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RESEARCH ARTICLE

Oxygen and temperature effect on continuous citric acid secretion in *Candida oleophila*

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Abbreviations: m_p:Specific productivity of the generic product, g product/(g biomass x h)
R_j: Volumetric productivity of the generic product, g product/(l x h)
RT: Residence time, hrs
Rs: Volumetric glucose consumption rate, g/(l x h)

The influence of air saturation and temperature on continuous citric acid secretion was studied in chemostat cultures of *Candida oleophila* ATCC 20177 (var.). Simultaneous measurements of intra- and extracellular concentration of glucose, citric and isocitric acid confirmed the involvement of a specific active transport system in citrate secretion, favouring citric acid over isocitrate. An optimum air oxygen saturation of 20% and temperature of 30-31ºC were determined for the continuous citric acid secretion. The highest values of citric acid concentration (98 g/L), citrate to isocitrate ratio (33.3:1), volumetric citric productivity (1.8 g/(L x h)), and specific citric acid productivity (0.1 g/(g x h)), were reached at 20% air saturation at a residence time of 54 hrs by the experiment's lowest biomass of 18 g/L. The highest isocitic acid volumetric productivity (55.6 mg/(L x h)) and specific productivity (0.99 mg/(g x h)) were identified at 50%, instead. The fastest citrate excretion rate of the generic product of 0.046 g/(g*h) was found at 30-31ºC. A concentration ratio between extra- and intracellular concentration of citrate of up to 9 was identified. The highest extra-/intracellular ratio of citrate and lowest intracellular concentrations of glucose, citric and isocitric acid were determined at optimum air saturation as a consequence of active citrate export.

Citric acid is a tricarboxilic organic acid of industrial importance (Naumenko et al. 2004), which is produced by fermentation using almost exclusively improved strains of the fungus *Aspergillus niger* in aerobe bioreactors (Saha and Takahashi, 1997; Wieczorek and Brauer, 1997; Wieczorek and Brauer, 1998). The simultaneous synthesis of citric, isocitric (undesirable by-product) and other organic acids by yeasts was extensively studied (Akiyama et al. 1973a; Akiyama et al. 1973b; Anastassiadis, 1994; Moresi, 2004; Grewal and Kalra, 1995; Anastassiadis et al. 1993; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Crolla and Kennedy, 2001; Kamzolova et al. 2003; Crolla and Kennedy, 2004; Venter et al. 2004), in particular by using strains of *Yarrowia* spp. and *Candida* spp. under shake flasks, batch or fed batch, continuous repeated batch, continuous repeated fed batch or chemostat fermentation conditions (Tabuchi et al. 1970; Lozinov and Finogenova, 1982; Rupcic et al. 1996; Chernyavskaya et al. 2000; Crolla

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and Kennedy, 2001; Papanikolaou and Aggelis, 2002; Morgunov et al. 2004; Anastassiadis and Rehm, 2005; Anastassiadis and Rehm, 2006).

Previous studies have demonstrated the feasibility of a semi-continuous production of citric acid by *A. niger* (Lesniak and Stawicki, 1979). An efficient continuous production of citric acid remained however for long time an unachievable dream. A multi-tank system was suggested for the continuous operation using *A. niger*, which would separate growth phase and idiophase from each other. This would essentially require higher investments and efforts, making a commercial production uneconomical (Lockwood, 1979; Milson, 1987; Crueger and Crueger, 1989; Klasson et al. 1989; Saha and Takahashi, 1997; Wieczorek and Brauer, 1997; Wieczorek and Brauer, 1998). Continuous single-stage operations using *A. niger* yielded low citric acid concentrations (Kristiansen and Sinclair, 1979; Saha and Takahashi, 1997; Wieczorek and Brauer, 1997; Wieczorek and Brauer, 1998). The main actual problems at citric acid industry today are the still low productivities of discontinuous *A. niger* processes, requiring long operational times, higher investments and production costs, compared with the novel yeast processes (Legisa and Gradisnik-Grapulin, 1995; Anastassiadis et al. 2001; Kumar et al. 2003; Kurbanoglu, 2004; Kurbanoglu and Kurbanoglu, 2004; Ikram-ul et al. 2004; Ali and Ikram-ul, 2005; Anastassiadis and Rehm, 2005). Wieczorek and Brauer (1997) reported about slowing down of continuous citric acid formation by *A. niger* in a reciprocating-jet-bioreactor at increasing fermentation time (more than 20 days), still without achieving of steady state conditions. Continuous operations for the production of citric acid by yeasts have increasingly received research interest in last years. However, the yields were insufficient for an economically competitive industrial operation (Enzminger and Asenjo, 1986; Kim et al. 1987; Kim and Roberts, 1991; Klasson et al. 1989; Bubbico et al. 1996; Arzumanov et al. 2000; Anastassiadis et al. 2005; Anastassiadis and Rehm, 2005).

