-	-	2.82 (24 h)	[40]
Erythrulose	Free	-	-	14.78 (24 h)	[40]
Glycolic acid	Free	1.59 (48 h)	1.95 (48 h)	-	[33]
	Immobilized	0.86 (48 h)	1.39 (48 h)	1.32 (48 h)	[33]
Dihydroxyacetone	Free	1.94 ± 0.09 (102 h)	-	9.41 ± 0.26 (32 h)	[67]
Acetoin	Free	3.25 (30 h)	-	4.61 (36 h)	[68]

A clear example of the benefits of this technology with both free and immobilized cells is described by Hua et al in the production of glycolic acid from ethylene glycol. Reactions were carried out using free or PVA-alginate immobilized *Gluconobacter oxydans* NL71, in three different reactor configurations: air-open stirred bioreactor, oxygen-open stirred bioreactor and oxygen-compressed stirred bioreactor [33]. Initial experiments of glycolic acid production were carried out in an air-open stirred bioreactor.

From the results, it became evident that free cell catalysis was more efficient, as immobilized cells only reached a glycolic acid yield of 41.3 g/L, which means 54% of the yield achieved with free cells (76.5 g/L) in the same 48 h time frame. The dissolved oxygen (DO) level in the free cell experiment was lower in comparison with the immobilized cell experiment, indicating that the demand and consumption of immobilized cells was lower than that of free cells due to mass transference problems. In order to improve the oxygen uptake, the glycolic acid biosynthesis was carried out in an oxygen-open stirred bioreactor, with a pure-oxygen feed rate of 0.5 vvm. In these conditions, not only the glycolic acid production for both free and immobilized cells increased (93.5 g/L and 66.9 g/L, respectively) but also oxygen consumption was increased. In both cases, the DO profile levels remained at nearly 35% during the biotransformation, meaning an improved mass transfer for the immobilized cells. However, as this oxygen supply strategy is unfeasible at a larger scale, the authors evaluated the use of an oxygen-compressed stirred bioreactor. The production of glycolic acid with this reactor reached 63.3 g/L in 48 h, virtually the same yield achieved with the immobilized cells in the oxygen-open stirred bioreactor but saving the oxygen consumption by a 40-fold.

2.3. Reaction media optimization.

Particularly, when working with bioconversions using whole cells in growing phase, the reaction media usually has a complex composition that complicates the downstream processing and elevates production costs. In order to solve this problem, Poljungreed and Boonyarattanakalin studied the production of DHA by *G. frateurii* BCC36199 in a simplified reaction media [69]. The replacement of yeast extract by an inorganic source of nitrogen in the media such as $(NH_4)_2SO_4$, increased the amount of DHA produced from 23.77 ± 1.30 g/L to 28.89 ± 0.17 g/L. Interestingly, after the biotransformation, the biomass yield achieved with the inorganic media was significantly lower than the one achieved with the organic media (0.31 0.04± $g_{biomass}$/L vs. 0.83 ± 0.17 $g_{biomass}$/L). Probably, in a complex reaction media, the portion of glycerol destined to produce ATP and biomass might be increased, having a direct impact on the DHA production yield [69]. In the same work, the authors modeled DHA production in order to further optimize the minimum reaction media composition. High concentrations of glycerol were found to be detrimental for DHA production, while the supplementation of the reaction media with

$(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $CaCO_3$ and $MgSO_4$ were found to have a positive effect on the biotransformation [69]. After optimization, the model developed by the authors could correctly predict the DHA yield of the conversion of 30 g/L of pure glycerol accurately. These findings could be further applied to crude glycerol bioconversions. In fact, in a recent study we have demonstrated that DHA production from crude glycerol is possible in minimal medium as resting cells of *G. frateurii* NBRC 103465 and *G. oxydans* NBRC 14819 were used for the conversion of crude glycerol from the biodiesel industry [70].

