The Effect of Iron on Gluconic Acid Production by *Aureobasidium pullulans*

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Abstract: New processes have been previously described for the continuous and discontinuous production of gluconic acid by *Aureobasidium pullulans* (de bary) Arnaud. Little is known about the regulatory mechanisms of gluconic acid production by *A. pullulans*. The response of growth and gluconic acid metabolism to a variable profile of iron concentrations was studied with *A. pullulans* in batch and chemostat cultures. A surprisingly high optimum N-dependent iron ion concentration in the feed medium, in the range between 0.5 mM and 3.0 mM Fe (optimum 1-2 mM), was found to be particular requirement for economically profitable continuous production of gluconic acid with 3 g/l NH_4Cl. Increased iron concentration promoted growth on defined glucose medium. 223.3 g/l gluconic acid were continuously produced at a formation rate of the generic product (R_j) of 16.8 g/(l∗h) and a specific gluconic acid productivity (m_p) of 2.5 g/(g∗h) at 13 h residence time (RT) with 1mM iron, compared with 182 g/l reached at 0.1 mM. The product selectivity (product yield based on glucose) increased continuously by raising iron concentration following a saturation curve, reaching a maximum of about 98% (mol/mol) at 2 mM Fe and 76.2% conversion, compared with only 84.3% determined at 0.1 mM. The process is not obligatory growth limiting or growth related and residual nitrogen was found in all of continuous experiments, e.g. 197 mg/l of nitrogen at 0.1 mM and 201 mg/l at 2 mM of iron.

Key Words: Gluconic acid, *Aureobasidium pullulans*, continuous, continuous fermentation, chemostat, iron effect.

INTRODUCTION

The physiological D-form of gluconic acid is the oxidation product of glucose usually formed by the microbial oxidation of glucose. Gluconic acid is a colorless, or nearly colorless, light brown syrupy liquid with a mild acid taste. As a multifunctional carbonic acid of great interest, naturally occurring in plants, fruits, wine (up to 0.25%), honey (up to 1%), rice, meat, vinegar and other natural sources, belonging to the bulk chemicals and due to its versatile physiological and chemical characteristics, gluconic acid (pentahydroxycaproic acid, $C_6H_{12}O_7$) itself, its salts (e.g. alkali metal salts, in especially sodium gluconate) and the gluconolactone form have found extensively various uses in the chemical, pharmaceutical, food and animal feed (improves growth performance), textile, detergent, leather, photographic, construction (it increases cement's resistance against fracture, frost and water) and other biological and chemical industries as well as for analytical purposes [1-7]. Gluconic acid is a mild neither caustic nor corrosive non toxic and readily biodegradable organic acid (98% after 2 days) with an excellent sequestering power [7].

Numerous gluconic acid manufacturing processes from glucose are described in the international bibliography and patent literature, including chemical and electrochemical catalysis, enzymatic biocatalysis and fermentation processes using bacteria such as *Gluconobacter*-spec., *Pseudomonas*, *Phytomonas*, *Achromobacter*, *Zymomonas mobilis* and *Acetobacter methanolicus*, fungi such as fungi like *Aspergillus* and *Penicillium* and yeast-like fungi such as *Aureobasidium pullulans* (de bary) Arnaud [2, 4, 6-13].

Essentially only *Aspergillus niger* (predominantly) based on the process developed by [14] or *Gluconobacter oxidans* have been applied as far for the industrial production of gluconic acid. Alternatively, new superior fermentation processes using *Aureobasidium pullulans* have been extensively described for the continuous and discontinuous production of gluconic acid by isolated strains of yeast-like mold, which reached gluconic acid concentrations of 230-450 g/l at residence times of about 12-20 hours and 504 g/l in fed batch mode, offering numerous advantages over the traditional discontinuous fungi processes of the last 100 years. In contrast to *A. pullulans* the multicellular fungus *Aspergillus niger* is unsuitable for a continuous production of gluconic acid by free growing cells, whereas *Gluconobacter* is sensitive to high osmotic pressures and produces a relative large quantity of keto-acids, complicating processing and product recovery [4, 7, 15, 16,].