Citric acid production takes places under growth limiting conditions (Crueger and Crueger, 1989; Grewal and Kalra, 1995; Anastassiadis et al. 2001; Anastassiadis et al. 2002; Anastassiadis et al. 2005; Anastassiadis and Rehm, 2006). The microbial system must be rigid enough, in order to withstand the sheerness and to resist against the stress conditions occurring during the long time continuous operation of chemostat cultures. Long sporulation times are necessary before the inoculation of the small inoculum bioreactor and thereafter of the larger production bioreactor. For this reason, *A. niger* is unsuitable for an efficient continuous operation using free growing cells without any biomass retention. However, in contrary to yeasts *A. niger* can also convert sucrose by invertase (Hossain et al. 1984; Milson, 1987). Because of the above indicated inadequacies and problems, *Candida oleophila* ATCC 20177 (var.) was identified during an extensive screening under many yeast strains (Anastassiadis, 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2002), enabling the development of new continuous fermentation processes, which would stand today's strong competition in citric acid industry (Anastassiadis, 1994; Anastassiadis et al. 1993; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2005; Anastassiadis and Rehm, 2005; Anastassiadis and Rehm, 2006). *C. oleophila* offers a good alternative for a continuous operation of citric acid production. Initially, only 10 g/L of citric acid were produced in preliminary continuous experiments by *C. oleophila*. 150 g/L and a formation rate for the generic product (Rj) of 2 g/(L x h) were reached after an extensive process development program carried out in chemostat (Anastassiadis et al. 1993; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis and Rehm, 2005; Anastassiadis and Rehm, 2006). 210-250 g/L citric acid [Rj of up to 5 g/(L x h)] have been continuously produced within 56-120 hrs in repeated batch experiments by a *Yarrowia lipolytica* strain at the Research in Biotechnology, Greece, (data not shown here). *A. niger* is difficult to handle, because of its multicellular mycelial growth behaviour, causing clogging problems. The use of yeasts instead of moulds represents an innovative approach, which is also of human health. In contrary to yeast *Candida oleophila*, *A. niger* possesses the formation potential of toxic compounds (micotoxins) (Anastasssiadis et al. 2005).

A little information exists in international bibliography regarding the mechanism of citric acid secretion in yeasts and fungi and the excretion mechanism of citrate from cytoplasm into the medium in *A. niger* still remains unclear (Grewal and Kalra, 1995; Netik et al. 1997; Anastassiadis and Rehm, 2005; Anastassiadis and Rehm, 2006). Looking at the literature, no results were found about the effect of oxygen and temperature on the continuous citric acid

Table 1. Temperature influence on the steady citric acid production continuous process. Chemostat was operated at a dilution D = 0.018 h^{-1} (3 g/L NH_4Cl, 240 g/L glucose, pH 4.5).

Temp.	[Citrate]	[Isocitrate]	Ratio	[Biomass]	Rcitrate	$m_{citrate}$
ºC	(g/l)	(g/l)		(g/l)	[g/(l x h)]	[g/(g x h)]
24	12.00	0.58	17.0	8.00	0.15	0.019
27	22.67	1.09	20.9	11.2	0.28	0.025
29	43.14	2.53	17.1	20.3	0.54	0.027
30	63.54	2.21	28.8	17.4	0.79	0.046
31	60.63	2.97	20.4	16.5	0.76	0.046

