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RESEARCH ARTICLE

Continuous gluconic acid production by *Aureobasidium pullulans* with and without biomass retention

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Keywords: biomass immobilization, continuous fermentation, cross over filtration, gluconic acid fermentation, reaction technique, residence time.

Abbreviations: Conversion (%): [(g consumed glucose/g feeding glucose) x 100], dilution of medium glucose by NaOH feeding was considered in the calculations
m_p: Specific gluconic acid productivity, [g gluconic acid/(g biomass x h)], Rj [g/(l x h)]/biomass concentration (g/l), g/(g x h)
R_j: Formation rate of the generic product (volumetric productivity), g gluconic acid/(l x h), gluconic acid concentration (g/l)/RT (h), g/(l x h)
Rs: Glucose consumption rate, g/(l x h), [(g feeding glucose-g consumed glucose)/RT (h)], g/(l x h)
RT: Residence time - hours, [Bioreactor volume (ml)/(medium feeding rate (ml/h) + NaOH feeding rate (ml/h))], h
Selectivity (%): [(g gluconic acid/g consumed glucose) x 100]
Yield (%): [(g gluconic acid/g feeding glucose) x 100]

New alternative processes for the continuous production of gluconic acid by *Aureobasidium pullulans*, using biomass retention by cell immobilization or cross over filtration, are described in the present work. 315 g/l gluconic acid was continuously produced in chemostat cultures at 21 hrs residence time without any biomass retention. 260 g/l gluconic acid was produced in fluidized bed reactor at 21 hrs residence time. The support carrier was overgrown resulting in limitations of oxygen transfer towards the inner layers of immobilized biomass. 375 g/l gluconic acid was produced under continuous cultivation at 22 hrs of residence time with a formation rate for the generic product of 17 g/(l x h) and a specific gluconic acid productivity of only 0.74 g/(g x h), using biomass retention by cross over filtration. 370 g/l were obtained at 19 hrs RT and 100% conversion with 25 g/l biomass and a formation rate of 19 g/(l x h). At 100% conversion, a selectivity of only 78% was determined at 22 hrs and of 77% at 19 hrs RT, because of the very high biomass concentration. Biomass retention makes it possible to break the existing link between growth and residence time.

As a multifunctional carbonic acid, belonging to the bulk chemicals and due to its physiological and chemical characteristics, gluconic acid itself, the gluconolatone form and its salts (*e.g.* alkali metal salts, in especially sodium gluconate) have found extensively versatile uses in the chemical, pharmaceutical (*e.g.* iron and calcium deficiency), food, beverage, textile and other industries (Hustede et al. 1989; Anastassiadis et al. 2003; Znad et al. 2004). Additionally, it can be exploited for cleaning purposes (*e.g.* diary industry) as well as for the extraction of trace elements like calcium, copper and iron. Gluconic acid can have further applications for the solubilization of phosphate (Fenice et al. 2000; Vassilev et al. 2001; Rodríguez et al. 2004) and as a cement additive in the

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Table 1. Results of the continuous gluconic acid fermentation with residence times of 21 and 25 hrs (3 g/l NH_4Cl, 450 g/l glucose, 1 mM iron, 5 mM manganese, pH 6.5, 30ºC and 155% oxygen saturation).

Residence time	hr	21	25
Biomass	g/l	6.8	6.8
Optical density	660 nm	37	37
Glucose	g/l	80	70
Gluconic acid	g/l	315	330
Rj	g/(l x h)	15	13.3
m_p	g/(g x h)	2.2	1.9
Conversion	%	81	82.5
Yield	%	71	76
Selectivity	%	92	92.1

construction industry, because it enhances the cement's resistance and stability under extreme climatic conditions, *e.g.* frost and water (Singh, 1976; Hustede et al. 1989).