Bioconversion of glucose to gluconic acid is a simple dehydrogenation reaction which takes place without any involvement of complex metabolic pathways. However, numerous parameters influence and regulate gluconic acid pro-

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duction like oxygen, pH, temperature and medium composition [4, 7, 17].

Glucose in the medium is oxidized extracellularly in a two-step reaction to gluconic acid even in the absence of cells through the action of glucose oxidase and catalase derived from *A. niger*, in where nearly 100% of the glucose is converted to gluconic acid under the appropriate conditions [18].

Gluconic acid production by *A. niger* and *A. pullulans* is a high oxygen (>100% air saturation), pH (pH above 6.5) and temperature depending process (30-31°C), also strongly influenced by the composition of bioreactor medium [3, 4, 7, 13, 17, 19].

Little is known about the regulatory mechanisms of gluconic acid production in *A. pullulans* and regarding the effect of trace elements and other medium compounds on the production of gluconic acid.

The response of growth and gluconic acid metabolism to a variable profile of iron concentrations was studied in batch and chemostat cultures of glucose-grown yeast-like *A. pullulans*, in order to elucidate the very significant role of iron ions in gluconate metabolism.

MATERIALS AND METHODS

Microorganism

Aureobasidium pullulans isolate Nr. 70 (DSM 7085) used in present studies has been isolated from wild flowers (Jülich, Germany) as has been previously described [20].

Pre-Culture

Glucose 30 g/l, NH_4Cl 3 g/l, KH_2PO_4 0.7 g/l, $MgSO_4.7H_2O$ 0.35 g/l, $MnSO_4.4\ H_2O$ 50 µg/l, $FeSO_4.7\ H_2O$ 50 µg/l, $CuSO_4.5\ H_2O$ 2 mg/l, $ZnSO_4.7\ H_2O$ 0.01 g/l, $CoSO_4.7\ H_2O$ 4 mg/l, H_3BO_3 40 mg/l, $CaCO_3$ 5 g/l, thiamine-HCl 2 mg/l, biotin 0.25 mg/l, 24 h at 30°C and 100 rpm. For further experiments 10 µM manganese and iron were taken for inoculum and the concentration of manganese and iron were varied in the fermentation media between 0.25 and 2 µM manganese and between 50 and 500 µM iron.

Batch Experiments

Glucose 155 g/l, NH_4Cl 0.3 g/l, KH_2PO_4 0.3 g/l, $MgSO_4.7\ H_2O$ 0.2 g/l, $MnSO_4.4\ H_2O$ 0 or 10 µM, $FeSO_4.7\ H_2O$ varied from 0 to 10 µM, Thiamine-HCl 2 mg/l, Biotin 0.025 mg/l. The pH was automatically attained at 6.5 and the temperature at 30°C.

CSTR Experiments

CSTR's cells were grown in a magnetically stirred 1 liter double glass fermenter as has been described previously [4]. The medium contained (g/l): Glucose 360 g/l, NH_4Cl 3 g/l, KH_2PO_4 0.7 g/l, $MgSO_4*7\ H_2O$ 0.35 g/l, $MnSO_4*4\ H_2O$ 5 mM, $FeSO_4*7\ H_2O$ 0.5 mM, $CuSO_4*5\ H_2O$ 4 µM (1 mg/l), $ZnSO_4*7\ H_2O$ 0.01 g/l, $CoSO_4*7\ H_2O$ 4 mg/l, H_3BO_3 0.04 g/l, CaCl 0.1 g/l, NaCl 0.1 g/l, citric acid 2.5 g/l, $Na_2MoO_4*\ 2\ H_2O$ 0.2 mg/l, thiamine-HCl 2 mg/l, biotin 0.25 g/l, pyridoxine-HCl 0.625 mg/l, Ca-D-pantothenate 0.625 mg/l, nicotinic acid 0.5 mg/l. The vitamins and NH_4Cl were added separately to the autoclaved medium, which was sterilized for 30-60 min at 121°C, by sterile filtration (Sartorius, Göttingen, Germany). All chemicals were of highest purity commercially available. The fermentations were carried out at 30°C, 1300 rpm, 5 l/h of pure oxygen and pH 6.5 automatically adding a 45% NaOH solution.

