Continuous citric acid secretion by a high specific pH dependent active transport system in yeast *Candida oleophila* ATCC 20177

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The pH influence on continuous citric acid secretion was investigated in *Candida oleophila* ATCC 20177 (var.) under NH_4^+ limiting state steady conditions, using glucose. Highest citric acid concentration of 57.8 g/l, citrate/isocitrate ratio of 15.6, space-time yield of 0.96 g/(l x hr) and biomass specific productivity of 0.041 g/(g x hr) were obtained at pH 5 and 60 hrs residence time. Only 22.8 g/l (39.4%) and a ratio of 9.9 were achieved at pH 6 pH and 12.4 g/l (21.5%) and a ratio of 3.7 at pH 3. Under non producing conditions, in excess of nitrogen, biomass concentration increased at raising pH. An iron concentration of 200 ppm was determined in biomass of *C. oleophila* at pH 5, compared with only 26 ppm found at pH 3 (factor 7.7). Intra- and extracellular concentrations of citrates and glucose confirmed the existence of a high specific, pH dependent active transport system for citrate secretion, while isocitrate isn't a high-affine substrate, displaying a strong correlation with ATP/ADP ratio. Differences between extra- and intracellular concentration of citrate higher than 1 and up to about 60 were determined. The active transport systemfor citrate excretion appears to be the main speed-determining factor in citrate overproduction by yeasts.

Although citric acid production using mutant strains of *A. niger* or yeast strains has almost been extensively optimised, there is still no comprehensive explanation for citrate overproduction, and many aspects related to citrate accumulation and secretion remain unclear. There are many similarities between *A. niger* and yeast strains in mechanism of citric acid synthesis, however, differences still exist in terms of triggering out and regulation of citrate overproduction. Many models have been developed describing the biochemistry of citrate synthesis, using glucose and other carbon sources, however a complete picture of formation pathway, regulation and secretion has not been described.

Overproduction of citric acid in moulds and yeast has been reported to be triggered out by limitations of certain elements, like N, P, Mn, Fe or Zn, essential for citrate accumulation in *A. niger* (Shu and Johnson, 1948a; Shu and Johnson, 1948b; Noguchi and Johnson, 1961; Kisser et al. 1980; Kubicek and Röhr, 1980; Kapoor et al. 1982; Kristiansen et al. 1982; Crueger and Crueger, 1989; Dawson and Maddox, 1989; Grewal and Kalra, 1995), as well as N, P, S and Mg in yeasts *Yarrowia lipolytica* and *Candida oleophila* (Lozinov et al. 1974; Behrens et al. 1987; Stottmeister and Hoppe, 1991; Anastassiadis, 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2002; Anastassiadis et al. 2004). The yeasts can use various carbon sources for the formation of citric acid (Ikeno et al. 1975; Stottmeister and Hoppe, 1991; Grewal and Kalra, 1995; Mansfeld et al. 1995; Crolla and Kennedy, 2001; Crolla and Kennedy, 2004; Venter et al. 2004) or lipid production (Papanikolaou and Aggelis, 2002). Intracellular nitrogen limitation and low intracellular nitrogen content (Briffaud and Engasser, 1979; Moresi, 1994; Anastassiadis et al. 2002; Anastassiadis et al. 2004), occurring after
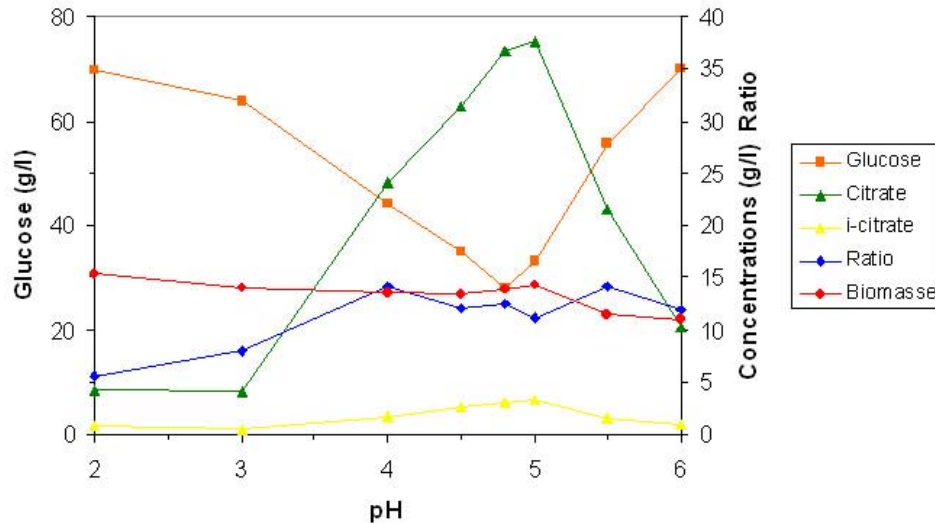
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Figure 1. pH Influence on continuous citric acid fermentation with iron supply at a residence time of about 40 hrs (3 g/l NH₄Cl, 120 g/l glucose and 5 µM iron).

extracellular nitrogen exhaustion and entering a transition phase, and the increase in intracellular NH_4^+ concentration, possibly caused by proteolysis, are the most important factors influencing and triggering out citric acid formation and secretion in yeasts (Anastassiadis et al. 1993; Anastassiadis 1994; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2002; Anastassiadis et al. 2004). Intracellular accumulation of NH_4^+ found in cytoplasm of *A. niger* (Röhr and Kubicek, 1981; Habison et al. 1983) and in *Candida oleophila* (Anastassiadis et al. 2002), possibly caused by a disturbances in protein or nucleic acid turn over, uncouples citrate feed back inhibitory effect on phosphofructokinase, enabling an unlimited flow through glycolysis. A further increase of glycolysis flow is obtained by the stimulation of pyruvate kinase through fructose bi-phosphate (Habison et al. 1979; Kubicek and Röhr, 1980; Habison et al. 1983; Kubicek et al. 1984; Milson and Meers, 1985). A negative effect on pyruvate kinase isn't known, so there is no need for any kind of control at this point (Meixner-Monori et al. 1984).

Utilizing glucose as carbon source, the basic principle, extensively investigated in *A. niger*, of oxaloacetate formation by anaplerotic sequences, *e.g.* by the key enzyme pyruvate carboxylase for citric acid production, (Kapoor et al. 1982; Milson, 1987; Peksel et al. 2002) and phosphoenol carboxykinase (Crueger and Crueger, 1989), the flux delay and inhibition of TCA cycle (α-oxoglutarate dehydrogenase inhibition) and high activity of citrate synthase, is also valid for yeast strains. Glyoxylate cycle is involved in citric acid synthesis in case that acetate, other C_2 sources or aliphatic compounds are used as carbon sources. A positive control of phosphofructokinase by ammonium ions, enabling supply of citrate synthase by acetyl-Co A and oxaloacetate, even a possible repression of α-ketoglutarate dehydrogenase through high glucose and ammonium

concentration (Kubicek and Röhr, 1978; Röhr et al. 1983), or inhibition of its activity by oxaloacetate (Meixner-Monori et al. 1985) doesn't explain completely intracellular accumulation and secretion of citric acid. Cis-aconitate has also been assumed to inactivate the only irreversible reaction of tricarboxylic acid cycle (Kubicek and Röhr 1986). The increasing concentration of α-ketoglutarate caused by oxaloacetate inhibition, inhibits isocitrate dehydrogenase and thus a further α-ketoglutarate formation. As a result, increasing citrate concentration inhibits isocitrate dehydrogenase and reaching a critical level it stops its further metabolism (Agrawal et al. 1983; Meixner-Monori et al. 1985; Grewal and Kalra, 1995), causing a complete block of TCA cycle. An additional block of citrate cycle occurs at succinate dehydrogenase level by oxaloacetate.