Numerous methods have been extensively described for the production of gluconic acid, including chemical and electrochemical catalysis, enzymatic biocatalysis in enzyme bioreactor, microbial production using free growing or immobilized cells of either *Gluconobacter oxydans* or *Aspergillus niger*. The immobilization of whole cells or glucose oxidase enzyme by various techniques has often been reported to be a useful approach to the production of gluconic acid or other microbial metabolites (Hartmeier and Döppner, 1983; Pronk et al. 1989; Sakurai et al. 1989; Gromada and Fiedurek, 1997; Nakao et al. 1997; Kara and Bozdemir, 1998; Velizarov and Beschkov, 1998; Anastassiadis et al. 1999; Sankpal et al. 1999; Bang et al. 1999; Ferraz et al. 2000; Bao et al. 2001; Blandino et al. 2001; Klein et al. 2002; Sankpal and Kulkarni, 2002; Anastassiadis et al. 2003; Godjevargova et al. 2004; Anastassiadis et al. 2005; Mukhopadhyay et al. 2005). Fluidized bed reactors have been often used for several fermentation applications, in especially for anaerobic processes. The separation of the biocatalyst is practically connected with high expenses, substantially influencing the economics of a fermentation process. Cell immobilization would therefore be an economically efficient alternative option for the production of gluconic acid under either batch or continuous cultivation. Sankpal and Kulkarni (2002) described gluconic acid fermentation by immobilized cells of *A. niger* on a highly porous cellulose support.

Biomass retention by immobilization on porous sinter glass or by cross over filtration was applied in present work in order to investigate the feasibility of a further acceleration of continuous gluconic acid production and of the maximization of product concentration by an isolated *Aureobasidium pullulans* strain, as a comparison with the continuous cultivation of free growing cells.

MATERIALS AND METHODS

Microorganism

Aureobasidium pullulans (de Bary) Arnaud isolate Nr. 70 (DSM 7085), which was isolated from wild flowers (Jülich, Germany) was used during the entire work (Anastassiadis et al. 1999; Anastassiadis et al. 2003; Anastassiadis et al. 2005). Yeast malt extract agar plates (YME), inoculated with *Aureobasidium pullulans,* were incubated for 2-3 days and stored at 4ºC. The inoculum (10%) was prepared by transferring of cells from agar plates into 500 ml shake flasks with buffels on a medium containing (g/l): Glucose 30 g/l, NH_4Cl 3 g/l, KH_2PO_4 1.4 g/l, $MgSO_4$ x 7 H_2O 0.35 g/l, $MnSO_4$ x 4 H_2O 5 mM, $FeSO_4$ x 7 H_2O 1 mM, $CuSO_4$ x 5 H_2O 4 µM (1 mg/l), $ZnSO_4$ x 7 H_2O 0.01 g/l, $CoSO_4$ x 7 H_2O 4 mg/l, H_3BO_3 0.04 g/l, $CaCl_2$ 0.1 g/l, NaCl 0.1 g/l, citric acid 2.5 g/l, Na_2MoO_4 x 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.

Culture conditions

For the investigation of continuous gluconic acid fermentation with or without biomass retention, cells were grown in a 5 litre fermenter (Biostat E, Braun-Diessel) at a working volume of 3 l, 1000 rpm, pH 6.5 and 30ºC in chemostat mode on a basal defined medium containing (g/l): varying glucose concentration (s. results), NH_4Cl 3 g/l, KH_2PO_4 1.4 g/l, $MgSO_4$ x 7 H_2O 0.35 g/l, $MnSO_4$ x 4 H_2O 5 mM, $FeSO_4$ x 7 H_2O 1 mM, $CuSO_4$ x 5 H_2O 4 µM (1 mg/l), $ZnSO_4$ x 7 H_2O 0.01 g/l, $CoSO_4$ x 7 H_2O 4 mg/l, H_3BO_3 0.04 g/l, $CaCl_2$ 0.1 g/l, NaCl 0.1 g/l, citric acid 2.5 g/l, Na_2MoO_4 x $2H_2O$ 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 [24,25,2]. Vitamins and NH_4Cl were added separately to autoclaved medium (30-60 min at 121ºC) by sterile filtration (Sartorius filter, Göttingen, Germany). The fermentations were carried out at 30ºC and pH 6.5 automatically adding a 45% NaOH solution.

Fermentation equipment

The fermentations were carried out in agitation 5 litre fermenters (Figure 1) (Biostat M, Diessel-Braun,