formation and secretion by free growing yeast cells. The intracellular accumulation and secretion of citric acid are obviously two different phenomena, which influence each other in a very complicated manner. A high specific active transport system for citric acid secretion has been detected for the first time in *Candida oleophila* based on simultaneous intra- and extracellular steady state measurements of citric and isocitric acid, glucose and the ATP/ADP ratio in the cells. It is acting as the speed determining factor well explaining overproduction of citric acid against a concentration gradient (Anastassiadis et al. 1993; Anastassiadis, 1994; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis and Rehm, 2005; Anastassiadis and Rehm, 2006). Netik et al. (1997) reported later about a ΔpH-driven H^+-symport dependent system for citric acid export in manganese-deficient cells of *A. niger*. They claimed that only a passive diffusion over the plasma membrane had been reported before for citrate excretion in yeasts in accordance with reports of Marchal et al. (1980) and McKay et al. (cited in Gutierrez and Maddox, 1993). For a very long time it has been thought that a continuous citric acid production using free growing cells wasn't feasible. Additionally, no information existed about the influence of air saturation on the continuous citric acid secretion and a little was known regarding the real effect of oxygen on the continuous citrate formation.

The central aspect of present work was to investigate the effect of important fermentation parameters, such as air saturation and temperature on the continuous secretion of citric acid by a specific active transport system in *C. oleophila* ATCC 20177 (var.).

MATERIALS AND METHODS

Microorganism

Candida oleophila, strain ATCC 20177 (var.) was used, which was obtained from Dr. Siebert, Jungbunzlauer Co. and from Haarmann and Reimer (Bayer Co affiliated company, Leverkusen, Germany) was used. This strain was selected in previous studies under many yeast strains (Anastassiadis, 1994). Yeast malt extract agar plates (YME) inoculated with *C. oleophila* were incubated for 2-3 days and stored at 4ºC. Cultures were refreshed every 2-3 months. Glycerine cultures (30% glycerine) were frozen at –20 or -80ºC and stock cultures were lyophilized as well.

Apparatus

The influence of temperature on continuous citric acid fermentation was investigated in 1 litter glass bioreactor (Research Center Jülich, Germany) at 1000 rpm and an aeration rate of 4 l/min pure oxygen for a working volume of 460 ml (corresponds to 0.145 vvm). Pure oxygen was used in order to satisfy the low oxygen transfer in small glass fermenter (Anastassiadis et al. 2005). The effect ofoxygen on continuous citric acid fermentation was investigated in a 2 litter bioreactor (Biostat E, Braun-Diessel) at a working volume of 1.9 l, 600 rpm, pH 4.5 and 30ºC. The air saturation (%) was measured and controlled as has been described in Anastassiadis et al. 2001 and Anastassiadis et al. 2005. This setting of air saturation control was found to be best solution for keeping constant air saturation over a very long period of time. The air saturation was adjusted to 100% using atmospheric air at 1 vvm flow rate, 1000 rpm agitation, 30ºC and pH 4.5 in sterilized fermentation medium. The 0% air saturation value was adjusted using 1 vvm nitrogen gas.