Another disadvantage of biotransformations with growing cells is that secondary reactions are bound to happen due to the active bacterial metabolism, further contaminating with by-products the already complex reaction media. By using resting cells, this is not an issue, as they are in a non-growing state in which secondary reactions are minimized. As a starting point, we carried out the biotransformation of pure glycerol to DHA and glyceric acid (GA) (another value-added product) in non-sterile conditions, with a minimum medium consisting of glycerol 25–170 g/L, KH_2PO_4 0.9 g/L, K_2HPO_4 0.1 g/L, $MgSO_4.7H_2O$ 1 g/L, pH 3–8. After studying different growth phases to collect the cells, initial pHs, inoculum and initial glycerol concentrations, the conversion of crude glycerol was carried out by both *Gluconobacter* strains. Conversion kinetics were studied after 45 hours starting from 50 g/L (*G. oxydans*) or 25 g/L (*G. frateurii*) of crude glycerol. *G. oxydans* was able to produce 45.62 ± 5.71 g/L of DHA, while *G. frateurii* produced 18.12 ± 1.94 g/L of DHA and 0.61 ± 0.19 g/L of GA, proving that crude glycerol conversion to DHA can be also carried out in a minimum media. The promising results obtained with *G. oxydans* prompted our group to further simplify the reaction media for the conversion of crude glycerol to DHA by using only water. Our first approach was the use of distilled water as reaction media, which resulted in the production of 42.10 ± 2.32 g/L of DHA in 20 hours. Similar results were obtained with regular water, achieving the production of 40.18 ± 0.04 g/L of DHA in the same time frame.

Crude glycerol composition may vary depending on the origin of oils and fats, and the reaction conditions used during the transesterification process [71]. Mainly, this by-product is contaminated with ashes, moisture, soap, methanol and chlorides [72]. These contaminants can affect the DHA production from this feedstock, so it is of great importance to develop simple processes that can remove these contaminants before bioconversions. In a recent study Jittjang et al treated crude glycerol derived from palm

oil by ion exchange chromatography in order to remove NaCl, with a 74% removal yield [73]. Their results indicate that a low-cost ion exchange technique increases the transformation yields of crude glycerol bioconversion to DHA. The reaction was successfully scaled up to a 3L volume in a 7L bioreactor operated in batch mode, achieving a DHA production of 61.9 ± 2.57 g/L after 138 hours [74]. Production of DHA in fed-batch mode demonstrated product inhibition. The DHA concentration achieved after 156 hours (65.05 ± 4.52 g/L) was not significantly different to that of the batch reaction.

3. Improvements through genetic modification

Gluconobacter has many qualities that turn it into a potential synthetic biology relevant chassis. This microorganism can oxidise a great number of carbohydrates, alcohols and other relational compounds [95], which can be further taken advantage for potential metabolic engineering purposes [96]. Therefore, this section will focus on reviewing the different strategies used to perform genetic modification in *Gluconobacter* in order to generate strains with increased bioprocess efficiency.

3.1. Random mutagenesis

3.1.1. Adaptive evolution

A great tool to generated strains with a high yield of production is the adaptative evolution. The adaptive evolution submits to the microorganism to an unfavorable environment to force the microorganisms to mutate. The adaptive evolution and selection of mutants with better characteristics has been successfully applied in *Gluconobacter* strains. Jin and collaborators intensified sugar simultaneous utilisation from the lignocellulose oxidation using an adaptive evolution approach [97]. They continuously alternated the medium with different hydrolysate inhibitors in order to develop more robust *Gluconobacter oxydans* fermentation. The expression of the membrane bound PQQ-dependent glucose dehydrogenase enzyme (mGDH) increased 40-folds in comparison to the parental strain. mGDH catalyse the conversion of lignocellulose-derived sugars to the corresponding sugar acids.