Analysis

Optical density ($OD_{660\ nm}$), dry biomass (filter method) and the concentration of glucose and gluconic acid were determined as has described in previous works [17].

RESULTS

The effect of iron on growth and gluconic acid of *A. pullulans* DSM 7085 (isolate 70) was investigated under batch and chemostat cultivation (CSTR's) applying constant medium feed rate at RT of about 13 and 18 hours, carried out in 1 liter magnetically stirred glass fermenters on media described in materials and methods. Iron concentrations between 0.25 and 2 µM were investigated in batch cultures and 0.1-2 mM iron in CSTRs.

Batch Experiments

Because the gluconic acid process using *A. pullulans* was still poorly investigated the effect of varying iron and (0 or 10 µM) and manganese concentration (0- 2000 µM) was initially examined in preliminary orientation batch experiments, carried out in 1 L glass fermenter with 500 ml working volume at 30°C und pH 6.5, 1000 rpm and 5 l pure oxygen/h, using a defined mineral medium described in material and methods. The pH was controlled automatically by the addition of 4 N NaOH.

It is possible to control growth and gluconic acid formation of *A. pullulans* by varying iron and manganese concentration. The highest product concentration and OD were reached in experiments with a medium containing both, iron and manganese as compared with experiments either without iron or manganese (Table **1**). An OD of only 0.2 and 2.3 g/l gluconic acid were reached after 24 h in experiment without manganese and iron in comparison to OD of 0.55 and 11.1 g/l gluconic acid reached with 10 µM iron and 10 µM of manganese. Only 18.7 g/l and an OD of 0.52 were reached after 48 h without Fe and Mn, compared with 127.2 g/l and 6.03 OD obtained with 10 µM Mn and 10 µM Fe. Very high molar selectivities were obtained under iron and manganese limitation and in experiments with 10 µM Fe and 10 µM Mn, approaching 98% without Fe and Mn due to very low pellet forming biomass. 32 g/l gluconic acid were produced after 54 h without Fe and Mn compared with 147.1 g/l reached with 0 µM Fe und 10 µM Mn and 118,9 g/l obtained with 10 µM iron und 0 µM Mn. Further increase of manganese concentration accelerated gluconic acid and biomass formation even in absence of iron displaying an optimum.

Furthermore, the effect of iron on growth and gluconate formation was investigated in batch experiments at a variable iron concentration between 0.05 and 0.5 mM. Whereas OD increased with increasing Fe differences in gluconic acid production were insignificant, reaching the highest gluconic acid concentration of 160 g/l (after 100 h) with 0.1 mM Fe at an OD of 7.9.

Table 1. Manganese and Iron Effect on Gluconic Acid Formation by *A. pullulans*

Fe (µM)	Mn (µM)	$OD_{660\ nm}$ (g/l)			Gluconic acid (g/l)		
Cultivation time (h)		24	28	48	24	28	48
0	0	0.21	0.22	0.52	2.3	5.0	19.6
0	10	0.45	0.71	1.85	10.9	23.7	88.8
10	0	0.29	1.49	1.96	6.8	28.3	111.4
10	10	0.55	1.52	6.03	11.1	30.2	127.2
10	250	3.95	4.99	6.32	29.1	40.3	69.2
10	500	3.59	4.36	5,77	44.3	61.8	129.3
10	2000	3.04	3.82	4.08	35.7	58.4	112.6

CSTRs Experiments

The effect of iron on growth and gluconic acid production was investigated in CSTR experiments at 30°C and pH 6.5 with a medium as described in materials and methods containing 3 g/l NH_4Cl and 360 g/l glucose. Fig. (**1**) shows the effect of varying iron concentration on growth and gluconic acid production of *A. pullulans* at 13 h RT.