Significant cytological, morphological and physiological changes (*e.g.* cell wall composition, cell compartmentalization, pellet formation, vacuolization and formation of storage compounds and polyols) take place in both microbial systems and clear variations are occurring in terms of electron transport and energy coupling (Kisser et al. 1980; Honecker et al. 1989; Papagianni et al. 1999; Paul et al. 1999; Pera and Callieri, 1999; Anastassiadis et al. 2002; Haq et al. 2002). Alternative respiration chains with a higher oxygen demand, functioning without yielding of ATP has also been reported to be involved in citrate accumulation (Kubicek et al. 1980; Zehentgruber et al. 1980; Röhr et al. 1983; Wallrath et al. 1991), leading to higher glucolysis rate and substrate phosphorylation (Wallrath et al. 1991). Byproducts (*e.g.* polyols) are produced in late fermentation phases that can be reconsumed forming citric acid.

The overall success of citric acid production depends to a large extent on the regulation of the TCA cycle. However,
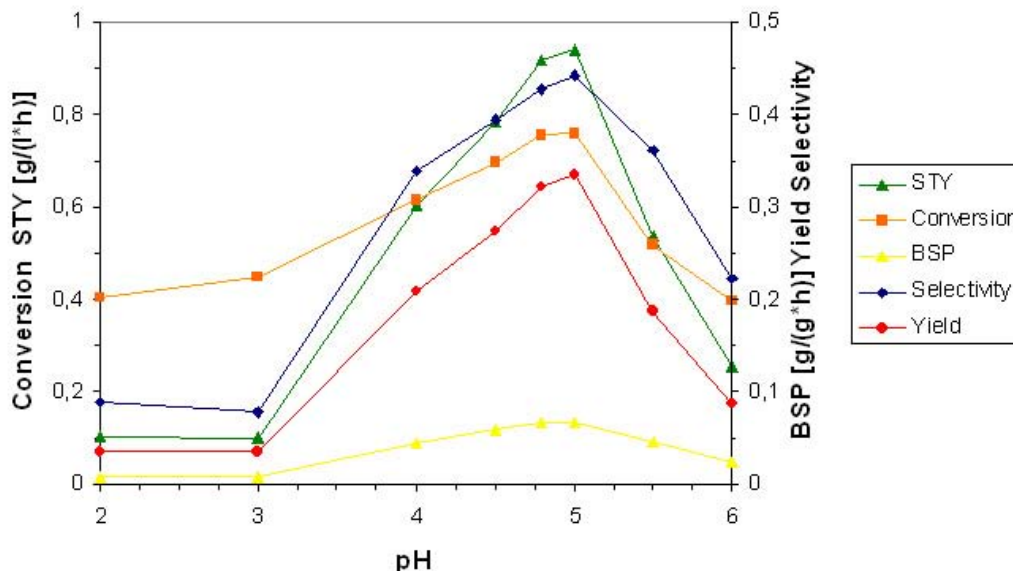
Figure 2. pH Influence on space-time yield, biomass specific productivity and continuous citric acid fermentation with iron supply at a residence time of about 40 hrs (3 g/l NH$_4$Cl, 120 g/l glucose and 5 µM iron).

the excretion mechanism in *A. niger* remained unclear (Grewal and Kalra, 1995).A little information is found in literature regarding the mechanism of citric acid secretion in yeasts and fungi. Whereas transportation of citrate from mitochondria into cytoplasm by citrate malate translocase is well known, excretion mechanism of citrate from cytoplasm into the medium still remained unknown. Marchal et al.(1980) found almost equal concentrations of citrate and isocitrate in the cells of *C. lipolytica* and in fermentation medium, suggesting a passive diffusion of citrate and isocitrate over cell membrane. McKayet al.(cited in Gutierrez and Maddox, 1993) assumed a passive diffusion of citrate and isocitrate over the cell membrane, in which the extracellular ratio between citrate and isocitrate corresponds to the intracellular ratio. Intracellular accumulation of citric acid and citrate secretion are obviously two different phenomena. A specific active transport system for citric acid secretion preferring citrate over isocitrate was found for the first time in *Candida oleophila*, based on simultaneous extra- and intracellular measurements of citrates and glucose acting as speed determining factor, well explaining overproduction of citric acid against a very high concentration gradient between the cell and fermentation medium (Anastassiadis et al. 1993; Anastassiadis, 1994; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2002). 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*, also claiming that only a passive diffusion through plasma membrane had been before reported for citrate excretion in yeasts.

The central aspect of present work was to investigate the influence of pH on continuous citric acid secretion by a specific active transport system, as well as on the elemental

biomass composition in free growing chemostat cultures of *Candida oleophila* ATCC 20177. A mechanism for citric acid secretion by a specific pH dependent active transport system is presented.

MATERIALS AND METHODS

Microbial system

Candida oleophila ATCC 20177 var. (obtained from Dr. Siebert, Jungbunzlauer Co. and later H and R, Bayer, Germany) was used through all experiments in this present work that was selected under many yeast strains during an extensive screening (Anastassiadis et al. 1993; Anastassiadis, 1994; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2002). 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 as well.

Chemostat experiments

The influence of pH on continuous citric acid fermentation and secretion was investigated in chemostat experiments carried out in 1 litter magnetically stirred double glass fermenter (Research Centre Jülich, RCJ, Germany) with a working volume of about 460 ml at 30ºC, 1300 rpm and a constant aeration rate of 0.145 vvm. Pure oxygen was used in order to satisfy the low oxygen transfer in small glass fermenter. At the beginning of experiments, the microorganism was simply transferred from a fresh agar plate into fermenter via a shake flask supplied with silicon tubing and quick connectors using a peristaltic pump.

Temperature was controlled using a circulation water bath (Lauda KG, Königshofen, Germany). pH was automatically controlled by the addition of 22.5% NaOH solution (w/v) using a pH controller (Ingold Meßtechnik GmbH, Steinbach) and a peristaltic pump. An automatic gravimetric dosing system (Sartorius, Göttingen, Germany) and a peristaltic pump (Watson-Marlow Ltd.) were used for constant medium feeding into fermenter. The residence time (hr) that corresponds to 1/dilution rate (1/D) was calculated under steady state conditions based on the working volume of fermenter via medium feed flux and NaOH flux. Residence time is the time required to replace once the working volume of fermenter. Achieving of steady state conditions was detected by monitoring the concentration of products, substrate and biomass and optical density (OD) on a daily basis. During entire fermentation and until achieving state conditions, samples were frequently taken out and analysed on a daily basis. Continuous fermentation was still carried out for a while under unchanged conditions, also after achieving steady state conditions. Fermentation times of several days or weeks were often required for achieving steady state conditions. During entire duration of fermentation, medium feeding rate, NaOH consumption and physical parameters (*e.g.* pH of fermentation broth was periodically measured externally and corrected) were measured and evaluated. Fermentation data presented here didn't result from just only one single measurement, however they were obtained based on long time measurements during continuous fermentation, meaning several days or weeks, until reaching steady state conditions. They represent an average of several measurements attained after achieving steady state conditions during a period of several days. Chemostat cultures carried out under controlled constant conditions are tending to reach steady state conditions, in which all fermentation parameters are remaining constant (unchanged) as time passes. In generally, between five and 10 generations (residence times, RT) are necessary for getting steady state conditions, depending on process and strain stability.

