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RESEARCH ARTICLE

Valorization of raw glycerol for citric acid production by *Yarrowia lipolytica* yeast

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Keywords: citric acid, fed-batch culture, *Yarrowia lipolytica*, raw glycerol.

Abbreviations: CA: citric acid
ER: erythritol
Gly: glycerol
HPLC: high pressure liquid chromatography
ICA: isocitric acid
MAN: mannitol
q_{CA}: specific rate of citric acid production, g g^{-1} h^{-1}
Q_{CA}: volumetric citric acid productivity, g L^{-1} h^{-1}
X: biomass
Y_{CA}: yield of citric acid, g acid g^{-1} glucose

In the present report, citric acid production from raw glycerol in two fed-batch systems by acetate negative-mutants of *Yarrowia lipolytica*: Wratislavia 1.31 and Wratislavia AWG7 was compared. In the system, in which the total glycerol concentration was 200 g·L^{-1}, the substrate was added by pulsed additions, and in the other, in which the total glycerol concentration was 300 g·L^{-1}, the substrate was added at a constant feeding rate of 1.4 g·h^{-1}. Despite high citric acid concentrations (155.2 and 157.5 g·L^{-1} with *Y. lipolytica* Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG7, respectively) obtained from 300 g·L^{-1} of glycerol, the yield of citric acid was similar, *i.e.* about 0.6 g·g^{-1}. The volumetric citric acid productivity was markedly higher (1.05 and 0.94 g·$L^{-1}h^{-1}$ with *Y. lipolytica* Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG7 strains, respectively) in the cultures containing 200 g·L^{-1} of carbon source.

Rapid development of biofuel industries, including biodiesel production, offers one of very few options for mobility needs and can provide geopolitical, environmental and economic benefits. However, generation of huge amounts of effluents, especially glycerin production is a major environmental problem. Before the launch of large-scale biodiesel processing, global glycerol production was well-balanced with its consumption by the food, pharmaceutical, cosmetics and many other industries.

Besides the synthetic glycerol derived from petroleum, glycerol from the oleochemical industries for the production of soaps, fatty acids, waxes and surfactants once dominated the market (Koutinas et al. 2007). Transesterification of plant oils to biodiesel - one of the principal biofuels currently produced in large-scale operations - is coupled with significant production of glycerol-rich water (so-called "crude" or "raw" glycerol), as an important by-product of the process. The increasing demand for biodiesel leads to abundant quantities of this glycerol-rich material on the market. Therefore, glycerol valorization has much to offer in the cost reduction of the overall biodiesel production process. To this end, various

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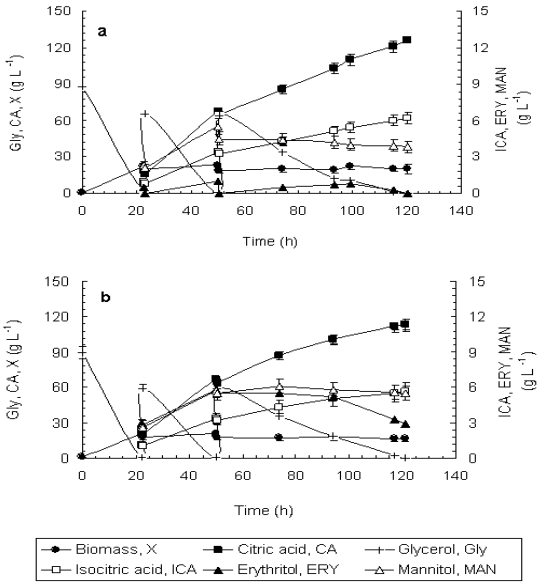

Figure 1. Time course of biomass, X (●), glycerol, Gly (+), citric acid, CA (■), isocitric acid (□), erythritol, ER (▲), and mannitol, MAN (∆), during fed-batch fermentation by *Y. lipolytica* Wratislavia 1.31 (a) and *Y. lipolytica* Wratislavia AWG7 (b) strain growing on crude glycerol (BDK Biodiesel GmBH, Kyritz, Germany). Cultivation conditions: culture broth medium was fed with two identical portions of glycerol after 24 and 48 hrs of cultivation in order to obtain a final glycerol concentration of 200 g·L^{-1}. Data are the average of duplicate cultures. Error bars indicate the standard deviations of mean data values.