More than 190 mg/l residual nitrogen was determined in all of CSTR experiments at 13 h RT; 197 mg/l nitrogen at 0.1 mM and 201 mg/l at 2 mM iron. Insignificant differences were observed in biomass formation ranging between 7.3 g/l biomass at 0.1 mM and 6.9 g/l at 2 mM iron (Fig. **1**). No correlation was observed between the optical density and biomass concentration at varying iron concentration, indicating the strong influence of iron concentration on cell morphology of dimorphic (single or multicellular) yeast-like fungus *A. pullulans*, meaning that single cells have a higher optical density. The higher OD/biomass at lower iron concentrations indicates the occurrence of smaller single cells (higher total cell surface, higher OD).

At 13 h RT, the highest formation rate of the generic product of 16.8 g/(l∗h), specific gluconic acid productivity (m_p) of 2.5 g/(g∗h) and gluconic acid concentration of 223.3 g/l were obtained at 1 mM iron. Comparatively, only 182 g/l (81.5%), 14.5 g/(l∗h) Rj and 1.82 g/(g∗h) mp were reached at 0.1 mM and 215.7 g/l (96.6%), 15.66 g/(l*h) Rj and 2.27 g/(g*h) mp at 2 mM iron. Surprisingly, major variations were found in product selectivity as a function of iron concentration at 13 h RT and incomplete conversion of glucose. Product selectivity increased continuously by increasing iron concentration and showing a saturation effect reached a maximum of about 98% at 2 mM and 76.2% conversion, compared with only 84.3% at 0.1 mM. At iron concentration higher than 0.5 mM, selectivity was above 90% (Fig. **2**).

No significant differences were observed in gluconate concentration at 18 h RT (Fig. **3**) in contrast to 13 h, because