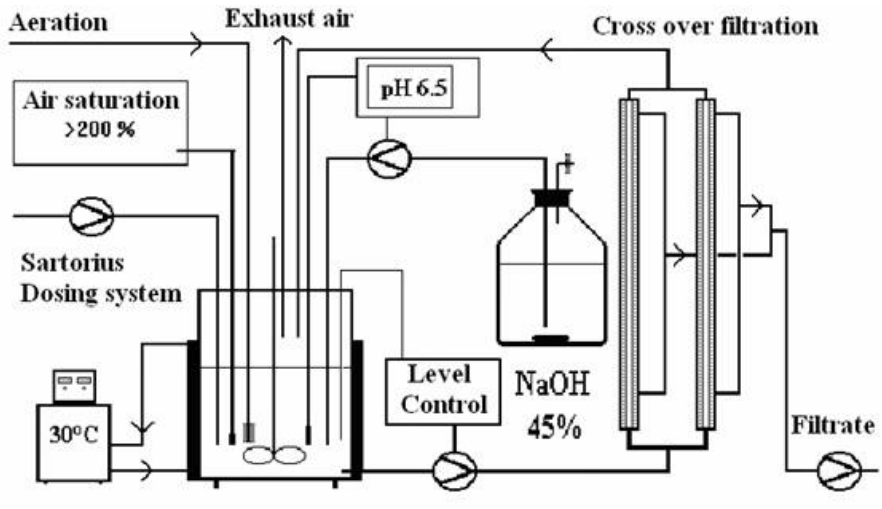

Figure 1. Flow scheme of a stirrer fermenter with intergraded cross over filtration.

Germany). From the concept, the fermenters were laid out for the continuous mode of operation. The fermenters worked according to the principle of chemostat, *i.e.* by delimitation of a growth factor and at a constant supply of nutritive solution into fermenter a stationary condition (steady state) can be adjusted. The temperature was controlled using laboratory thermostats Lauda M3 (Meßgerätewerk Lauda, D6970-Lauda-Königshofen). The air saturation (%) was measured as has been reported in Anastassiadis et al. (2005). A sufficient oxygen supply was ensured adding pure oxygen over two parallel attached sterile filters (T-fitting) into the supply line, in order to maintain air saturations higher than 100% in fermenter (Anastassiadis et al. 2005). The oxygen was distributed by means of a sinter glass frit in the culture liquid. The exhaust gas stream left likewise over two parallel sterile filters the fermenter. A condenser prevented the discharge of water over the exhaust upstream of air. The experimental setup of such a fermenter is schematically represented in Figure 1.

The fermentation medium was continuously added into the fermenter using a precision gravimetric dosing system (Sartorius, Göttingen, Germany) and a peristalticpump (Watson Marlow). Periodically, an antifoaming agent was pumped to the fermenter using a time switch clock (15 sec performance every 1.5 hrs). The residence time was determined based on the fermenter working volume via the total medium and NaOH flux. More than five residence times were required for achieving steady state conditions and the cultivation in each mode was continued until the culture medium in fermenter was replaced at least five times.

Biomass retention by cross over filtration

The experiments for the continuous production of gluconic acid by biomass retention by means of cross over filtration were carried out in a 5 litre stirrer fermenter. For the cross over filtration, two parallel connected autoclavable microfiltration tubing modules (cross over filtration) were attached to the vessel (Figure 1). Fermenter, filters, medium and NaOH solution were externally sterilized in a large autoclave. In case of clogging appearances, the modules were exchanged or were cleaned by turning periodically the pumping direction backwards. The goal was implemented by taking off a part of the fermentation broth as filtrate and thus it is increased the stationary biomass concentration in bioreactor. At the same time, the discharge remainder (Bleed), containing fermentation broth and biomass, was taken out of the fermenter by means of a second peristaltic pump. Both pumps were simultaneously controlled by a level control system. By this way, it became feasible to adjust a constant relationship between both types of discharges. The level control probe (Weathston's bridge) was calibrated, enabling the proportional adjustment of desired level of filling under constant fermentation conditions. For safety reasons, the substrate pump was also interconnected to this control system and at reaching a certain (adjustable) level of bioreactor broth the pump was shut down automatically. The fermentation was performed at 30ºC, pH 6.5 and 290% air saturation.

Biomass retention by biomass immobilization

The experiments with biomass immobilization were carried out in a fluidized bed reactor at a working volume of about 0.9 litre (Figure 2). For the immobilization of biomass, about 350 g of porous sinter glass beads (SIRAN^R) (Schott AG, Mainz, Germany) was added in fluidized bed reactor,

resulting in the ideal case to a total working volume of 0.9 litre. Sinter glass balls with a three-dimensional cavity structure and the

following characteristics were used: ball form with 1-2 mm diameter, 60% porosity, distribution of pore sizes of 60-300 µm, from borosilicate. The carrier drawn away from fermenter was collected in a cyclone (precipitation vessel) in order to avoid clogging of tubing lines. The oxygen electrode was placed in an external glass vessel and the pH electrode was placed in a conical glass vessel, which was located between the large circulation pump and the fluidized bed fermenter, in order to avoid fouling by biofilm formation. Furthermore, the pH was periodically checked using an external pH meter and readjusted. The reactor was securely placed and stabilized on a stand. An automatic dosing system (Sartorius, Germany) was used for the constant feeding rate of fermentation medium (Figure 2). Because of the very high relationship between substrate flow rate and recirculation flow rate, the fluidized bed reactor can be considered as a regular stirrer fermenter.