Feed medium (basic medium, BM)

A basic fermentation medium of following composition was used in a series of experiments for the preliminary orientation's investigation of pH influence on iron uptake and citrate formation (BM): 3 g/l NH_4Cl, 120 g/l glucose, 0.7 g/l KH_2PO_4, 0.35 g/l $MgSO_4$ x $7H_2O$, 0.11 g/l (0.5 mM) $MnSO_4$ x $4H_2O$, 5 µM $FeSO_4$ x $7H_2O$, 0.001 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, 0.1 mg/l potassium iodide (KJ), 2.5 g/l citric acid, 0.2 mg/l Na_2MoO_4 x $2H_2O$, 2 mg/l Thiamine-HCl, 0.25 mg/l Biotin, 0.625 mg/l Pyridoxine-HCl, 0.625 mg/l Ca-D-Pantothenate, 0.5 mg/l Nicotinic acid.

Non producing medium (BM with 6 g/l NH_4Cl and 120 g/l glucose)

The basic medium, however with 6 g/l NH_4Cl, 120 g/l glucose and 0.5 mM iron, was used for the investigation of pH influence on biomass formation under non producing conditions.

Production medium

In a second series of experiments, a production medium with 4.5 g/l NH_4Cl, analogously increased concentrations of residual compounds (at factor 1.5 higher than in medium with 3 g/l NH_4Cl), however with 1.125 mM manganese and without iron supply, and with 250 g/l glucose was used for the investigation of pH influence on citric acid secretion.
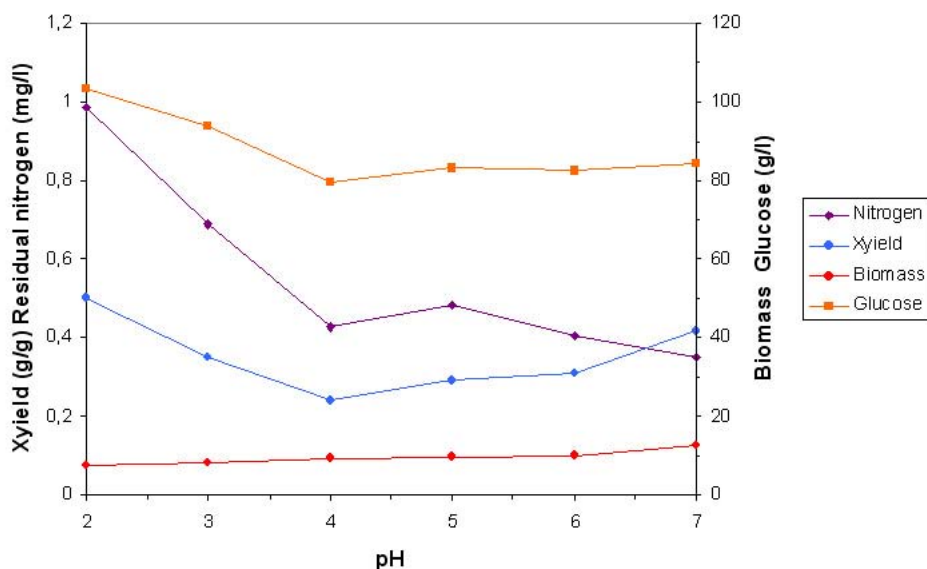

Figure 3. pH Influence on continuous biomass formation and biomass yield (X_{yield}) under non producing conditions (6 g/l NH_4Cl, 120 g/l glucose and 5 µM iron).

Medium composition

4.5 g/l NH_4Cl, 250 g/l glucose, 1.05 g/l KH_2PO_4, 0.525 g/l $MgSO_4$ x $7H_2O$, 0.2475 g/l (1.125 mM) $MnSO_4$ x $4H_2O$, 0 µM $FeSO_4$ x $7H_2O$, 0.0015 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, 0.15 mg/l potassium iodide (KJ), 2.5 g/l citric acid, 0.3 mg/l Na_2MoO_4 x $2H_2O$, 2 mg/l Thiamine-HCl, 0.375 mg/l Biotin, 0.9375 mg/l Pyridoxine-HCl, 0.9375 mg/l Ca-D-Pantothenate, 0.75 mg/l Nicotinic acid.

Silicon oil or polypropylene glycol was used as antifoaming agent. The 20 litter medium was sterilized in autoclave for 30-60 min at 121ºC. Vitamins and NH_4Cl were added separately into media following filtration through a 0.2 µm membrane filter (Sartorius, Göttingen, Germany). The glucose concentration and specific weight (g/ml) of each new medium and the specific weight of each new NaOH solution were determined and considered in all of following evaluations (*e.g.* determination of RT, real glucose concentration of feeding medium, specific parameters).

Optical density (OD)

Optical density (OD) was measured at 660 nm using a spectrophotometer (CPS-240, Shimadzu, Japan) and it was used for daily monitoring of achieving steady state conditions in fermenter.

Dry biomass

Dry biomass was measured using the filter method. 10 ml of fermentation broth were quickly filtered through a 5 cm diameter filter with 0.2 µm pores. The biomass was washed 3 to 4 times with 0.9% NaCl solution and distilled water and dried for 10 min in a microwave oven at a low power between 200 and 400 watt in order to prevent burning of biomass. After stabilizing of weight in a desiccator for 15 min, dry biomass was determined by the difference of weight before and after filtration and drying of filter.