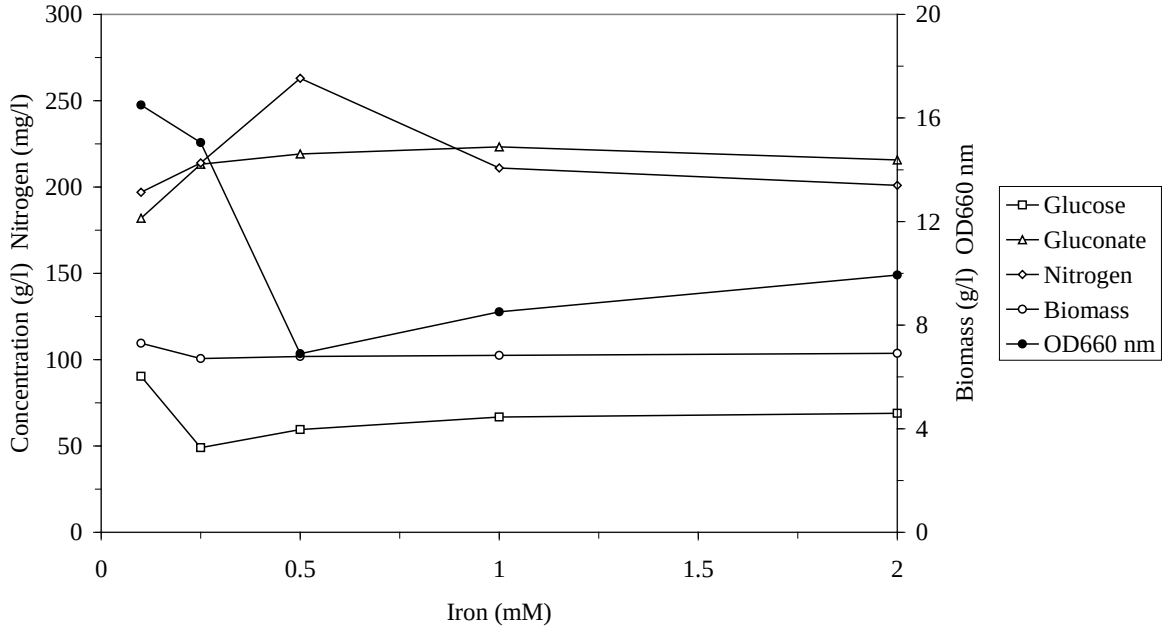

Fig. (1). Iron effect on growth and gluconic acid production at about 13 h RT (3 g/l NH_4Cl, 360 g/l glucose, 5 mM Mn, 30°C and pH 6.5).

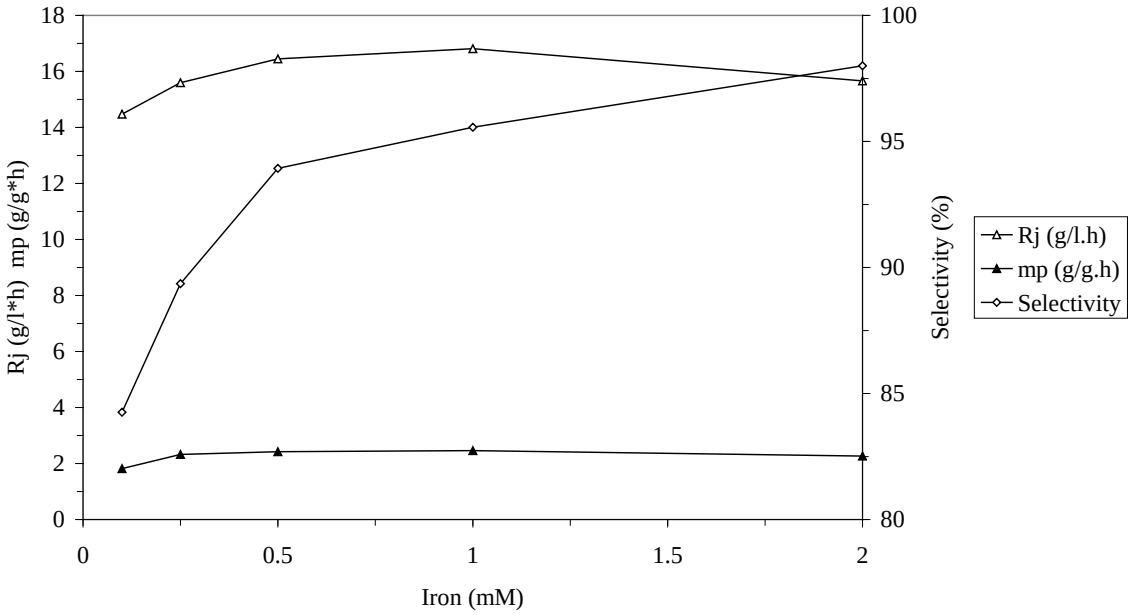

Fig. (2). Iron effect on R_j, m_p and selectivity at a residence time of about 13 h (3 g/l NH_4Cl, 360 g/l glucose, 5 mM Mn, 30°C and pH 6.5).