Media

The inoculum (10%) was prepared by transferring cells from agar plates into 500 ml shake flasks with buffles on a medium containing BM medium with 3 g/L NH_4Cl and 30 g/L glucose. The shake flasks were incubated for 1 day at 30ºC and 200 rpm. A basal medium (BM) was used for the investigation of temperature effect containing: 3 g/L NH_4Cl, 240 g/L glucose, 0.7 g/L KH_2PO_4, 0.35 g/L $MgSO_4$ x $7H_2O$, 0.11 g/L $MnSO_4$x $4H_2O$, 0.002 g/L $CuSO_4$ x $5H_2O$, 0.021 g/L $ZnSO_4$ x $7H_2O$, 0.004 g/L $CoSO_4$ x $7H_2O$, 0.04 g/L H_3BO_3, 0.1 g/L $CaCl_2$, 0.1 g/L NaCl, 0.0001 g/L KJ, 2.5 g/L citric acid, 0.0002 g/L Na_2MoO_4 x $2H_2O$, 0.002 g/L Thiamine-HCl, 0.00025 g/L Biotin, 0.000625 g/L Pyridoxine-HCl, 0.000625 g/L Ca-D-Pantothenate, 0.0005 g/L Nicotinic acid. An optimized medium was used for the investigation of oxygen influence containing: 250 g/L glucose, 4.5 g/L NH_4Cl, 1.05 g/L KH_2PO_4, 0.525 g/L $MgSO_4$ x $7H_2O$, 0.2507 g/L $MnSO_4$ x $4H_2O$, 0.00015 g/L $CuSO_4$ x $5H_2O$, 0.0315 g/L $ZnSO_4$ x $7H_2O$, 0.006 g/L $CoSO_4$ x $7H_2O$, 0.06 g/L H_3BO_3, 0.15 g/L $CaCl_2$, 0.15 g/L NaCl, 0.00015 g/L KJ, 2.5 g/L citric acid, 0.0003 g/L Na_2MoO_4 x $2H_2O$, 0.003 g/L Thiamine-HCl, 0.000375 g/L Biotin, 0.0009375 g/L Pyridoxine-HCl, 0.0009375 g/L Ca-D-Pantothenate, 0.00075 g/L Nicotinic acid. Vitamins and NH_4Cl were added separately into autoclaved medium (30-60 min at 121ºC) by sterile filtration through 0.2 µm filters (Sartorius filter, Göttingen Germany). No Fe^{+2} salt has been intentionally added in to the media. Silicon oil or polypropylene glycol was used as antifoaming agent. In all cases, nitrogen was a limiting factor of yeast growth. The culture flow rate D was 0.0185 h^{-1}.

Chemostat experiments

The temperature was maintained automatically at 30ºC and the pH at 4.5 by the automatic titration of 45% NaOH solution. The pO_2 level was maintained within 5-133% of saturation. The experiments were carried out as has been described in Anastassiadis and Rehm, 2005 and Anastassiadis and Rehm, 2006. Fermentation data presented here didn't result from just only one single measurement. They represent an average of several measurements attained after achieving steady state conditions during a period of several days. In generally, between five and 10 generations (residence times, RT) were necessary for achieving steady state conditions at each of air saturation levels. When the flow rate and fermentation

Table 2. Intracellular concentration of critical metabolites (glucose, citrate and isocitrate), and intra- (i) and extracellular (e) ratios between the citrate species as a function of temperatures.

	Intracellular concentration					Ratios			
Temp	Citrate	Isocitrate	Glucose	Citrate + Isocitrate	Citrate + Isocitrate + Glucose	Citrate/ Citrate	Isocitrate/ Isocitrate	Citrate/ Isocitrate	Citrate/ Isocitrate
(ºC)	(mg/g)	(mg/g)	(mg/g)	(mg/g)	(mg/g)	(e/i)	(e/i)	(i/i)	(e/e)
27	21.6	90.6	2.6	112.2	114.8	2.4	0.03	0.24	20.9
29	10.2	54.3	45.8	64.5	110.3	9.3	0.10	0.19	17.1
31	19.8	25.9	0	45.7	45.7	6.7	0.25	0.76	20.4

parameters had achieved a stationary level, the cultivation in each mode was continued until the culture medium in bioreactor was replaced five times. There was no difference in experimental data obtained under the same operation conditions in dependence of starting randomly or not a new chemostat experiment from a new Agar plaque or continuing last fermentation.

Analysis

Optical density ($OD_{660\ nm}$) and dry biomass (filter method) were measured as described (Anastassiadis et al. 2002). Organic acids, glucose, ammonia nitrogen and intracellular concentrations were analyzed as described in Anastassiadis, 1993; Anastassiadis, 1994; Anastassiadis et al. 1993; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2002; and Anastassiadis et al. 2005.

Intracellular measurements of citric and isocitric acid and glucose

Intracellular concentrations of citric, isocitric acid and glucose were precisely evaluated using the above HPLC methods (High Pressure Liquid Chromatography) after their extraction following the procedure as has been described in Anastassiadis and Rehm, 2005 and Anastassiadis and Rehm, 2006.

Residual nitrogen

The residual nitrogen was measured as has been described in Anastassiadis et al. (2002), however no nitrogen was detected in all of experiments.