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 (Anastassiadis, 1993; Anastassiadis et al. 1999; Anastassiadis et al. 2003; Anastassiadis et al. 2005).

Ammonium analysis

Ammonium nitrogen was analyzed as has been described in Anastassiadis et al. (2002).

RESULTS

Novel superior processes were developed and optimized for the continuous and discontinuous production of gluconic acid by isolated strains of *Aureobasidium pullulans* during an extensive process development program (Anastassiadis et al. 1999; Anastassiadis et al. 2003; Anastassiadis et al. 2005). A defined fermentation medium was composed and optimized, which makes it easy to reproduce those data. Completing the process optimization program, reaction-technical investigations were accomplished, in order to examine the transferability of obtained results and to test different fermentation process variants (*e.g.* batches, fed batches, continuous fermentation without and with biomass retention) for the production of gluconic acid by *Aureobasidium pullulans* isolate 70 (DSM 7085) (Anastassiadis et al. 1999).

Table 2. Continuous gluconic acid fermentation with biomass retention by means of cross over filtration.

Residence time	hr	22	19
Biomass	(g/l)	23	25
Gluconic acid	(g/l)	375	370
Rj	(g/l x h)	17	19.5
m_p	(g/g x h)	0.74	0.78
Conversion	(%)	100	100
Selectivity	(%)	78	77

Continuous production of gluconic acid by free growing cells in stirrer fermenter

The continuous gluconic fermentation by free growing cells of *A. pullulans* and without any biomass retention was carried out in a 5 litre fermenter under optimized conditions (pH 6.5, 30ºC, air saturation 120-180%), using an extensively optimized defined medium (Anastassiadis et al. 1999). More than 220 g/l were produced under continuous steady state cultivation in previous chemostat studies (Anastassiadis et al. 2005). With 450 g/l glucose in feeding medium and at 155% air saturation, 315 g/l gluconic acid were continuously produced under steady state conditions at 21 hrs residence time with 6.8 g/l biomass and a formation rate for the generic product of 15 g/(l x h). Figure 3 shows the continuous production of gluconic acid at 25 hrs residence time, reaching steady state conditions about 170 hrs after the inoculation of fermenter. Table 1 illustrates the results that have been achieved at residence times of 21 and 25 hrs. 330 g/l were continuously produced at a conversion of 82.5% and 92% selectivity with a formation rate for the generic product of 13.2 g/(l x h) and a biomass of 6.8 g/l, corresponding to a specific productivity of 1.94 g/(g x h). For comparison, a maximum specific productivity of about 9.3 g/(g x h) and formation rate of about 18.6 g/(l x h) were reached in a chemostat under optimized conditions without any biomass retention at very short times of about 11.8 hrs (data not shown here). The fermentation continued to stably run without any technical and microbial stability problems for a very long period of time. A continuous gluconic acid fermentation using a new strain of *A. pullulans*, which was isolated at the Research in Biotechnology Co. (Greece) was running for about 1 year without any microbial stability problems, showing again the stability of *Aureobasidium* process.

Continuous gluconic acid production by immobilized cells

The continuous production of gluconic acid by cell immobilization on porous sinter glass (Schott, Germany) was studied in a fluidized bed reactor under optimum fermentation condition, using an optimized defined medium with 450 g/l glucose. The fermenter was initially inoculated with a two days culture of *Aureobasidium pullulans* isolate 70 (DSM 7085). The fermenter performed under batch conditions at the beginning of fermentation and the