Zhu and collaborators used adaptative evolution to enhance the growth of *Gluconobater oxydans* membrane-bound glucose dehydrogenase-deficient strain (GDHK) [98]. The results showed an increment of 1.4-fold in the maximum specific growth and biomass yield compared with the GDHK strain. Also, the modified strain had the capacity to grow in low glucose concentration (10g/L) and could catalyse the biotransformation of ethylene glycol to glycolic acid and glycerol to dihydroxyacetone. The adaptive evolution is a great method to create strains with a high yield and adapting to unfavourable environment. However, this process usually requires a long time and constantly transfer of medium to generate these strains, an alternative of this disadvantages is the implementation UV mutagenesis.

3.1.2. UV mutagenesis

The UV mutagenesis is effective genetic technique to create mutants with biotechnology application. This method consist in induces mutation by UV irradiation, after that select the mutant with the characteristic desired. Ke and collaborators exposed *Gluconobacter oxydans* to UV irradiation for generating a strain with improved membrane-bound d-sorbitol dehydrogenase (mSLDH) catalytic activity, reaching a 1.5-fold catalytic activity improvement and an increase of 34% for the production of 6-(*N*-hydroxyethl)-amino-6-deoxy-a-L-sorbofuranose [99]. Furthermore, Zhu and collaborators combined the UV-mutagenesis technique with advanced transcriptional analysis to demonstrate that the membrane-bound aldehyde dehydrogenase (mALDH) and membrane-bound alcohol dehydrogenase (mADH) were involved in the 3-hydroxy propionic acid (3-HP) production in *Gluconobacter oxydans* [100]. The authors then successfully generated two strains overexpressing *adh*AB and *aldh* in pBBR1MCS-5 increasing production for 3-HP up to 46 g/L when the strains were mixed at a 1:2 (cell density) ratio respectively. Lin and collaborators used a combination of UV irradiation and ion beam implantation in order to generate aleatory mutations in *Gluconobacter oxydans* allowing to increase the production of the 1,3-dihydroxyacetone (DHA) to 104 g/L, 115.7% higher than the wild type strain [101].

Although the previously described methods are fast and efficient to generate strains with desired characteristics, theses methodologies have several disadvantages, like the necessity for large-scale screening or negative effects in the enzymatic activity [102]. An

alternative for this is taking advantage of the latest developments in rational plasmid and genome engineering techniques described in the next sections.

3.2. Rational plasmid and genome editing

3.2.1. Promoter characterization

Several plasmid expressions vectors have been successfully constructed to be used in *Gluconobacter species to* confer new phenotypes or alter gene expression. Shen and collaborators overexpressed the gene *trx*, which encoded the thioredoxin enzyme responsible for maintaining the internal redox potential which is essential in converting xylose to xylonate [103]. In this study, the gene *trx* was designed to be controlled by the heterologous *lac* promoter using vector pBBR1MCS-5. The production of Trx increase the resistances of *G. oxydans* to formic acid and PHBA generated in the xylonic acid production. Also, Zhang and collaborators overexpressed the gene *adhS* using vector pBBR1MCS-5 that encoded the subunit of the membrane-bound alcohol dehydrogenase (mADH) [104]. This overexpression influenced the increase of mADH activity up to a 1.96-fold, also the glyceric acid production increased by an 8-fold. In another study, the mADH was overexpressed using plasmid vector pBBR1MCS-5. The result of this was increased resistance to glycolic acid and increased biomass by around 30% [85].

Wang and collaborators overexpressed the gene cluster (*pqqA, pqqB, pqqC, pqqD* and *pqqE*) implicated in the pyrroloquinoline quinone (PQQ) synthesis using plasmid vector pBBR1MCS-2 [105]. For this, they used two different promoters to regulate each single gene; these promoters were *pqqA* and *tufB.* The PQQ extracellular production was enhanced 39% (67.6 μg/L), 68% (82.2 μg/L), 20% (58.5 μg/L), 30% (63.3 μg/L) and 8% (52.8 μg/L) respectively for each gene when using the promoter *pqqA.* In comparison when using the promoter *tufB,* the enhanced PQQ production was increased by 59% (77.8 μg/L), 86% (90.6 μg/L), 31% (63.9 μg/L), 42% (69.4 μg/L) and 19*% (58.2 μg/L)* respectively. Yuan and collaborators tested different constitutive promoters (P0169, PtufB, P0264 and P0452) to overexpress the sorbitol dehydrogenase enzyme using plasmid vector pUCpr [92]. The greater production was obtained with the P0169 promoter (122 g/L of 5-keto-D-gluconic), followed by promoters P0264, PtufB and