chemical or biotechnological strategies have been developed to obtain added-value products using crude glycerol as substrate (Wen at al. 2009). Production of 10 kg of biodiesel from esterified rapeseed oil corresponds to the production of 1 kg of glycerol (Meesters et al. 1996). There is an urgent need to revolutionize the processing strategies to synchronize biodiesel production with the consumption of crude glycerol. Three general approaches, aqueous-phase reforming, chemical conversion and bioconversion, form the core focus of current research into the innovative utilization of glycerol. Bioconversion provides broader opportunities for the production of a list of platform and fine chemicals that could be used either as end-products or as precursors for the production of other chemicals, under much milder conditions (Koutinas et al. 2007). At present, microbial fermentations of glycerol are used for the production of succinic acid with higher yield than that obtained from common sugars, such as glucose (Lee et al. 2001). Glycerol has been used for the production of polyhydroalkanoates, 3-hydroxypropionaldehyde, hydrogen, butanol, propionic acid, 1,3-propanediol and single cell oil - SCO (Biebl, 2001; Doleyres et al. 2005; Koller et al. 2005; Cheng et al. 2007; Papanikolaou and Aggelis, 2009; Sabourin-Provost and Hallenbeck, 2009; Wen et al. 2009; Zhang and Yang, 2009). According to Takeshita et al. (2005) glycerol could be used as a carbon source and energy source for the growth of recombinant microorganisms that could convert methane into methanol. Although it is known that the yeast of Yarrowia lipolytica are able to utilize glycerol, there are little data in literature on the synthesis of citric acid (CA) from this substrate (Papanikolaou et al. 2002; Papanikolaou and Aggelis, 2003; Imandi et al. 2007; Levinson et al. 2007; Papanikolaou et al. 2008; Rywińska and Rymowicz 2009). Additionally, some strains of the yeast *Y. lipolytica* when growing on crude glycerol under nitrogen-limited conditions can synthesize SCO together with CA (or instead of CA). Since the biochemical pathways of synthesis of both compounds are identical in their first steps and occur due to intra-cellular exhaustion. The only difference in these processes is identified in the level of the existence (or the activity) of the enzyme ATP-citrate lyase, that catalyzes the cleavage of intra-cellular citric acid into oxaloacetate and acetyl-CoA, the latter being used as base-unit for the synthesis of intra-cellular fatty acids by inversed β-oxidation (Papanikolaou and Aggelis, 2009).

The results obtained in our earlier studies showed that glycerol was a good substrate for citric acid biosynthesis by acetate mutants of *Y. lipolytica* (Rymowicz et al. 2006; Rywińska et al. 2009). Batch-biosynthesis of citric acid has been studied extensively by many authors (Arzumanov et al. 2000; Kamzolova et al. 2003; Papanikolaou et al. 2006; Moeller et al. 2007). In batch cultures, carbon source concentrations usually range from 10 to 15%, since higher concentrations (200-300 g·L^{-1}) of the substrate are likely to prolong the lag-phase, which has been observed in the processes of citric acid biosynthesis from glucose by the strain of *Candida oleophila* ATCC 20177 (Anastassiadis and Rhem, 2006).

Modifications of batch culture (fed-batch and repeated fed-batch) or continuous cultures can enhance the parameters of biosynthesis, such as yield and productivity (Kim et al. 2007). Continuous fermentations usually offer higher productivities of metabolites only at low dilution rates (long residence times). For practical reasons, therefore, some continuous operations have been replaced by fed-batch processes.

Generally, fed-batch cultures were used for citric acid production from n-paraffins and vegetable oils as carbon sources (Aurich et al. 2003; Crolla and Kennedy, 2004; Kamzolova et al. 2005; Kamzolova et al. 2008). The results obtained by these authors in earlier studies showed that the fed-batch systems were also very effective for citric acid biosynthesis from glycerol (Rywińska et al. 2009; Rymowicz et al. 2005). Other authors also report that fed-batch systems, in which a substrate is added by pulsed additions or at a constant feeding rate, are very good for high substrate concentrations (Sousa and Almeida, 2001; Levišauskas et al. 2006; Cheng et al. 2007).

The purpose of the present investigation was to compare the yields and productivity of citric acid biosynthesis from crude glycerol by two strains of Y. lipolytica: Wratislavia 1.31 and Wratislavia AWG7 in fed-batch cultures, in which

the total glycerol concentrations were 200 and 300 g·L-1 and the substrate was added either with pulsed additions or at a constant feeding rate.