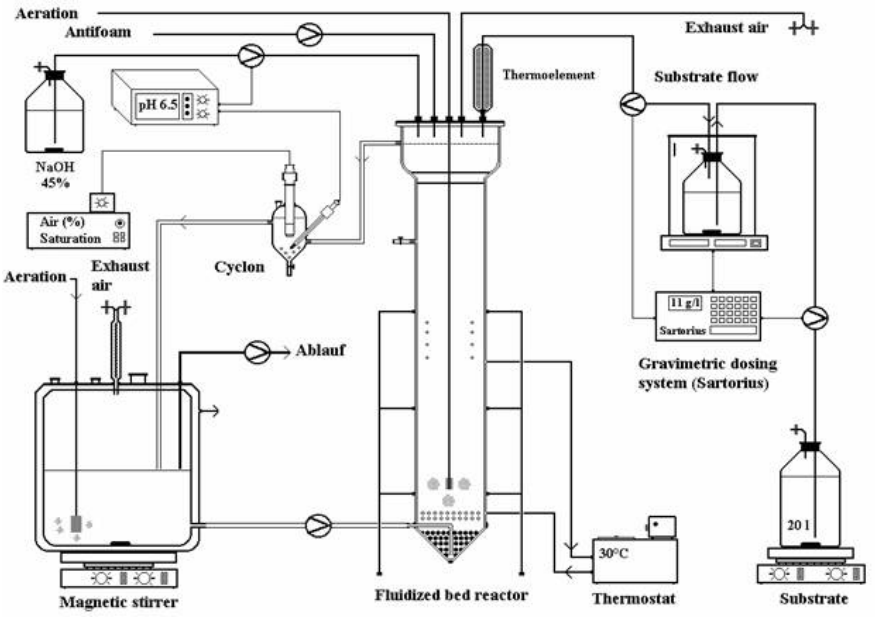

Figure 2. Flow scheme of a magnetic stirrer fermenter with intergraded fluidized bed reactor.

continuous feeding of medium started after the biomass reached a certain concentration of more than 3 g/l, in order to increase the load of biomass on the porous glass beads. The glass beads were overgrown from cells of *A. Pullulans*. 260 g/l gluconic acid could be continuously obtained in fluidized bed reactor under steady state conditions at a retention time of 21 hrs with a formation rate for the generic product gluconic acid of 12.4 g/(l x h). The adhesive behaviour of *A. pullulans* resulted in diffusion problems for the oxygen transfer and thus in oxygen limitation of the immobilized biomass.

Continuous gluconic acid fermentation with biomass retention by means of microfiltration

A good alternative approach for the acceleration of product concentration maximization is the partial retention of biomass by means of microfiltration (cross over filtration). Biomass retention enables the breaking of the link between growth (biomass formation) and residence time and between growth and production. It makes feasible the performance of the process at very high oxygen concentrations, which are toxic for cell growth, resulting in higher biomass specific productivities. It should also be examined, whether very high gluconic acid concentrations can be reached at shortened retention times, using very high oxygen concentrations. In order to receive comparable data, the experiments were accomplished under the same conditions as the preceding continuous fermentations without biomass retention. About 80% of filtrate (filtered fermentation solution) and 20% bleed (unfiltered fermentation solution) were adjusted and maintained constant during the entire duration of the experiment. Applying a glucose concentration of 450 g/l, 375 g/l gluconic acid was continuously produced at a residence time of about 22 hrs, 100% conversion of glucose, 30ºC, pH 6.5 and 290% air saturation by 23 g/l of accumulated biomass. 370 g/l of gluconic acid were also produced at 19 hrs and complete glucose conversion by a biomass of 25 g/l (Table 1, Figure 4).

In previous studies, it has been extensively studied the influence of residence time on continuous production without biomass retention (Anastassiadis et al. 2003; Anastassiadis et al. 2005). In the present work, the maximization of gluconic acid concentration has been achieved by applying biomass retention in bioreactor by means of cross over filtration. According to present results, it seems very possible to still convert glucose completely at even very short residence times *i.e.* less than 7 hrs. The investigation of the performance of continuous gluconic acid production by biomass retention at very low residence times was not possible because of technical inadequacies.

DISCUSSION

Microbial production of metabolites of primary and intermediary metabolism usually takes place under stress conditions. The medium composition plays a crucial role for a successful metabolite production. Fermentation parameters such as pH, oxygen, temperature and medium composition, influencing continuous and discontinuous gluconic acid production by isolated strains of *A. pullulans*, have been identified and optimized in previous chemostat studies (Anastassiadis et al. 1999; Anastassiadis et al. 2003;

Anastassiadis et al. 2005). In a conventional chemostat culture without any biomass retention, where growth and production occur simultaneously, the wash out effect of biomass is the limiting factor in terms of achieving very high product concentrations at very low residence times (high dilution rates). The highest formation rate and specific productivity for the generic product gluconic acid have been achieved at lower residence times. They decrease continuously at increasing residence time and very high gluconic acid concentrations of more than 230 g/l (Anastassiadis et al. 1999; Anastassiadis et al. 2003). The formation rate of the generic product (Rj) is a compensation effect between biomass concentration and biomass specific production rate. In continuous process, growth and production run parallel, influencing each other. For example, different optimum dissolved oxygen concentrations have been determined for growth, gluconic acid production and for various specific fermentation factors such as conversion, formation rate for the generic product, specific gluconic acid productivity, selectivity and yield. Hence, the optimum fermentation parameters that had been found for example for the pH, temperature and oxygen saturation are the compensation result between growth and production.