RESULTS

The effect of temperature on continuous citric acid secretion by *C. oleophila*

The influence of temperature on citric acid production by *C. oleophila* was investigated in chemostat experiments at a residence time of about 80 hrs and pH 4.5. 20 days were necessary for achieving steady state conditions after the temperature was increased from 27 to 29ºC. The highest citrate concentration of 63.5 g/L, citrate/isocitrate ratio of 28.8 and formation rate of the generic product (Rj) of 0.8 g/(L x h) were achieved at 30ºC, compared with only 12 g/L (18.9%), 17 and 0.15 g/(L x h) found at 24ºC. The highest specific citric acid productivity of 0.046 g/(g x h) was determined at 30-31ºC. The highest biomass concentration (X) of 20.3 g/L was measured at 29ºC. Isocitric acid concentration increased with the exception of 30ºC (2.21 g/L) at raising temperature (Table 1). Only 80 g/L of citric acid was achieved at a RT of more than 100 at 30ºC under still sub-optimal fermentation conditions. The intracellular steady state concentrations of citrates and glucose were determined at selected temperatures. Intracellular isocitric acid concentration decreased drastically with increasing temperature reaching 10.2 mg/g dried biomass at 31ºC. In contrary, citric acid concentration remained roughly constant, indicating in accordance to previous studies (Anastassiadis and Rehm, 2005) that a minimum concentration is required for functioning of active transport system. Thus, a greater intracellular ratio between citrate and isocitrate is to record at higher temperatures, which is still lower than 1, in contrary to an almost identical extracellular ratio (Table 2). An intracellular glucose concentration of 2.6 mg/g was identified at 27ºC and of 45.8 mg/g at 29ºC (Table 2).

The effect of oxygen on continuous citric acid secretion by *C. oleophila*

The effect of air saturation on continuous citric acid secretion by *C. oleophila* was examined at a residence time around 55 hrs, applying an optimised chemostat medium, optimum temperature of 30ºC and pH 4.5. Citric acid secretion has been found to be strongly influenced by air saturation, resulting at 54 hrs residence time (D = 0.0185 h^{-1}) to maximum values of 98 g/L citric acid, 70% molar selectivity (Mol citrate/Mol glucose), 1.81 g/(L x h) formation rate of the generic product (R_j), 0.1 g/(g x h) specific citric acid productivity (m_p) of and 33.3 ratio between citrate and isocitrate (3% isocitrate) at the optimum saturation of 20%. Only 71.4 g/L citric acid (72.6%) was produced at 133% and 77.2 g/L (78.6%) at 5%. Hereby, the experiment's lowest biomass of 18 g/L was measured at 20% compared with 29.6 g/L found at 80%. The maximum Rj of 55.6 mg/(L x h) for isocitric acid was identified at 50% and m_p of 2.9 mg/(g x h) at 20%. The very high ratio found between citrate and isocitrate, which

corresponds to m_p(citrate)/m_p(isocitrate) ratio, specifies the high affinity of active transport system towards citric and over isocitric acid, claiming a specificity maximum at 20% saturation. The highest glucose consumption rate of 2.6 g/(L x h) was specified at 78% air saturation, whereas specific glucose consumption rate reached a maximum of 0.13 g/(g x h) at 20%. Citrate concentrations of up to 150 g/L have been achieved under the same fermentation conditions at longer RT using *C. oleophila* (Anastassiadis, 1994; Anastassiadis et al. 1993; Anastassiadis et al. 1994; Anastassiadis et al. 2001).