MATERIALS AND METHODS

The acetate-negative mutants of *Yarrowia lipolytica*: Wratislavia 1.31 and Wratislavia AWG-7 used in this study were from the collection belonging to the Department of Biotechnology and Food Microbiology at the Wrocław University of Environmental and Life Sciences, Poland. Acetate-negative mutants *Y. lipolytica* Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG-7 were isolated after exposure to UV irradiation - *Y. lipolytica* Wratislavia 1.31 from a wild strain *Y. lipolytica* A-101 and *Y. lipolytica* Wratislavia AWG7 from the strain *Y. lipolytica* Wratislavia 1.31 (Rywińska et al. 2003). The colony phenotype of *Y. lipolytica* Wratislavia AWG7 is smooth as compared to the *Y. lipolytica* Wratislavia 1.31 strain. The yeast strains were maintained on agar slants (YM), containing 3 g of yeast extract, 3 g of malt extract, 5 g of bactopeptone, 10 g of glucose and 20 g of agar in 1 L of distilled water, under the layer of paraffin oil, at 4ºC.

The growth medium for inoculum production contained 50 g of pure glycerol, 3 g of yeast extract, 3 g of malt extract and 5 g of bactopepton in 1 L of distilled water. The cultures were grown in 0.3 L flasks containing 0.1 L of growth medium on an Elpan (Poland) rotary shaker at 30ºC for 3 days. An inoculum of 0.2 L was introduced into the bioreactor.

The following types of glycerol were used as carbon and energy sources in the media for citric acid production: pure glycerol (98% wt/wt) from POCH Gliwice Poland, crude glycerol from methyl ester production (SG BODDINS GmbH, Germany) containing 86% wt/wt of glycerol and 6.5% wt/wt of NaCl and crude glycerol from BDK Biodiesel GmbH, Kyritz, German, containing 92% wt/wt of glycerol, the impurities in this glycerol solution were sodium salts [2-3% (wt/wt)], methanol [0.01% (wt/wt)], metals [Cu 0.2, Mg 96, Fe 12.7, Zn 2.5 and Ca 44 ppm], heavy metals [Cd, Cr, Hg not detected], other organic materials [0.5% (wt/wt)] and water [6% (wt/wt)].

Biosynthesis media in 1 L of tap water contained: 200 or 300 g of carbon source, 3.0 g of NH_4Cl, 0.2 g of KH_2PO_4, 1.0 g of $MgSO_4$ $7H_2O$ and 1.0 g of yeast extract.

In the first method, in which the initial glycerol concentration was 80 g·L^{-1}, the total glycerol concentration of 200 g·L^{-1} was obtained by adding two portions of glycerol after 24 hrs and 48 hrs of cultivation. They were identical portions, 60 g L^{-1} of glycerol. In the second method, the initial glycerol concentration was 150 g·L^{-1}. After 50 hrs of cultivation, the remaining 150 g·L^{-1} glycerol was added until the total glycerol concentration of 300 g·L^{-1} and the total working volume of 2.0 L were reached at a constant feeding rate of 1.4 g·h^{-1}.

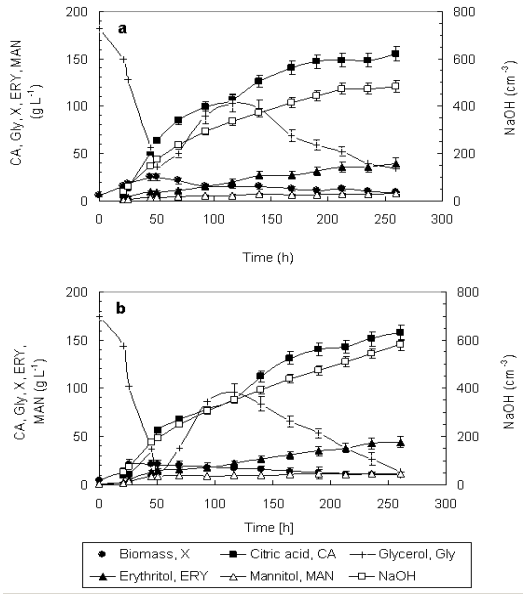

Figure 2. Time course of of biomass, X (●), glycerol, Gly (+), citric acid, CA (■), erythritol, ER (▲), mannitol, MAN (Δ), and NaOH (□) during fed-batch fermentation by *Y. lipolytica* Wratislavia 1.31 (a) and *Y. lipolytica* Wratislavia AWG7 (b) strain growing on crude glycerol (SG BODDINS GmbH, Germany). Cultivation conditions: glycerol (150 g·L^{-1}) was fed to the culture broth medium after 50 hrs of cultivation with a constant feeding rate of 1.4 g·h^{-1} to obtain a final glycerol concentration of 300 g·L^{-1}. Data are the average of duplicate cultures. Error bars indicate