P0452. Also, Tan, Yang and Lu compared the promoters PtufB, Pgmr, Pglp1 and Pglp2 to overexpress glycerol transporter (GlpFp) in pBBR1MCS-5. The overexpression with PtufB or Pgdh promoter increased the 1[106],3-Dihydroxyacetone production by 13% [107].

3.2.2. Heterologous gene expression

Gluconobacter has also been engineered for the heterologous gene expression to confer new characteristics to the strain. Herweg and collaborators expressed a fructose dehydrogenase using the plasmid pBBR1p264- FDH-Strep from *G. japonicus* into *G. oxydans* with the goal of producing 5-ketofructose as a promising sweetener [106]. The expression of this gene resulted in 489 g/L 5-ketofructose concentration. Also, Ke and collaborators produced the *sldA* and *sldB* subunits of membrane-bound D-sorbitol dehydrogenase (mSLDH) [108]. *sldAB* was regulated by the promoter gHp0169 and was synthesized and cloned into pBBR1MCS-5. The result of the production of these recombinants subunits generated increasing enzyme activity of mSLDH by a 2-fold. Wang and collaborators expressed the heterologous genes *luxI* and *luxR* (from *Vibrio fischeri*) and *ccdB* (from *E. coli*) into *G. oxydans* using vector pBBR1MCS-5 and *lux pR* promoter and *tufB* promoter for controlling the *G.oxydans* growth and formed consortia with *K.vulgaris* and *B.megaterium* to produce 2-keto-L-gulonic acid (2-KGA) fermentation [109]. The highest 2-KGA titer producer for the consortia as the 69 g/L.

Recently, Kim et al. noticed the great efficiency of *G. oxydans* sorbitol dehydrogenase (GoSLDH) to produce L-sorbose [110]. However, in the L-sorbose production also the NADPH was produced which is an inhibitor of the GoSLDH. Therefore, the authors express LreNOX (NAD(P)H oxidase from *Lactobacillus reuteri* in pBBR1MCS-2 to convert the NADPH into $NADP^+$. The resultant strain produced 2.9-fold L-sorbose in comparison with the wild type strain.

Although important success has been achieved using plasmids for the heterologous expression of genes in *Gluconobacter* species, using plasmid has several disadvantages, including a high metabolic burden, the need to use antibiotics as resistance markers and the high heterogeneity in copy number among others, which is pushing the preference of the biotechnology industry for plasmid-free strains [111]. A solution to this is applying