Qualitative and quantitative analysis

Organic acids, glucose, ammonia nitrogen and intracellular concentrations were analysed as described in Anastassiadis, 1993; Anastassiadis et al. 1993; Anastassiadis, 1994; Anastassiadis et al. 2001 and Anastassiadis et al. 2002. A new HPLC application was developed for the analysis of carbonic acids that can separate the chemically related citric and isocitric acid (Anastassiadis, 1993; Anastassiadis et al. 1993). A solvent with 5 mM TBA (tetrabutyle ammonium hydrogen sulphate) and 2% acetonitrile was used as mobile phase at a flow rate of 1 ml/min. The column temperature was kept at 37ºC. An 5 C_{18} AB column (Macherey and Nagel, Düren, Germany) was used as stationary phase. A pressure of 0.4-0.5 bar was applied in the eluent bottle using helium gas. The substances were detected by a UV detector at 210 nm and the concentrations were evaluated by a computer program (AXXIOM). The same method was also used by Antonucci et al. (2001) for the separation and analysis of citric and isocitric acid (no reference to above method). For glucose analysis, an HPLC application was developed that also enables separation glucose from gluconic acid. A Nucleosil NH_2 column and a mobile phase with 75% acetonitrile were used at 40-45ºC and 1-1.5 mL/min flow rate (Anastassiadis et al. 1993; Anastassiadis, 1994; Anastassiadis et al. 1994; Anastassiadis et al. 2001; Anastassiadis et al. 2002). The eluent was able to use for longer time, because of the eluent's high toxicity (no microbial growth occurred in the eluent at 75% acetonitrile). Glucose was detected using an RI detector (S4110/S 1000, Sykam).
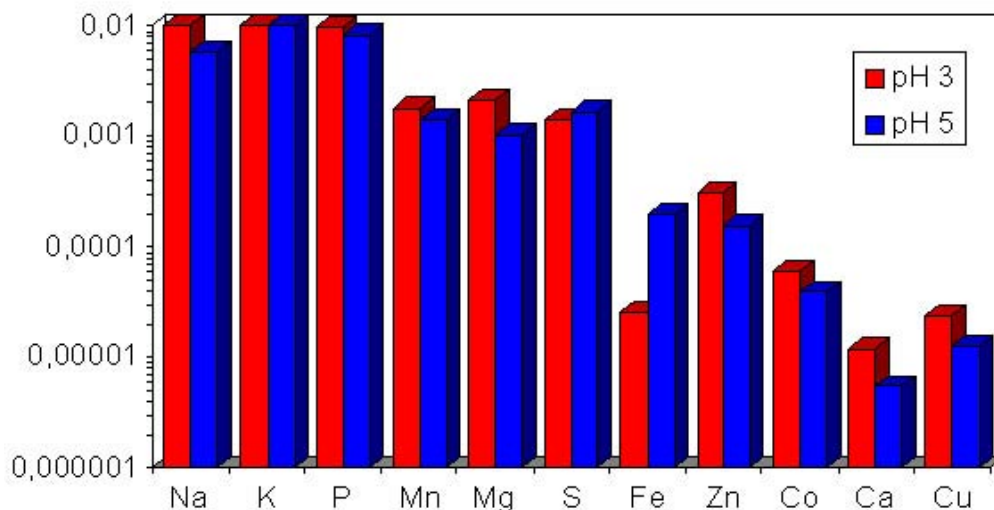

Figure 4. Logarithmic presentation of elemental composition of biomass of *Candida oleophila* at pH 3 and 5 (Chemostat, basic medium with 3 g/l NH_4Cl, 120 g/l glucose and 5 µM Fe).

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 after their extraction following next procedure.

1. 10 ml of fresh fermentation broth of known biomass concentration (tubes were hold in ice) were immediately filtered using a vacuum pump and washed 3-4 times using a cold 0.9% NaOH solution.

2. Intracellular acids and glucose were extracted by placing the filter with biomass in boiling ethanol for 5 min. Thereafter, filter and solids were dried using a vacuum rotator and resolved in 5 ml distilled water.

3. The sample was filtered using a filter with 0.2 μm pores and analysed. No destruction of metabolites was found in the controls submitted to the same treatment method.

4. Intracellular concentrations of various metabolites were determined and presented in g per gram dried biomass or in mM (under the consideration that cell volume corresponds to an average value of about 2.2 μl/mg dry weight that was determined) based on the report of Marchal et al. (1980) for cell volume of *Saccharomycopsis lipolytica*. The authors give an average value of 0.43 ml x (g wet weight)$^{-1}$. The above value of 2.2 μl/(mg dry weight) was calculated according to the ratio between wet and dried weight of about 5 determined for cells of *C. oleophila* (used in present work).

Similar values were determined for cell volume of *C. oleophila* as well, using a method involving a radioactive polymer, which can not diffuse into the internal volume of yeast cells (D. Brücher, RCJ). In generally, cell volume of microorganisms arranges between 2 and 4 μl/(mg dry weight) (Knowles, 1977). Höfer et al. 1985 determined a value of 2 μl/(mg dry weight) for the cell volume of *Rhodotorula gracilis (glutinis)*.

ATP and ADP analysis

ATP and ADP ratio was determined at the Institute of Biotechnology 1 of Research Centre Jülich (Jülich, Germany) using the luciferin/luciferase method for ATP measurement. After measurement of ATP, ADP was first transformed into ATP using PEP and pyruvate kinase and measured as ATP (Lundin et al. 1976, modified; Schimz et al. 1981).

Ammonium nitrogen analysis

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

RESULTS

The influence of pH on the growth and elemental biomass composition of *C. oleophila* and citric acid secretion was investigated in different series of chemostat experiments.

pH influence on continuous citric acid formation (3 g/l NH$_4$Cl and 120 g/l glucose)

The influence of pH on growth of *C. oleophila* and continuous citric acid formation was investigated in a series of preliminary orientation's experiments, carried out in chemostat cultures at a residence time of about 40 hrs (D = 0.025 h^{-1}) using the basic fermentation medium (BM) with 3 g/l NH$_4$Cl and 120 g/l glucose. Figure 1 illustrates the course of biomass, glucose, citrate and isocitrate concentration as well as the ratio between citrate and isocitrate as a function of pH. Citric acid was continuously produced at a pH range between 2 and 6. Only 4.22 g/l of citric acid were measured at pH 2 and 4.02 g/l at pH 3. Increasing the pH from 3 to 4, citric acid concentration increased continuously by a factor of about 6 reaching a steady state concentration of 24.1 g/l after several days. Isocitric acid reached in generally a stationary steady state concentration within shorter times compared with citrate. Highest citrate concentration of 37.6 g/l was achieved at pH 5, compared with 24.1 g/l and only 10.2 g/l reached at pH 4 and pH 6, respectively. Isocitric acid reached concentrations between 0.5 and 3.36 g/l. A ratio between citrate and isocitrate of around 12 was determined at most of pH values. Residual glucose concentrations between 27 g/l (pH 5) and 70.2 g/l (pH 6) or 69.7 g/l (pH 2) were measured under steady state conditions, corresponding to conversions (conversion means the consumed glucose/feeding medium glucose, scale 0-1, which corresponds to 0-100%; 100% conversion corresponds to total conversion of glucose) between 40% and about 76% (Figure 2). The decreasing of pH, resulted to continuous increase of biomass concentration, starting with about 11 g/l biomass at pH 6 and reaching 15.3 g/l at pH 2 (factor 1.4) (Figure 1). A correlation factor between 0.27 (pH 5.5) and 0.34 (pH 2) has been determined between biomass and OD (Biomass/OD).