A gluconic acid concentration higher than 230 g/l was continuously produced at residence times of 12 hrs without any biomass retention (Anastassiadis et al. 2005). New alternative processes for the continuous production of very high gluconic acid concentrations using free growing cells or biomass retention of *A. pullulans* are described in the present work. Biomass retention by immobilization or cross over filtration enables the application of production optima in a continuous fermentation process even at very short times that would normally wash out the biomass from the fermenter. Thus, the maximum specific productivity of generic product can be maintained, uncoupling product formation from growth rate, although it differs from the optimum growth conditions. Biomass retention by means of cross over filtration makes it possible to break the existing link between growth and the residence time. *A. pullulans* was easily immobilized on porous sintered glass and suitable for a continuous gluconic acid production by immobilized cells. A good alternative for the acceleration of product concentration maximization offers the partial biomass retention by means of microfiltration (cross over filtration). Continuous fermentation can thus perform at optimum production parameters, which would not necessarily be optimal for the formation of biomass, thus breaking partially the interaction between growth and production. A very high formation rate and concentration of generic product can be obtained at simultaneously keeping very high specific productivities. Although continuous fermentation performing in a fluidized bed reactor with immobilized biomass on a support carrier may be more convenient from a process point of view, fermentation with non-supported microorganisms performing without or with biomass retention by cross over filtration has been found to be advantageously. In both cases, very high dissolved oxygen concentrations higher than 200% air saturation are applied successfully.

Early fungal fermentation processes for gluconic acid production employed species of *Penicillium* (Herrick and

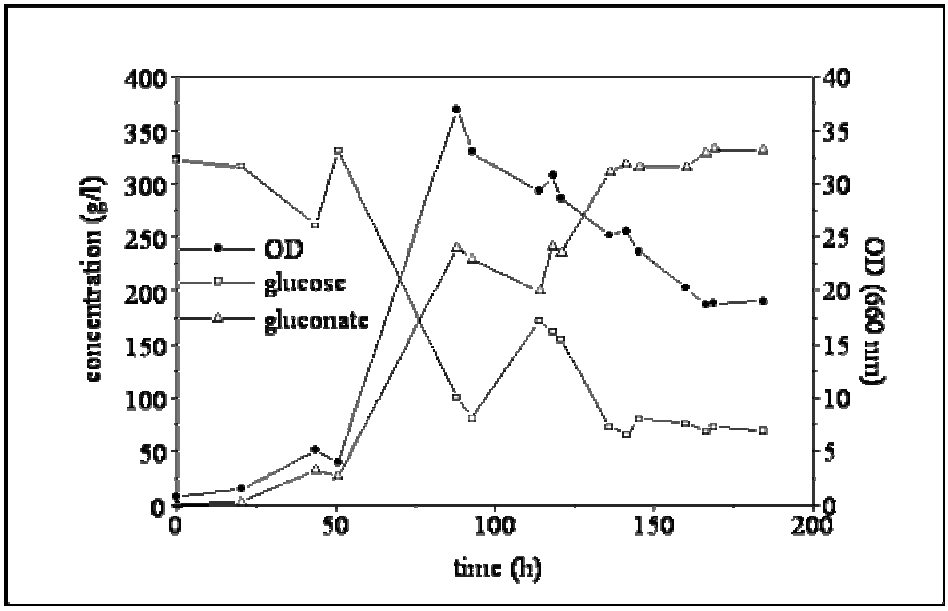

Figure 3. Continuous gluconic acid fermentation by free growing cells of A. pullulans at a residence time of 25 hrs (3 g/l NH_4Cl, 450 g/l glucose, 1 mM iron, 5 mM manganese, pH 6.5, 30ºC and 155% oxygen saturation).