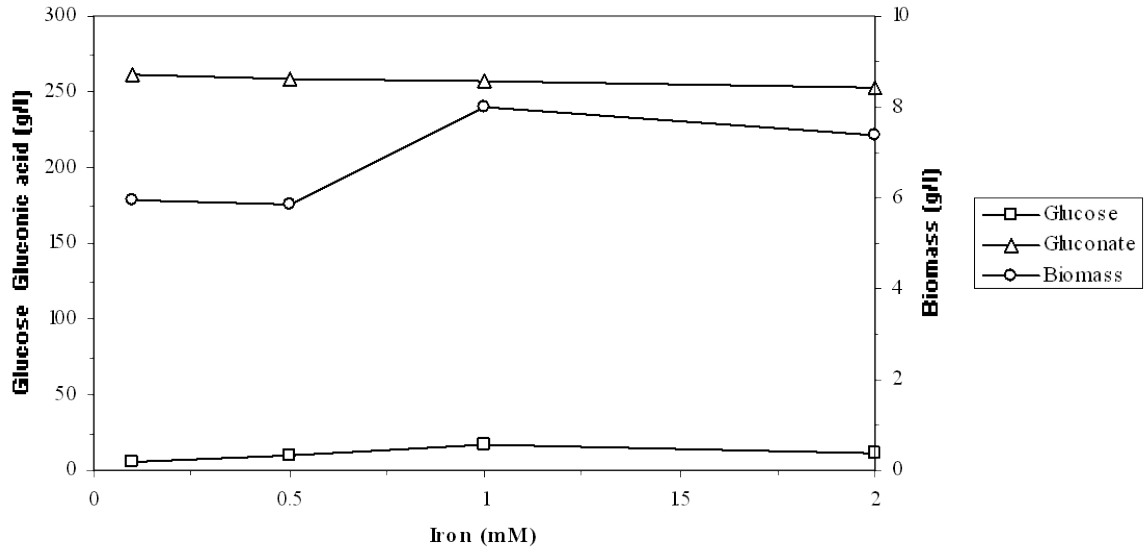

Fig. (3). Iron effect on growth and gluconic acid production at about 18 h RT (3 g/l NH_4Cl, 360 g/l glucose, 5 mM Mn, 30°C and pH 6.5).

of the nearly complete consumption of glucose. Optimization CSTRs studies are supposed to be operated at about 80% of conversion (g converted glucose/g feed glucose). XT diagrams as a function of varying residence time or dilution rate have been published in previous work [16].

The highest biomass of 8 g/l was achieved at 18 h RT with 1 mM iron, compared with 5.95 g/l reached with 0.1 mM. More than 250 g/l gluconic acid was produced with all of investigated iron concentrations, reaching the highest concentration of 260.9 g/l and a Rj of 14.5 g/(l*h) at 0.1 mM iron compared with 252.4 g/l and 14.02 g/(l*h) measured at 2 mM (Fig. **3**). Selectivity dropped with increasing iron concentration at 18 h, because of intensifying nitrogen limitation which causes byproduct formation, like pullulan and other organic acids (e.g. fumaric acid). Fumaric acid concentrations between 0.026 mM (presence of nitrogen, 6.8 h RT) and 15.2 mM (absence of nitrogen, 50.1 h RT) have been found as a byproduct in gluconic acid production by *A. pullulans* as a function of RT and nitrogen limitation (data not shown here). A molar selectivity of 85.1% (92.7% g/g) was calculated at 1 mM and 82% at 2 mM iron (89.3% g/g). Glucose was not consumed completely and a residual glucose of 16.5 g/l was measured at 2 mM iron and about 95% conversion.

DISCUSSION

The development of any new multi-step biotechnological process requires three basic steps, namely: the identification and characterization of a suitable biological system (microorganism, biocatalyst), the increase of bioreactor productiv-

ity by sophisticated media optimization and adaptation of fermentation technology to the developed process and downstream processing (cell separation by centrifugation or ultrafiltration, product separation, evaporation and drying).

Chemostat cultures are dynamic systems for sophisticated medium optimization and process development, in where single parameters can be investigated in detail under steady state conditions, identifying and optimizing various optima for microbial growth and production. Very high glucose concentrations can be applied in feeding medium because of the wash out effect of chemostat due to continuous microbial glucose consumption, resulting to low residual steady state glucose concentrations. Alternatively, high initial glucose concentrations slow down the growth of *A. pullulans* in batch cultures. Additionally, chemostat results obtained under steady state conditions are easily reproducible and compensation effects between growth and production are detected based on volumetric and biomass-specific productivity of the generic product [4, 16].