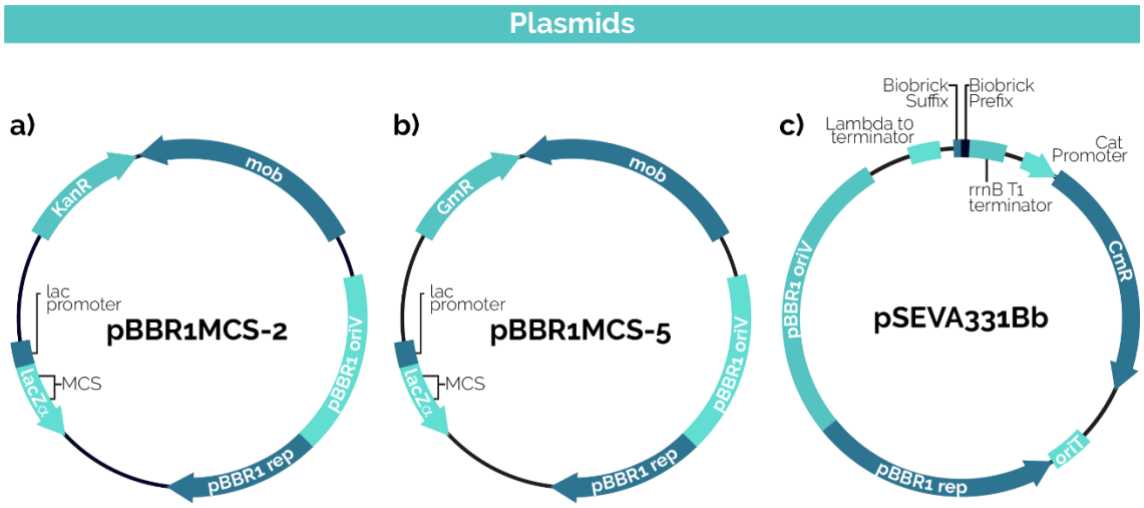
the novel genome engineering tools developed for *Gluconobacter* species which will be discussed in the next section.

3.2.3. Genome engineering

The industrial impact and further potential of *Gluconobacter* generated the necessity of the development and adaptation of efficient genome engineering technologies to of *Gluconobacter* genome for metabolic engineering applications. Plasmid transformation has a high relevance for editing the *Gluconobacter* genome. For this organism, the transformation method more widely used is the triparental conjugation or transmating. The bacterial conjugation is the genetic material mobilization from a bacterium to another cell in a process where there needs to be cell-to-cell contact [112]. For *Gluconobacter* the triparental technology consists in the plasmid mobilization from a donor organism (*E. coli* DH5α) to a receptor organism (*Gluconobacter* strain) assisted by a helper organism (*E. coli* HB101). The helper organism has a mobilization plasmid which for *Gluconobacter* strains it is usually the pRK2013 vector, which is the responsible for the mobilization of the plasmid by cell-to-cell contact.

The genome modification/insertions by homologous recombination have also been successfully achieved in *Gluconobacter* species, using the plasmid pK18 and homologous sequences to facilitate the insertion of a target gene into the microorganism [113]. A critical part of genome modification in *Gluconobacter* is the selection of the mutant. Schäfer and collaborators designed the plasmid pK-mobSacB which contains the mutant marker selection *sacB* [113]. However, this system did not work well in *Gluconobacter*. For this reason, Peters and collaborators developed a deletion system in *Gluconobacter oxydans* based on the method of conjugation, consisting in the use of *upp* mutant marker selection [95]. *Upp* encode the hosphoribosyltransferase that catalyse the reaction of 5-fluorouracil (FU) to 5- fluorouridinemonophosphate (F-UMP). They applied this method for the successful deletion of the pyruvate decarboxylase which was responsible for acetate formation. The authors also deleted all the membrane-bound dehydrogenases and evaluated its impact in the carbon utilisation and morphology when using fructose, mannitol and glucose as carbon source [114].

Zeng and collaborators used the same methodology for eliminating the *ga5dh-1* gene and overexpressing the ga2dh-A gene [91]. The result of these modifications was the increase of 2-keto-D-gluconic acid (2-KGA) production by 91%. Furthermore, Yan and collaborators (2018) utilised this methodology successfully for deleting the major membrane-bound alcohol dehydrogenase (GOX1068) and polyol dehydrogenase (GOX0854) with the goal of demonstrating the implication of these enzymes in the use of *glycerol* as a carbon source in *Gluconobacter*. However, is important keep developing techniques to edit the *Gluconobacter* genomes to create more ambitious projects and harness the biotechnological application of this organism.



d)

Promoters
Constitutive
J23100
J23101
J23102
J23104
J23105
J23108
J23109
J23110
J23115
J23119
J23119-A27T
PpqqA
Plufb
P0169
P0264
P0452
Inducible
Plux
PTet
PLac
PBad

e)