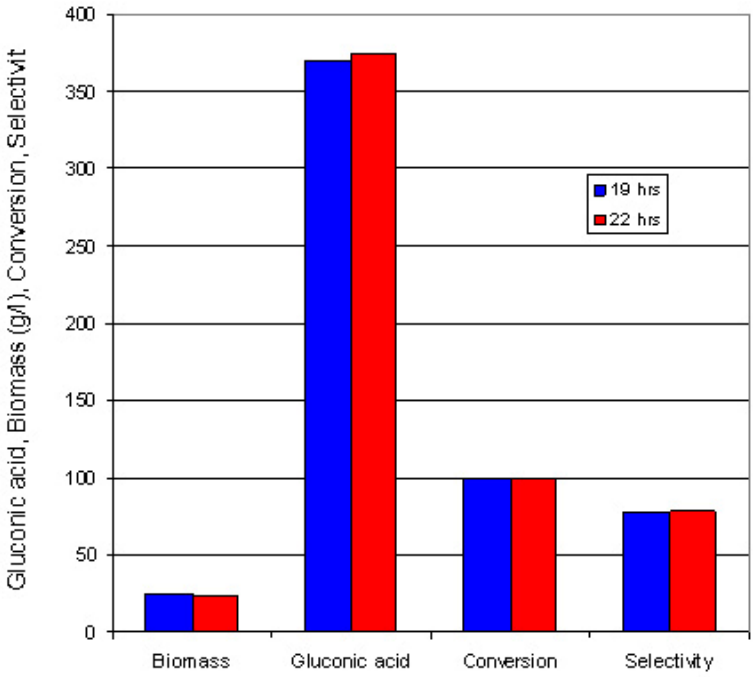

Figure 4. Continuous production of gluconic acid by cross over filtration.

May, 1928). Improved strains of *Aspergillus niger* (predominantly) with recycled mycelia or *Gluconobacter suboxidans* are used in discontinuous submerged fermentations in gluconic acid industry (Röhr et al. 1983). Gluconic acid formation carried out in shake flasks by the yeast-like mold *Aureobasidium* (former *Dematium* or *Pullularia*) *pullulans*, using various carbon sources, has been known since very long time (Pervozvansky, 1939; Takao and Sasaki, 1964; Sasaki and Takao, 1970). However, gluconic acid production by *A. pullulans* on an industrial scale, which is well characterized for the production of pullulan, stayed out of question and investigation.

To our knowledge, no comparable results reaching those high product concentrations (Anastassiadis et al. 1999; Anastassiadis et al. 2003; Anastassiadis et al. 2005; present work) can be found in the international literature, referring to the continuous gluconic acid production by free growing or immobilized cells of *Aspergillus* and *Penicillium* strains or other microorganisms, without any biomass retention. 260 g/l of gluconic acid were continuously produced without any biomass retention under optimized conditions by *A. pullulans* at 15.3 hrs RT and 305-315 g/l at about 21 hrs. 370 g/l of gluconic acid were also continuously produced at complete glucose conversion at 19 hrs residence time and up to 504 g/l were achieved in fed batch experiments (Anastassiadis et al. 1999; Anastassiadis et al. 2003; present work). Sankpal et al. (1999) reported about continuous gluconic acid production by immobilized mycelia of *A. niger* achieving 120-140 g/l gluconic acid. The repeated reuse of cell mycelia has been reported as an option of semi-continuous production of gluconic acid by *A. niger* (Hatcher, 1972). Alternatively, high gluconate concentrations and very high productivities were achieved by the fed-batch-wise addition of large amounts of glucose into fermenter using *A. niger*. However, the productivity became unattainable after a few repetitions, whereas 220 g/l gluconate were repeatedly produced 14 times, using immobilized mycelia on a non-woven fabric within a period of about 1,000 hrs (Sakurai et al. 1989). A product inhibition has been observed in *A. pullulans* process at gluconic acid concentrations above 250 g/l in good agreement to reports of Gastrock et al. (1938), Lee et al. (1987) and Sakurai et al. (1989) for *A. niger* (300 g/l).