As Figure 2 shows, product concentration, space-time yield (STY, volumetric productivity that means g product/[litter x hr]), biomass specific productivity (BSP, it means g product/[g dry biomass x hr]) and additional specific parameters were very poor at lower pH values (*e.g.* pH 2 and 3) in opposite of higher biomass concentrations. Highest STY of 0.94 g/(l x hr) was achieved at pH 5 and BSP of 0.067 g/(g x hr) at pH 4.8 compared with 0.11 g/(l x hr) and 0.0069 g/(g x hr) found at pH 3. Highest selectivity (selectivity means g of product/g of converted glucose; scale 0-1 or in %) of 44.3%, yield (yield means g of product/g of feeding glucose; scale 0-1 or in %) of 33.6% and conversion of 75.9% were determined at pH 5 as well. About 7.7% selectivity, 3.5% yield and 45% conversion were determined at pH 3 instead (Figure 2). Similar results were observed in further experiments with 3 g/l NH$_4$Cl, 120 g/l glucose and without any iron supply (data not shown
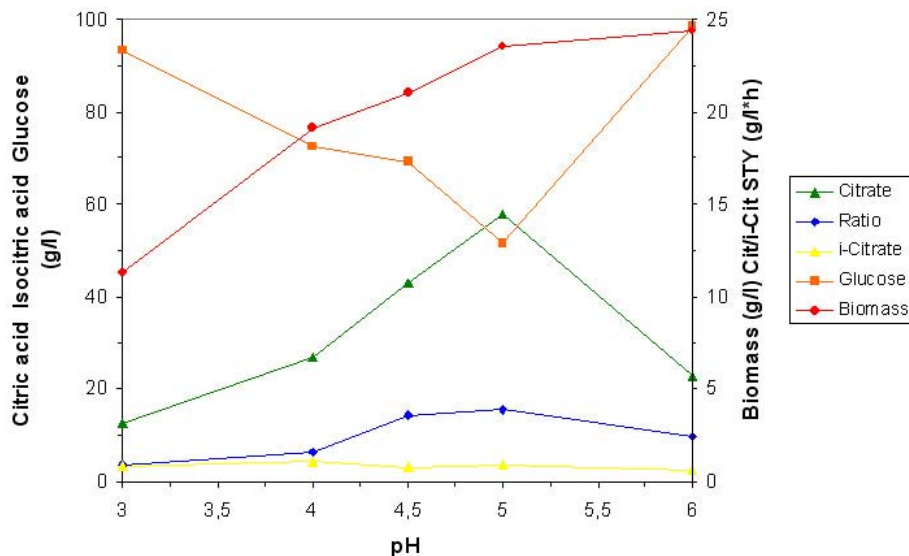
Figure 5. Influence of pH on continuous citric acid fermentation under optimised conditions (4.5 g/l NH₄Cl, 250 g/l glucose and a residence time of 60 hrs).

here), indicating that traces of iron are present in other media components.

pH influence on continuous biomass formation under non producing conditions (6 g/l NH₄Cl and 120 g/l glucose)

A series of chemostat experiments was carried out at pH between 2 and 7 for the investigation of pH influence on continuous growth of *C. oleophila*, independent from citric acid production. The basic medium as has been described in the material and methods was used, which contained an excessive NH₄Cl concentration of 6 g/l and 0.5 mM iron. Residual nitrogen was measured in all of the experiments, which decreased from 0.99 g/l (pH 2) to 0.35 g/l (pH 7). No citric acid was formed by *C. oleophila* in the presence of nitrogen in agreement of previous reports (Anastassiadis et al. 2002; Anastassiadis et al. 2004). The residual glucose reached values between 79.7 g/l (pH 4) and 103.3 g/l (pH 2), corresponding to glucose conversions (g of consumed glucose/g glucose of feeding medium) between 32.6% and 12.7%, respectively. Biomass concentration dropped continuously at raising pH between 2 and 6, from 7.4 g/l to 12.3 g/l (factor 1.67). Biomass yields (g biomass/g of consumed glucose) between 0.24 (pH 4) and 0.5 (pH 2) were determined dependent on pH in fermenter (Figure 3).

Elemental biomass composition of *C. oleophila* at pH 3 and 5 (basic medium with 3 g/l NH₄Cl and 120 g/l glucose)

The elemental biomass composition in biomass of *C. oleophila*, obtained from chemostat cultures grown at pH 3 and pH 5, was analysed by the Analytical Chemistry Department of RCJ (Jülich, Germany). Figure 4 shows the elemental composition at pH 3 and 5, which is presented as

the logarithm of element concentration. No significant differences were found in the content of macroelements (P, S) between pH 3 and pH 5. However, a higher content of the trace elements zinc, cobalt, calcium and copper (excess factors, needed at higher concentrations than the biomass contains for optimum production) was found in biomass of *C. oleophila* at pH 3. Slightly higher are the concentrations of sodium, magnesium and manganese at pH 3. The iron content of 200 ppm found in biomass of *C. oleophila* at pH 5 was at about factor 8 higher than at pH 3 (26 ppm), indicating a facilitating iron uptake in *Candida oleophila* at higher pH.

pH influence on continuous citric acid secretion by the specific active transport system (4.5 g/l NH₄Cl and 250 g/l glucose)

In a new series of chemostat experiments, the pH influence on intracellular concentrations of citrates and glucose and on citric acid secretion was investigated at a residence time of about 60 hrs (corresponds to a dilution rate of about D=~0.017 h⁻¹), using an optimised production medium with 4.5 g/l NH₄Cl, 250 g/l glucose, 1.125 mM of manganese and without iron supply (traces of iron are present in other chemical compounds). The addition of iron has been found to enhance biomass formation and to affect continuous citric acid production significantly. As Figure 5 shows, a maximum citric acid concentration of 57.8 g/l was achieved at pH 5, compared with 43.1 and only 22.8 g/l reached at pH 4.5 (factor 1.34 or 74.6%) and pH 6 (factor 2.54 or 39.5%), respectively. Only 12.4 g/l were obtained at pH 3 (factor 4.7 or 21.5%). The highest ratio between citrate and isocitrate of 15.6 was also found at pH 5, compared with only 3.7 or 9.9 identified at pH 3 and 6, respectively. Highest isocitric acid concentration of 4.2 g/l was measured at pH 4 instead. Biomass concentration increased steadily

with raising pH and achieved 23.54 g/l at pH 5 and 24.42 g/l at pH 6, corresponding to a factor of 2.16 between pH 6 and pH 3 (11.3 g/l).

Figure 6 shows space-time yield (STY), biomass-specific productivity (BSP), the ratio between ATP and ADP and the specific parameters product selectivity, conversion and product yield as a function of pH. Highest STY of 0.96 g/(l x hr) was obtained at optimum pH 5, compared with only 0.21 g/(l x hr) or 0.38 g/(l x hr) reached at pH 3 (21.5%) and pH 6 (39.5%). Highest BSP of 0.041 g/(g x hr) was also found at pH 5, in comparison to only 0.018 g/(g x hr), 0.0156 g/(g x hr) or 0.034 g/(g x hr), achieved at pH 3 (44.7%), pH 6 (38%) and pH 4.5 (83.3%), respectively. Highest product selectivity of 35%, conversion of 76%, yield (yield = selectivity x conversion) of 26.6% and ratio between ATP and ADP of 2.65 were determined at optimum pH 5 as well. In comparison, a selectivity of only 10%, conversion of 57%, yield of 5.7% and an ATP/ADP ratio of 1.31 were determined at pH 3 (Figure 6). The ratio between ATP and ADP correlated very strongly with the extracellular citrate concentration and the ratio between citrate and isocitrate, displaying a maximum at optimum pH 5, whereas isocitric acid showed a flat course, indicating a very strong connection between overproduction of citrate and energy state of yeast cells. Optimisation attempts running in chemostat experiments are favourably carried out at conversions lower than 80%, because the complete conversion wouldn't enable the identification of complex and interacting effects occurring during an optimisation approach.

The existence of an active transport system for citric acid secretion in *C. oleophila* became obvious, based on citric acid transport over the cell membrane against a very high concentration gradient between intra- and extracellular citrate concentration. The active transport system was influenced by various parameters such as air saturation, temperature, medium composition and growth state of chemostat cells (residence time, growth or dilution rate). It showed a very high specificity for citrate over isocitrate (specificity factor of 33). Furthermore, the highest intracellular concentrations of citrate, isocitrate and glucose and simultaneously the lowest extracellular citric acid concentrations were determined under none or low producing conditions. In contrary, maximum extracellular citric acid concentrations were reached under conditions, where the lowest intracellular concentrations of citrate and isocitrate appeared. Intracellular isocitric acid concentration exceeded citric acid concentration significantly. Under producing conditions, isocitrate was drawn out from aconitase equilibrium towards citrate, resulting to a higher glycolysis rate and to lowering of intracellular concentration of glucose and isocitric acid (Anastassiadis et al. 1993; Anastassiadis, 1994; Anastassiadis et al. 1994; Anastassiadis et al. 2001). Based on intracellular measurements of glucose, citrate, isocitrate as well as of ATP and ADP (ATP/ADP ratio) has been investigated,

whether there would be a pH dependency for citric acid secretion by active transport system.