In contrary to highest extracellular citric acid values, the lowest intracellular isocitrate concentration of 24.4 mg/g dry biomass, citrate + isocitrate of 53.1 mg/g (125.6 mM), glucose of 17.6 mg/g (8 g/L or 44.4 mM) or total acid plus glucose concentration of 70.72 mg/g and the highest intracellular ratio between citrate and isocitrate of 1.18 were determined at optimum air saturation of 20%. In comparison, a total intracellular acid concentration (citrate plus isocitrate) of 135.9 mg/g (256% compared with 20%) and total acid + glucose concentration of 163.2 mg/g (230.8%) were determined at 5% and 96.8 mg/g (182.3%) and 123.1 mg/g (174.1%) at 133% saturation. The lowest intracellular citrate concentration of about 9.5 mg citrate/g dried biomass (corresponds to 4.3 g/L or 22.5 mM) was determined at 29% compared with 28.7 mg/g (13.1 g/L or 67.96 mM) or 53.63 mg/g (20.7 g/L or 107.58 mM) measured at 20% or 5%, respectively. With exception of 20% a higher intracellular concentration of isocitrate has been found along the entire range of air saturation compared with citrate (intracellular ratio between citrate and isocitrate was lower than 1). Comparing the intracellular citrate/isocitrate ratio of 1.18 with the extracellular ratio of 33.3 found a very high citrate secretion rate becomes obviously at 20% that drives out isocitrate from aconitase equilibrium towards citrate. A concentration ratio of 7.5 was calculated at 20% between extra- and intracellular citric acid, based on measured extracellular concentration of 98 g/l and the determined intracellular citrate concentration of 13.1 g/L or 67.96 mM.

DISCUSSION

There are only a few reports related to the continuous production of citric acid. Kinetic data for growth and production from continuous chemostat cultures offer very important information for process development, optimization and scale up. Present work describes for the first time the very significant effect of oxygen and temperature on continuous production and secretion of citric acid using free growing cells of *Candida oleophila*. In comparison, most of the reported works regarding citric acid fermentation were carried out without any oxygen measurement and control.

Temperature effect

Significant differences and different optimum temperatures were determined for growth and production in mesophilic yeast *C. oleophila*, although they are somehow coupled to each other. *C. oleophila* grows and produces in good agreement with literature reports for the discontinuous citric acid fermentation by yeasts (Rane and Sims, 1993; Crolla and Kennedy, 2001) at temperatures between 24 and 31ºC, displaying an optimum temperature of 30-31ºC. Temperatures lower than 27ºC slowed down growth and production substantially. Tabuchi (1973) noted growth and citrate production in *C. lipolytica* even at 35ºC using glucose medium, whereas no growth took place in paraffin medium. Intracellular analysis data of present work confirmed in a good agreement with previous data regarding the continuous citrate secretion through a high specific pH-dependent active transport system in yeasts (Anastassiadis et al. 1993; Anastassiadis, 1994; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis and Rehm, 2005; Anastassiadis and Rehm, 2006). A higher glucose uptake rate, glucose consumption through the glycolysis and citrate secretion rate is proposed at optimum temperatures, based on the very low intracellular concentrations of glucose, citric and isocitrate acid. The specific productivity (m_p), which corresponds to the secretion rate, is the best parameter to choose in order to identify, whether there is any possible connection between the intracellular concentration of citrates and their secretion rate. A 3.5-fold intracellular isocitrate concentration was found at 27ºC and a 2.1-fold at 29ºC compared with 31ºC, which is 1.7 times higher at 27ºC compared with 29º C (3.5/2.1 = 1.7). For comparison, 2.67 times higher citric acid was produced at 31ºC compared with 27ºC, and 1.41 times higher compared with 29ºC (2.67/1.41 = 1.9). Intracellular concentration of isocitric acid is directly influenced by active transport system, reversely revealing extracellular concentration of citric acid in almost the same proportion. The speed of removal of intracellular isocitric acid from aconitase equilibrium towards citrate and citrate secretion reflects the transport system's activity. The lowering of citrate excretion by specific active transport system would consequentially result in the accumulation of isocitrate and glucose in cytoplasm. In contrary, intracellular citrate showed less fluctuation and a certain minimum concentration of about 20 mM is necessary for the proper functioning of active transport system. A 17.6-fold higher glucose concentration was found at 27ºC compared with 29ºC, meaning a much higher glucose consumption rate at 29ºC, again as a consequence of active transport system's activity. Lapujade et al. (1999) found a similar effect of temperature on the secretion of glutamic acid in *Corynebacterium glutamicum*, reporting about the highest secretion at 40ºC and the lowest intracellular glutamate accumulation. Enzyme activities as well as regulation and transport systems are in generally affected enormously by the temperature in microbial systems.