Knowingly, *A. niger* is difficult to handle because of causing clogging problems during the fermentation and unsuitable for a continuous operation using free growing cells. *Gluconobacter* has been reported to produce a relative large quantity of keto-acids during the production of gluconic acid (Elfari et al. 2005), thus complicating down streaming processing and the isolation of gluconic acid. Velizarov and Beschkov (1998) reported about a rapid decrease of conversion in *Gluconobacter oxydans* at glucose concentrations higher than 0.56 M. Increasing additions of gluconic acid to the medium resulted in extended lag phases of growth and the maximum possible

gluconic acid concentration has been approximated at 0.7 M (Velizarov and Beschkov, 1998). For comparison, the continuous operations using *A. pullulans* show many advantages compared with the traditional discontinuous fungi and bacterial processes. Under optimized fermentation conditions, it proved to be a superior gluconic acid producer, which can be used in continuous culture for a very long period without any stability problems. The yeast like fungus *Aureobasidium pullulans* integrates the advantages of fungal and bacterial systems at once. It is enabled to utilize astonishingly very high glucose concentrations in continuous single or two stage mode reaching very high gluconic acid concentrations of 200-433 at residence times between 10 and 38 hrs and a maximum biomass specific productivity of about 10 g/(g x h) (Anastassiadis et al. 1999; Anastassiadis et al. 2003; Anastassiadis et al. 2005). In comparison, Roukas and Harvey (1988) reached 16 g/l gluconic acid by free growing cells of *A. niger* at 83 hrs residence time. Babel and Müller (1987) obtained 208 g/l of gluconic acid at 40 residence time using *Acetobacter methanolicus*, whereas up to 295 g/l have been continuously produced by permeabilized cells of *Zymomonas mobilis* in about 75 hrs (Rehr et al. 1991). In a continuous process for the simultaneous production of sorbitol and gluconic acid, the use of cell-free glucose-fructose oxidoreductase from *Z. mobilis* requires the efficient stabilization of the enzyme (Gollhofer et al. 1995).

The use of genetically engineered microorganisms and the immobilization of isolated glucose oxidizing enzymes or whole cells in specialized reactors appear in literature reports as the possible future developments regarding the advanced continuous production of gluconic acid (Hartmeier and Döppner, 1983; Milson, 1987; Szajani et al. 1987). Park et al. (2000) cloned and expressed for example glucose oxidase from *A. niger* in *Saccharomyces cerevisiae* using a yeast shuttle vector. Specialized reactors (Basseguy et al. 2004; Godjevargova et al. 2004) and genetic programming, as well as multicriteria optimization strategies (Cheema et al. 2002; Halsall-Whitney et al. 2003) have been applied for the production of gluconic acid as well. The fact that the present superior results were reached using a wild strain as it has been isolated from the nature and without the involvement of any classical or genetic engineering mutagenesis approaches clearly emphasize the nature's great latent and still unknown potential for further future biotechnological achievements. The development of the present superior fermentation processes has accomplished the highest expectations of an expertise researcher in the field of industrial microbiology and biotechnology, extending the frontiers of known microbial capabilities and emphasizing the importance of the deep understanding of classical and industrial microbiology in the field of biotechnology science. The novel continuous and discontinuous fermentation processes of *Aureobasidium pullulans*, applying or not biomass retention, offer new opportunities and are very promising for future applications for the commercial production of gluconic acid. The new processes can last in today's high competitiveness in industry, claiming numerous advantages over the traditional fungi or bacteria processes of the last 100 years. A continuous industrial production of the responsible glucose oxidizing enzyme from *A. pullulans* appears feasible as well.

The process operation is very stable, under the condition that filter modules are cleaned (periodical reverse flow of filtrate). A precondition for an unproblematic long time operation is also keeping the system safe from contaminations, because some contaminants can overgrow the production strain. No strain instabilities have been observed so far during the extensive process optimization and development program, showing the genetic stability of the new yeast-like mold system.

CONCLUDING REMARKS

Such data of reaching very high gluconic acid concentrations, which are stably obtained for a very long time under continuous cultivation using free growing chemostat cells without any biomass retention, have not been published in the international literature before. The very high product molar and mass selectivities, reaching more than 100% (g/g), show that the new process would be a favourable alternative for the industrial production of gluconic acid compared with the discontinuous fungi processes of the last 100 years. Even 504 g/l of gluconic acid were achieved in fed-batch fermentations using *A. pullulans* isolate 70 (Anastassiadis et al. 1999; Anastassiadis et al. 2003; Anastassiadis et al. 2005). Interesting future research works would include the investigation of continuous gluconic acid production, using biomass retention by cross over filtration, at different residence times and at varying the proportion ratio between filtrate and bleed stream. Mathematical models would enable the prediction of experimental results at various stream ratios and facilitate future research designs and experimental plans.

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DECLARATION

The experiments of the present manuscript comply with the currant laws of the country Germany (Institute of Biotechnology 2 of Research Center Jülich 2, RCJ) where the experiments were performed.

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