Terminators
Natural
ECK120033736
ECK120010818
ECK120010799
ECK120051401
BBa_B0010
Synthetic
L3S2P21
L3S3P21
L3S2P24
L3S2P44
L3S1P47

f)

RBS
BBa_B0034
BBa_B0034-1AC
BBa_B0034-1AG
BBa_B0034-1AT
BBa_B0034-2AC
BBa_B0034-2AG
BBa_B0034-2AT
BBa_B0034-3AC
BBa_B0034-3AG
BBa_B0034-3AT
BBa_B0034-4GA
BBa_B0034-4GC
BBa_B0034-4GT
BBa_B0034-5AC
BBa_B0034-5AG
BBa_B0034-5AT
BBa_B0034-6GA
BBa_B0034-6GC
BBa_B0034-6GT
BBa_B0034-7GA
BBa_B0034-7GC
BBa_B0034-7GT
BBa_B0034-8AC

RBS
BBa_B0034-8AG
BBa_B0034-8AT
BBa_B0034-9GA
BBa_B0034-9GC
BBa_B0034-9GT
BBa_B0034-10AC
BBa_B0034-10AG
BBa_B0034-10AT
BBa_B0034-11AC
BBa_B0034-11AG
BBa_B0034-11AT
BBa_B0034-12AC
BBa_B0034-12AG
BBa_B0034-12AT

g)

Degradation tag
LAA
AAV
LVA
DAS
DAS+2

Figure x: *Gluconobacter* toolkit. a) pBBR1MSC-2 plasmid for protein expression in *Gluconobacter*. b) pBBR1MSC-5 plasmid for protein expression in *Gluconobacter*. c) pSEVA331-Bb plasmid for protein expression in *Gluconobacter*. d) Promoters used for protein expression in *Gluconobacter*. e) Terminators used for protein expression in *Gluconobacter*. f) RBS used for protein expression in *Gluconobacter*. g) Degradation tag used for protein expression in *Gluconobacter*.

4. Conclusions and future perspectives

Microbial biotransfromations, though having matured after years of investigation, still offer no universal solutions for an increasingly greener synthetic industry. However, advancement on bioprocess and microbial engineering and use of non-model organisms have started to establish a toolbox for the development of new and improved biotransformations. *Gluconobacter* has remarkable advantages as a biocatalyst and recently its study has established its potential as microbial chassis for numerous biotransformations. These advances have proven the value of its genetic modification, its immobilization and the adequate design of its catalysed transformations for improved productivities, stabilities or new bioconversions.

We anticipate that subsequent contributions in the kinetic modelling of *Gluconobacter* catalyzed reactions will help better identify critical parameters to solve poor or non-industrially feasible transformations. As for the stability, a much-desired quality in biocatalysts, a primary challenge involves the development of immobilization strategies that avoid the impact on the conversion rates of the biocatalyst. Moreover, a deeper understanding of the genetics of *Gluconobacter* will open possibilities to modify its genome *via* more efficient and advanced tools involving CRISPR as it has been proved with other acetobacter[115]. The build up knowledge in all these fields will undoubtedly enhance the biotechnological application of *Gluconobacter* strains.

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Declaration of interest statement

The authors report no conflict of interest.

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Tables (with captions, on individual pages)

Figures

Figure captions (as a list)

Info para hacer la Cover letter:

En la revista hay dos review sobre *Gluconobacter* publicados en el año 2001 (doi: 10.1080/20013891081665) y en el año 2007 (10.1080/07388550701503584), este último es sólo de *G. oxydans*.

Existen reviews recientes de acetobacterias, pero son generales (doi: 10.1111/1541-4337.12440 (2019); 10.14456/sehs.2018.13 (2018), 10.1016/j.biotechadv.2014.12.001 (2015); 10.1007/s12088-013-0414-z (2013); 10.1080/10826068.2011.563400 (2012). Son además de otras revistas.