Oxygen effect

The intensity of citric acid secretion has been shown to be strongly influenced by air saturation under batch, repeated batch and continuous cultivation of *C. oleophila*, indicating the importance of oxygen demand and oxygen mass

transfer (Anastassiadis and Rehm, 2005; present results). It had a remarkable effect on growth, citric acid accumulation, ratio between citrate and isocitrate and product yield, displaying a sharp production optimum in chemostat cultures at 20% in contrary to highest biomass found at 80%. Batch and continuous repeated batch process claimed a higher productivity and selectivity at 80% saturation instead (Anastassiadis and Rehm, 2006). Citric acid production by yeasts and fungi is an obligatory aerobic process, strongly depending on the oxygen supply and air saturation in bioreactor. Most of published works in literature were carried out at unknown air saturation or dissolved oxygen concentration. In present work the air saturation was controlled, enabling the reproduction of results in further future studies. Excess of oxygen is necessary during entire fermentation, whereby even short time interruptions of oxygen supply can cause irreversible changes or a complete production lost (Rehm, 1980; Anastassiadis, 1994; Grewal and Kalra, 1995; Sakurai et al. 1996; Crolla and Kennedy, 2004). Optimum air saturations higher than 50% and up to 60 ppm (equals to about 800% air saturation) were reported in literature for discontinuous citric acid production by yeasts (Stottmeister et al. 1981; Stottmeister et al. 1986; Okoshi et al. 1987).

The very high extracellular citrate/isocitrate ratio, the very low intracellular citrate/isocitrate ratio (excepted of 20% air saturation lower than 1) and the insignificant variations of isocitrate concentration found along the entire range of air saturation indicated the high specificity of active transport system towards citrate over isocitrate. In this context it is worthy to mention that specific citric and isocitric productivity corresponds to the secretion rate of active transport system. The formation rate of the citric acids is the compensation result between biomass formation and specific productivity. The lowest intracellular concentration of glucose, isocitrate, citrate plus isocitrate, total acid plus glucose, maximum intracellular citrate/isocitrate ratio and the highest extracellular citric acid concentration were found at optimum air saturation of 20% as a result of most intensive secretion and consequentially glycolysis rate. The high glycolysis rate is possibly resulting in very high ATP levels (higher energy charge) thus intensifying citrate secretion by active transport system as has been reported in Anastassiadis and Rehm (2005). A kind of a Crabtree effect could occur in this case, simulating an anaerobic glycolytic pathway under aerobic conditions. The higher intracellular isocitrate accumulation found at suboptimal air saturations (*e.g.* 7% and 29%) as a result of lower active secretion of citrate is possibly caused by the lower energy charge, indicating a higher aconitase activity. This would mean an inhibition of aconitase by ATP in contrary to AMP, which activates the enzyme, aiming the gain of energy through the respiratory chain. Dissolved oxygen concentration appears to play a very important role in terms of influencing the activity of glycolysis and respiration chain. Lower air saturation would promote ATP formation by substrate phosphorylation along the glycolysis, whereas at higher air saturation ATP synthesis would be essentially regenerated by the respiratory chain phosphorylation.

The existence of specific active transport system for citrate secretion and the strong correlation between ATP/ADP ratio and the formation rate of the generic product citrate found in *C. oleophila* (Anastassiadis, 1994; Anastassiadis and Rehm, 2005) goes well together with reports of Lozinov and Finogenova (1982) about the existence of a non-phosphorylating alternative oxidase found in yeasts that completes electron flow without ATP regeneration competing with the production of citric acid. Active citric acid producing strains showed lower alternative oxidase activity, instead (Lozinov and Finogenova, 1982). Whether the energy charge is the driving force for citrate excretion in *A. niger* is still unclear. In contrast to yeasts an active non-phosphorylating alternative oxidase has been discussed in relation with the overproduction of citric acid in *A. niger* (Wallrath et al. 1991). In this case, reduction equivalents are regenerated by the alternative oxidase, rather than by ATP formation. Meyrath (1967) has discussed on the other side the energy demand for growth and citrate excretion in stationary cells of *A. niger*. A significant amount of energy is required since the acid is excreted against a concentration gradient.