As Figure 7 shows, the highest intracellular glucose concentration of 372 mg/g dried biomass, citrate concentration of 57.6 mg/g (~136,3 mM, under the consideration that the cell volume of *C. oleophila* corresponds to 2.2 µl/mg dry weight, calculated from the cell volume for wet weight; Marchal et al. 1980, under the condition that 1 g dried biomass of *C. oleophila* corresponds to about 5 g wet weight) and isocitrate concentration of 156.4 mg/g (370.1 mM) were measured at non producing pH 3. However, the lowest intracellular citrate concentration of only 18.6 mg/g dried biomass (~44.1 mM) and total acid concentration (citrate + isocitrate) of 51.3 mg/g dried biomass (~121.4 mM) were found at optimum pH 5. The lowest intracellular isocitrate concentration of 30.2 mg/g dried biomass (32.7 mg/g biomass at pH 5) and glucose concentration of 43 mg/g biomass (52 mg/g biomass at pH 5) were determined at pH 4.5 instead (Figure 7). Present results are a very strong confirmation, that not the intracellular citrate accumulation alone, however the active secretion of citrate over plasma membrane is the speed determining factor for citric acid excretion, displaying an optimum at pH 5. Highest extracellular citrate concentration was found under conditions, where the lowest total acid and glucose concentration occurred, as a result of intensive citrate secretion. A higher glucose uptake and glycolysis rate is proposed at optimum pH 5.

DISCUSSION

Many reports exist related to the influence of initial or operating pH and other fermentation parameters on citric acid fermentation. Basically, most investigations were carried out in batch experiments running in stirrer fermenter or shake flasks (Nubel et al. 1971; Briffaud and Engasser, 1979; Kozlova et al. 1981; Lozinov and Finogenowa, 1982; Enzminger and Asenjo, 1986; Behrens et al. 1987; Rane and Sims, 1993; Antonucci et al. 2001; Crolla and Kennedy, 2001). However, no information was found about the influence of pH on the continuous citric acid secretion and a little is known regarding the real pH effect on citrate formation. Kinetic data obtained in chemostat cultures give essential information for sophisticated process design, process development and scale up. This type of information for continuous citric acid fermentation is rather rare in literature. For instance, looking in the internet about 15,200 results was found as compared with 89 for chemostat (Anastassiadis et al. 2004). Tisnadjaja et al. (1996) reported about a higher productivity of citric acid using *C. guilliermondii* in continuous culture compared with the batch process. About a four stage process for continuous production of citric acid using *A. niger* has been reported by Wieczorek and Brauer (1998).

The active citric acid export found for the first time in yeasts has been shown in present work to be a strongly pH
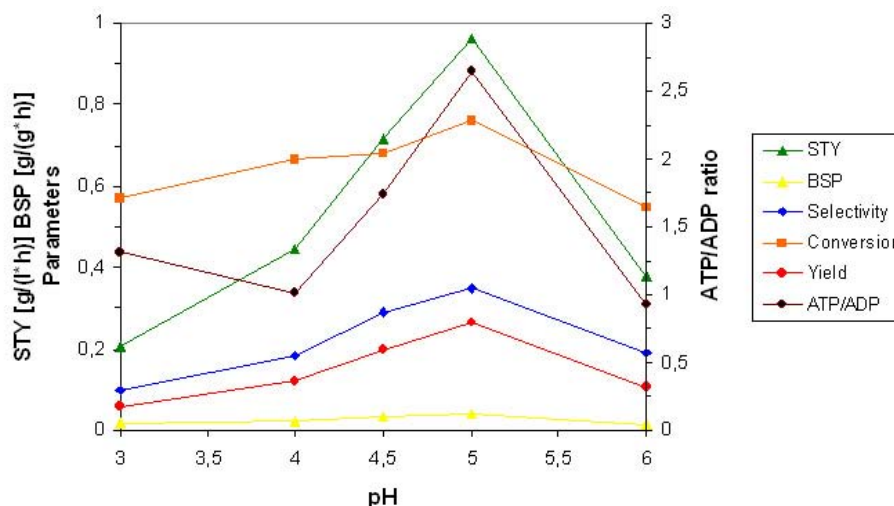
Figure 6. Influence of pH on STY, BSP, ATP/ADP ratio and the specific parameters selectivity, conversion and yield (4.5 g/l NH₄Cl, 250 g/l glucose and a residence time of 60 hrs).

dependent process. The pH had a remarkable effect on the growth, the elemental biomass composition and the secretion of citric acid in *C. oleophila*, displaying a production optimum and higher biomass iron content at pH 5. Already very low iron (cofactor of aconitase) concentrations (<20 μM) affect dramatically citric acid formation and ratio between citrate and isocitrate. On the other side, Crolla and Kennedy (2001) reached highest citrate production in *C. lipolytica* using 10 mg/l ferric nitrate. Iron is essential for yeast growth, however a little is known about the mechanism of its assimilation. Shavloskii et al. (1988) identified an active iron uptake system in *Pichia quilliermondii* with an optimum at pH 5.3 and 37ºC, whereas small pH alterations caused a dramatic lost of its uptake activity (90%). Kamzolova et al. (2003) reported about the dependency of oxygen requirements for the growth of *Y. lipolytica* and of citric acid synthesis on iron concentration. Oexle et al. (1999) reported about the modulation of the expression of the critical citric acid cycle enzyme aconitase via a translational mechanism involving iron regulatory proteins in human cell lines and the iron effect on other citric acid cycle enzymes.

Most of published works regarding citric acid production by yeasts were performed at pH between 4.5 and 5.5 (Briffaud and Engasser, 1979: pH 4.7; Enzminger and Asenjo, 1986: pH 5.0; Klasson et al. 1989: pH 5.5; Grewal and Kalra, 1995: pH >5.5; Anastassiadis et al. 2004). Nubel et al. (1971) reported on the other side about citric acid production in *Candida* (*Yarrowia*) *lipolytica* ATCC 20228, occurring even at lower pH. The yeast process differs from *Aspergillus niger* in terms of optimum pH operation, where pH lower than 2 is necessary for optimum citric acid production (Rehm, 1980; Grewal and Kalra, 1995; Netik et al. 1997). An optimum pH of 1.7 has been reported for continuous citric acid fermentation by *A. niger* (Kristiansen and Charley, 1981). On the other side, Roukas and Harvey (1988) claimed an optimum pH of 4 for continuous citric

acid production in *A. niger*. A sharp maximum around pH 3.4 has been found for continuous citric acid fermentation by *A. foetidus* (Kristiansen and Sinclair, 1979). Alternatively, *Penicillium* strains are producing citric acid at pH between 4 and 7 (Schinner and Burgstaller, 1989; Franz et al. 1991). Some variations in intracellular compartmentalization, pH topographies and differentiations in pH gradient (inside/outside) or regulation mechanisms may provide the explanation for those differences between various microbial systems. Roos and Slavik (1987) found two separate layers in cytoplasm of *P. cyclopium* (citrate producer), a thin outer layer with a pH of 5 and a greater core layer with approximate neutral pH.