Enzymatic glucose conversion to gluconic acid is enabled either by fungal enzyme glucose oxidase or bacterial glucose dehydrogenase (e.g. bacteria such as *Gluconobacter*, *Acetobacter*). The enzymatic complex glucose oxidase/catalase is present in several microorganisms belonging e.g. to the classes *Aspergillus* and *Penicillium* or alternative in yeast-like mold *Aureobasidium pullulans* (Pullularia pullulans). Bacteria like *Acetobacter* and *Gluconobacter* use different mechanisms for converting glucose into gluconic acid. *G. oxydans* contains two types of glucose dehydrogenase which convert glucose into gluconolactone without the formation of hydrogen peroxide. Enzymes derived from cloned genes of GMO organisms may also be involved in the production of gluconic acid production or alternative economical host systems can be developed [7, 15, 21, 22]. Strain improvement has been reported to be an essential step in developing industrial microbial production processes [23].

Yeast-like mould *A. pullulans* is well known for pullulan formation, a polysaccharide [24-28]. About gluconic acid's production by *A. pullulans* (formerly known as *Dematium* or *Pullularia pullulans*) has been previously reported using various carbon sources [29-32], however it hasn't been considered as a potential gluconic acid producer before. No information was available regarding fermentation conditions or process optimization and development and those pioneer works were mainly restricted to shake flask experiments [16]. Little is known about the regulatory mechanisms of gluconic acid production by yeast like mold *A. pullulans*, which has been shown to be a superior gluconic acid producer [4, 7, 15-17, 33]. *A. pullulans* isolate 70 (DSM 7085) was isolated from wild flowers in Germany and no genetic works were carried out, in order to increase gluconic acid production, in opposite of today's industrial gluconic acid production, which uses improved mutant strains of several generations, predominantly recycled mycelia of *Aspergillus niger*, or *Gluconobacter suboxidans* in discontinuous submerse fermentations [34-36]. Today's sodium gluconate fermentation using *A. niger* in submerse fermentation bases on the process developed by [14].

The response of growth and gluconic acid metabolism to a variable profile of different iron concentrations was studied with glucose-grown batch and chemostat cultures of yeast like mold *A. pullulans*; the emphasis was focused on the physiological parameters of yeast growth and gluconic acid production, demonstrating the tremendous significance of iron ions in this process. In preliminary orientation batch cultures, iron as well as manganese ions have been identified to be critical nutrients, strongly influencing growth and gluconic acid formation *in A. pullulans*, whereby manganese showed a stronger effect than iron. Serving as cofactors of essential enzymes for the production of gluconic acid (Glucose-Oxidase, Catalase) iron as also manganese are significant trace elements for optimal gluconate production in *Aspergillus niger* and *A. pullulans*. In commercial fermentations they are added into bioreactor in undefined amount with maize steep water and other medium compounds.

It is also well known that iron is the integral component of many metalloenzymes; such as aconitate-hydratase, catalase, peroxidases and components of mitochondrial electron transfer chain [37, 38] and often iron concentration was used as variable factor to control microbial metabolism [39-41]. Iron concentration had in accordance to batch experiments no relevant effect on the growth of *A. pullulans*. A very strong iron effect on gluconate production was observed in batch cultures at the lower concentrations of 0-10 μM. Substantial differences of iron effect on gluconic acid fermentation occurred at the lower RT (lower conversion) of 13 h, whereas no significant differences were observed in gluconate production at 18 h RT, because of the nearly complete consumption of glucose. Optimization CSTRs studies are supposed to be operated at about 80% conversions (g converted glucose/g feed glucose) for identifying the real effects. A surprisingly high particular requirement of iron ions (0.5-3.0 mMol at 3 g/l NH_4Cl) has been detected for profitably performing gluconic acid fermentation in *A. pullulans*, displaying an optimum iron concentration between 1-2 mM for product concentration, product yield, selectivity, R_j and m_p. With increasing iron concentration from 0.1 to 2 mM, product selectivity increased continuously showing a saturation behavior and reached almost 100%. A more intensive respiration (respiration chain) as well as byproduct formation (e.g. fumaric acid and pullulan formation) are eventually the main reasons for low selectivity at lower iron concentrations. For example, [42] reported that iron supply enhances mycelial growth form of dimorphic *A. pullulans* and decreases pullulan formation. Mycelial form of *A. pullulans* favors gluconic acid formation in our investigations, suppressing pullulan formation, instead, showing the relationship between morphology and gluconic acid production.