Iron appears to have a significant interacting influence in this content, compensating the oxygen effect by responses of cellular metabolism. Kamzolova et al. (2003) found for example a strong dependence of oxygen requirements for yeast growth and citric acid synthesis on the iron concentration in medium. Increasing iron concentration was found to result in higher biomass concentration following up Michaelis's Menden kinetics, whereas iron limitation promoted citric acid secretion by *C. oleophila* (Anastassiadis, 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2002). The iron effect has been reported to be related to the activity of mitochondrial aconitase and other TCA cycle enzymes (Oexle et al. 1999), acting as a cofactor. Knowingly, based of the legendary reports of Akiyama et al. (1973a) and Akiyama et al. (1973b) aconitase activity has been considered abroad in literature to directly influence citric acid accumulation in yeasts. Analyzing present results it appears that the iron effect on citrate secretion is more complex and it is also related to its influence on respiratory activities. In these terms, iron limitation forces a higher glycolytic activity, which is resulting in excessive formation of ATP and subsequently to higher citric acid secretion by active transport system. In excess of iron and carbon source, even under nitrogen limitation, citric acid is broken down to smaller molecules, whilst ATP is used for the formation of biomass instead of lipids (lipogenous yeasts) or citric acid (non-lipogenous yeast) (Anastassiadis and Rehm, 2005). Energy consuming fatty acid synthesis and citric acid secretion can be considered as a means of cutting down energy overload and surplus amount of $NAD(P)H_2$. Isocitric acid could somehow interact with ATP and AMP and regulate both, aconitase and transport system activity, thus also explaining its very low affinity to the transport system. This means,

that a higher dissolved oxygen concentration is necessary under iron limitation for an efficient respiratory activity and biomass formation. Cellular metabolism at lower oxygen concentrations is supported by a higher iron concentration in medium (Kamzolova et al. 2003). 10-fold higher fluxes of pyruvate into the tricarboxylic acid cycle branch and 20 times greater TCA cycle fluxes has been reported by Hua and Shimizu (1999) for cells of *Torulopsis glabrata*, grown at dissolved oxygen higher than 10% compared with 1%.

Previous studies have demonstrated a low-rate fed-batch and continuous production of citric acid under nitrogen-limiting conditions by the yeast *Candida lipolytica* or other *Candida* strains (Aiba and Matsuoka, 1979; Kim et al. 1987; Klasson et al. 1989; Tisnadjaja et al. 1996; Crolla and Kennedy, 2004). About a four stage process has been reported by Wieczorek and Brauer (1998) for the continuous citric acid production using *A. niger* and recirculation of fermentation broth. In comparison, present results for the continuous production of citric acid by free growing cells are the best that have been published in international bibliography. Significant citric acid concentrations have also been reached by Kamzolova et al. (2003). 98 g/L and 125 g/L citric acid were continuously produced by *C. oleophila* in chemostat cultures at about 54 hrs RT and up to 150 g/L citric acid at higher RT (even 200 g/L using a *Y. lipolytica* strain, unpublished data). 170 g/L were reached in continuously operating repeated batch experiments by *C. oleophila* (Anastassiadis, 1994; Anastassiadis et al. 1993; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis and Rehm, 2005; Anastassiadis and Rehm, 2006) and 250 g/L by a *Y. lipolytica* strain. 250 g/L were produced in continuous mode in fed batch experiments using *C. oleophila*.

CONCLUDING REMARKS

Citric acid fermentation is a very complex process. Numerous events including growth limitations, enzyme activities, energy gain and energy state, intracellular acid accumulation, as well as uptake and transport systems display different optima and regulation mechanisms, which are somehow interconnected and interrelated in a synergistic mode. The active transport system is the main speed-determining factor in citrate overproduction by yeasts. Isocitrate doesn't seem to be a high-affine substrate for the active transport system. In contrary to previous thoughts, the active transport system is regulating the intracellular accumulation of citric acid and its secretion, rather than aconitase activity. Present results for continuous citric acid production by free growing cells are the best that have been published in international bibliography. The effect of air saturation was significant, which would also influence the costs of an industrial fermentation process enormously.

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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 Centre Jülich 2 (RCJ); formerly known as Nuclear Research Centre Jülich (KFA), where the experiments were performed.