The lower citric acid production occurring at lower pH in yeasts could be theoretically attributed to reduced intracellular citrate formation or/and inhibition of citrate transport over the mitochondrial or cytoplasm membrane. Furthermore, citric acid secretion in yeasts has been reported to occur only as a result of passive diffusion of the undissociated acid through plasma membrane (Marchal et al. 1980; Netik et al. 1997). However, present results showed clearly that citrate secretion by the specific pH dependent active transport systemand not intracellular citrate accumulation alone is the crucial speed determining factor for overproduction in yeasts (Figure 8). The highest extra-/intracellular ratio of 6.8 for citrate and of 0.24 for isocitrate that have been determined at optimum pH 5 revealed the high specificity of transport system for citrate over isocitrate. The difference between extra- and intracellular isocitrate concentration is lower than 1, indicating that it doesn't seem to be an affine substrate for the active transport system and a diffusive excretion of isocitrate into medium may also take place, forced by gradient imbalances. Netik et al. (1997) reported about an active secretion of citric acid in *A. niger*, explained by small gradient differences of slightly above 2 (42% in cells of *A. niger*) found between extra- and intracellular
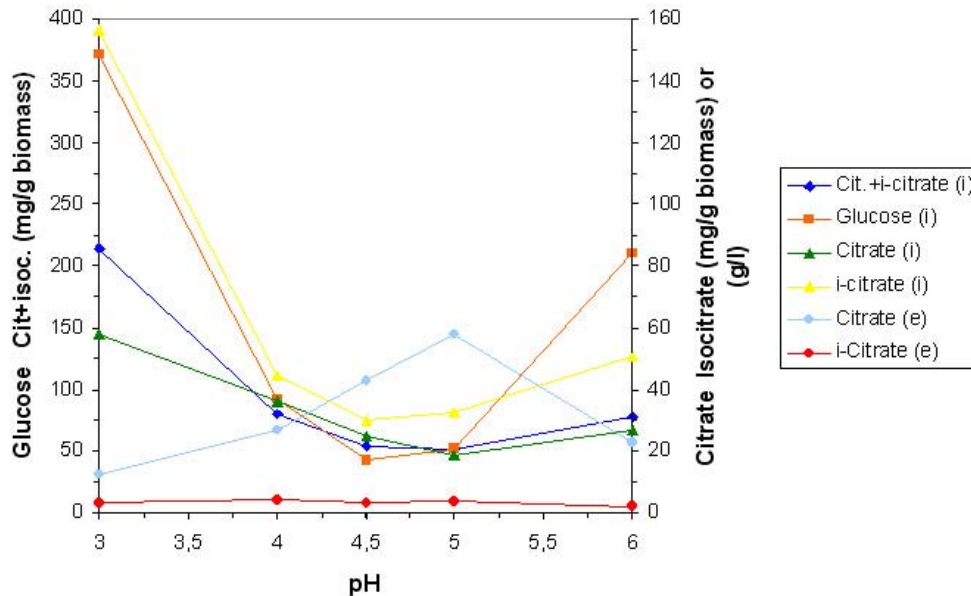
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Figure 7. Intracellular concentrations (g/g) of citrate, isocitrate, total acid and glucose, compared with the extracellular concentration (g/l) of citric and isocitric acid, as a function of pH (4.5 g/l NH₄Cl, 250 g/l glucose and a residence time of 60 hrs).

concentrations of citric acid. A very high difference between extra- and intracellular citric acid concentration of about 60 has been calculated at citric acid concentration of 250 g/l that has been achieved using *C. oleophila* and *Y. lipolytica*.

Intracellular accumulation and secretion of citrates are obviously two totally different phenomena, influencing each other, every time in a different way based on varying environmental conditions. Highest extracellular citrate concentrations were identified under optimised conditions (*e.g.* optimum air saturation, optimum temperature, optimum pH, optimum concentrations of important trace elements), where under also the lowest intracellular concentrations of citrates were identified (Anastassiadis et al. 1993; Anastassiadis, 1994). Thus, the active transport system seems to be induced by other factors, either than by the intracellular accumulation of citric acid. However, a certain critical intracellular level of citrate (~20 mM), determined at optimum air saturation of 20%, was found to be necessary for functioning of active transport system (Anastassiadis et al. 1993; Anastassiadis, 1994; Anastassiadis et al. 1994; Anastassiadis et al. 2001). Higher intracellular concentrations of citrates were also found in chemostat cultures of *C. oleophila* under non producing conditions at lower residence times, compared with higher producing residence times, confirming once again that citrate secretion by the specific energy consuming active transport system is the main speed determining event in citric acid overproduction (Anastassiadis et al. 1993; Anastassiadis, 1994), rather than intracellular citrate accumulation alone, as has been broadly thought before in international scientific community. Such transport systems are generally carrier proteins (Krämer and Sprenger, 1993).

Isocitric acid is taken out from aconitase equilibrium towards citric acid, followed up by citrate's secretion, resulting under optimum fermentation conditions to lowest intracellular concentrations of citric and isocitric acid. The lower biomass iron content found at pH 3 would mean a lower aconitase activity. However, lower aconitase activity caused by iron deficiency wouldn't explain the 3-fold intracellular isocitrate concentration compared with citrate at pH 3. In order to understand citric acid production as an event of ''over flow metabolism'', it is essential to consider citric acid overproduction as a whole complex biological system, starting at the initial growth and ending with the active product secretion. Passive diffusion of citric acids at lower pH-values seems hardly to be possible (citrate $pK_1 =$ 3.13, $pK_2 = 4.76$ and $pK_3 = 6.40$; isocitrate $pK_1 = 3.29$, $pK_2 = 4.71$ and $pK_3 = 6.40$). Because the pK_s values of citrate and isocitrate are almost identical, only the involvement of a transport system for citrate could explain the specificity of citrate over isocitrate. Cassio and Leao (1991) identified a low- (facilitated diffusion) and a high-affinity transport system (presumably a proton-citrate symport) for citrate uptake in yeast *Candida utilis*. Isocitrate competitively inhibited citrate uptake by the high-affinity system, suggesting that both of tricarboxylic acids used the same system (Cassio and Leao, 1991). An inducible uptake system for lactate with a pH optimum around 5 has been identified in *Candida utilis* by Leao and Van Uden (1986) as well.

The mechanism of citrate accumulation in yeasts has been studied for several years (Marchal et al. 1980; Gutierrez and Maddox, 1993; Anastassiadis, 1994; Netik et al. 1997; Anastassiadis et al. 2002). While transport of mitochondrial citrate and isocitrate into cytoplasm is well known, a little

was known about excretion of citrate into culture medium. Vacuolisation of cytoplasm has also been discussed in connection with citrate production in *C. lipolytica* (Kozlova et al. 1981; Behrens et al. 1987). However, in case that citrate excretion would be mediated by vacuoles, specificity between excretion of citrate and isocitrate would be attributed to aconitase equilibrium. An argument against this hypothesis is the lower intracellular iron concentration that indicates lower aconitase activity and in the mean time the higher intracellular isocitrate concentration found in present work at pH 3.