The increase of mp and and Rj at almost constant biomass at 13 h RT at increasing iron concentration up to 1 mM, indicates that iron stimulates synthesis or/and activity of glucose oxidizing enzymes in *A. pullulans*. [43] reported about the stimulation of glucose oxidase synthesis in *A. niger* by the supply of iron sulfate and KCl. Similar effects found [44,45] in *Penicillium* strains. [45] as well as [46] reported on the other side that supply of 0.001% $FeSO_4*7\ H_2O$ or of 2.1-40.4 μM iron didn't stimulate production of calcium gluconate by *P. chrysogenum* or with *A. nige*r, respectively. Furthermore, presence of iron has been reported to favor, in addition to sodium salts, accumulation of oxalic acid and of yellow-green pigments in mycelia of *A. niger*, affecting product separation [47].

The discrepancy between biomass and OD can be explained by the influence of iron on dimorphic growth behavior of yeast-like *A. pullulans*. Single cell growth (higher total cell surface, higher OD) promotes pullulans formation instead of gluconic acid. Nitrogen and Iron limitation have been reported to enhance pullulans production in *A. pullulans*. Iron limitation possibly promotes fumaric acid by-production as well.

RT is also of major importance for optimum production and must be taken into consideration, since gluconic acid is partly utilized at long RTs, because of almost complete glucose consumption (100% conversion). This is also confirmed by the reduction of selectivity at increasing iron concentration at 21 h RT, due to almost complete glucose consumption and progressive product redirection or reconsumption.

The present results of very high gluconic acid concentrations reached at very low RT (13 h) by free growing cells of *A. pullulans* are the best known that have been published in the international bibliography, encouraging the use of *A. pullulans* processes for future industrial applications in gluconic acid business as innovative alternatives to the discontinuous fungi processes.

CONCLUSIONS

Yeast-like *A. pullulans* offers the positive characteristics of both groups of eucaryotic (fungi and yeasts) and prokaryotic microorganisms (bacteria) at once, enabling a continuous process operation by free growing cells running at very high glucose and product titers. Medium optimization carried out in chemostat resulted in strong increase of yield and selectivity, productivity and product concentration at very short residence times. Present results of very high gluconic acid concentrations reached at very low RT (13 h) by free growing cells of *A. pullulans* without any biomass retention are the best known that have been published in the international bibliography, encouraging the use of new *Aureobasidium pullulans* processes [4, 5, 16, 17, 20, 33] for future industrial applications in gluconic acid business as innovative alternatives to the discontinuous fungi processes of the last 100 years. Based on our deep investigations on the influence of important fermentation parameters on gluconic acid production by *A. pullulans*, such as pH, temperature, air saturation, medium composition, residence time, biomass retention, cascading of two fermenters etc., further investigations would accelerate the continuous and discontinuous production of gluconic acid decreasing production costs to minimal levels. Present results show nature's latent potential for still unknown high producing microbial wild strains as a comparison to extensive publicity of genetic engineering research and development for future applications in gluconic acid research.

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ABBREVIATIONS AND DEFINITIONS

R_j	=	Formation rate of the generic product, g gluconic acid/(l*h) (volumetric productivity)
m_p	=	Specific gluconic acid productivity, g gluconic acid/(g biomass*h) (Biomass specific productivity)
RT	=	Residence time (h)
CSTR	=	Continuous stirred-tank reactor
OD	=	Optical density
Selectivity	=	G product/g converted glucose (%)
Yield	=	G product/g initial glucose (%)
Conversion	=	G converted glucose/g initial glucose (%)
XT diagram	=	Parameters as a function of residence time

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