Marchal et al. (1980) and McKay et al. (cited in: Gutierrez and Maddox, 1993) proposed a passive diffusion of citrate and isocitrate over yeast plasma membrane into fermentation medium, in a ratio reflecting their intracellular equilibrium, and the existence of a selective transport of mitochondrial isocitrate to cytoplasm, based on intra- and extracellular concentrations of citrate found in experiments using *C. lipolytica* and paraffins as sole C-source. The higher intracellular isocitrate concentrations indicate a high aconitase activity and in accordance to Marchal et al. (1980) a selective accumulation of isocitrate in cytoplasm. Citric acid production by yeasts seems to be a paradox, because cell accumulation of citric acid occurs under high

ratio between ATP and ADP (Anastassiadis, 1994), although the process is considered to be non-growth related, triggered out by nitrogen limitation. The strong correlation found between ATP/ADP ratio and citric acid accumulation varying the pH can be considered as a consequence of a high glycolytic flow under nitrogen limitation and intracellular NH_4^+ accumulation. A relatively high intracellular NH_4^+ concentration of about 1.2 mg/g biomass (~37.4 mM) was found in *C. oleophila* during the production phase (Anastassiadis, 1994; Anastassiadis et al. 2002). The pH dependent specific active transport is providing the explanation for citrate overproduction in yeasts. Active transport seems to be a way for regenerating reduction equivalents and converting excessive ATP, gained by intensive glycolysis under growth limiting conditions.

Yeasts are divided in two categories, namely the lipogenous and non-lipogenous citric acid accumulating strains, which can also include strains of the same genera. Under nitrogen limitation, yeasts belonging to the first category predominantly form fatty acids from intracellular citric acid by ATP:citrate lyase (located in cytosol), whereas yeasts belonging to the latter category produce citric acid (Evans and Ratledge, 1985). Thus, fatty acid synthesis and citric
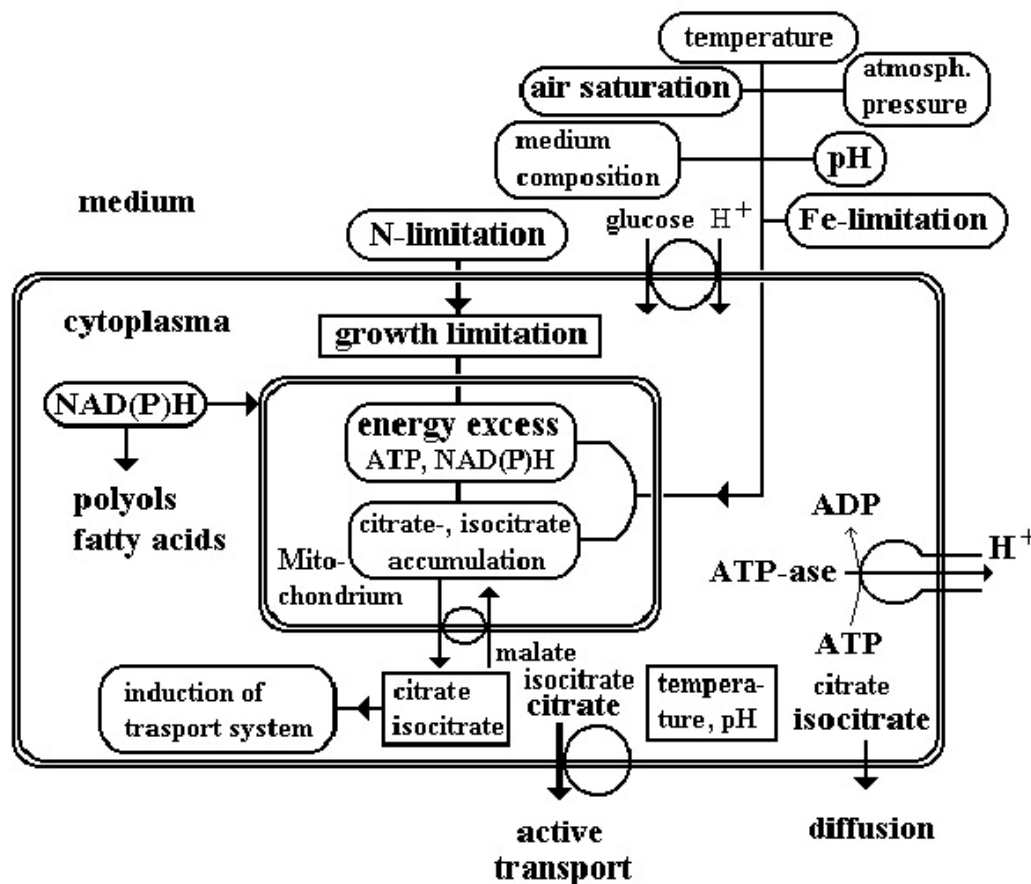

Figure 8. Proposed flow scheme of citric acid overproduction in yeasts based on experimental results obtained in chemostat cultures of *C. oleophila* and literature reports.

acid secretion by active transport system can be considered as a means to cut down energy overload and surplus amount of NAD(P)H$_2$. It seems also useful to consider polyol formation under the aspect of regulation, because polyol formation is discussed in relation to regeneration of reduced pyridine nucleotides in yeasts (Lozinov and Finogenova, 1982) as well as in *Aspergillus niger* (Röhr et al. 1987; Honecker et al. 1989; Wallrath et al. 1991).

The overproduction of precursors for di- and polysaccharide formation (*e.g.* trehalose, glycogen and pullulan) under various nutrient limitations by certain yeasts and moulds may be regulated by feedback control of an elevated cytosolic pool of citrate (Evans and Ratledge, 1985; Anastassiadis, 1994). The existence of active transport system for citrate secretion and the strong correlation between ATP/ADP ratio and citrate overproduction, found in *C. oleophila*, goes well together with reports of Lozinov and Finogenova (1982) about a non phosphorylating alternative oxidase, identified in yeasts, that completes electron flow without ATP regeneration, competing citrate production. Active citric acid producing strains showed lower alternative oxidase activity instead. Whether the energy charge is the driving force of citrate excretion in *A. niger* is still unclear. In contrast to yeasts an active non-phosphorylating alternative oxidase is discussed in relation to 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 relation to citrate overproduction with not growing cells of *A. niger*. A significant amount of energy is required since the acid is excreted against a concentration gradient. Kristiansen and Sinclair (1979) proposed an other option explaining citrate production in *A. niger* under consideration of cytoplasm streaming.

CONCLUDING REMARKS

Citric acid production is obviously a very complicated process, whereby numerous events such as 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 however somehow interconnected and interrelated in a synergistic mode. Essentially higher intracellular isocitrate concentrations found in producing cells of *C. oleophila* in comparison to citrate indicate a high aconitase activity. However, isocitrate doesn't seem to be a high-affine substrate for active transport system. Thus, the specific active transport of citrate is resulting to decreasing intracellular whole acid and cytoplasmatic isocitrate concentration under optimum fermentation conditions. This phenomenon is a further evidence for the existence of a specific active transport system for citrate secretion in yeasts, well explaining the overproduction of citric acid against a very strong concentration gradient. Figure 8 resumes most crucial evens influencing citric acid overproduction in yeasts.

ACKNOWLEDGMENTS

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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) and Greece (Research in Biotechnology Co., Avgi/Sohos, 57002 Thessaloniki), where the experiments were performed.

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