The cultivations were carried out in a 5 L stirred-tank reactor (BIOSTAT B-PLUS, Sartorius, Germany) at a working volume of 2.0 L at 30ºC. The aeration rate was fixed at 0.36 vvm. The stirrer speed was adjusted to 800 rpm and the pH was maintained automatically at 5.5 by the addition of 40% (w/v) NaOH solution.

Biomass was determined gravimetrically after drying in a drier at 105ºC. Isocitric acid was determined using an enzymatic method as described by Goldberg and Ellis (1983). The concentrations of citric acid, glycerol, erythritol and mannitol were determined by HPLC (Beckman Gold System, USA) on an Aminex HPX87H Organic Acid column coupled to a UV detector at 210 nm and RI detector. The column was eluted with 20 mM of H_2SO_4 at room temperature at a flow rate of 0.6 cm^3 min^{-1}.

RESULTS

Two types of crude glycerol, presenting differentiations in their purity, were used in present study. In our preliminary trials, using different types of crude glycerol, showed similar growth and production of metabolites for *Y. lipolytica* Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG7. Additionally, similar biochemical behavior was observed by these mutants and no significant differences in the values of kinetic parameters were found between different kind of crude glycerol and pure glycerol with the yeast strains. Therefore the different concentrations of the

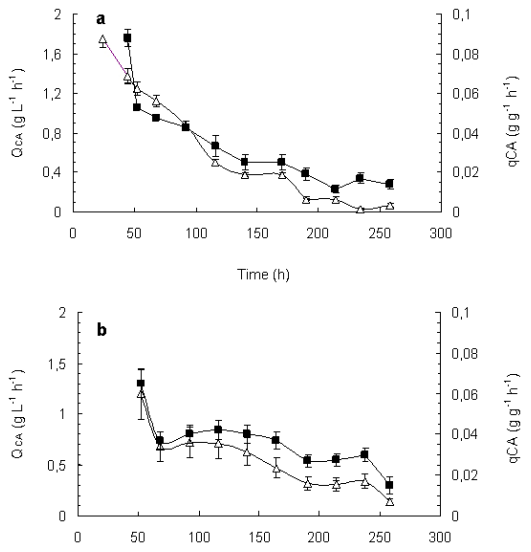

Figure 3. Dynamics of volumetric citric acid productivity, Q_{CA} (Δ), and specific citric acid production rate, q_{CA} (■), in fed-batch culture of *Y. lipolytica* Wratislavia 1.31 (a) and *Y. lipolytica* Wratislavia AWG7 (b) strain growing on crude glycerol (SG BODDINS GmbH, Germany). Cultivation conditions: glycerol (150 g·L^{-1}) was fed to the culture broth medium after 50 hrs of cultivation with a constant feeding rate of 1.4 g·h^{-1} to obtain a final glycerol concentration of 300 g·L^{-1}. Data are the average of duplicate cultures. Error bars indicate the standard deviations of mean data values.

impurities in feedstock do not have serious impact upon the production of CA (Rymowicz et al. 2006; Rywińska et al. 2009; Rywińska and Rymowicz, 2009). Additionally, the concentration of salts is not stated in the crude glycerol obtained by BDK Biodiesel plant (see Material and Methods) which was used in these investigations.

Moreover, in present investigations two types of feeding strategies were used in order for the fed-batch process to be performed. In our preliminary study we have found that initial concentration of glycerol in the range from 50 to 150 g L^{-1} have no affected on the growth and production of metabolites for *Y. lipolytica* Wratis*l*avia 1.31 and *Y. lipolytica* Wratislavia AWG7 used (data not presented). Therefore, in the present study different initial glycerol concentration was used (80 and 150 g L^{-1}).

Fed-batch culture with 200 g·l^{-1} of glycerol

In the cultures in which the total glycerol concentration was 200 g·L-1, crude glycerol containing 915 g·L-1 of glycerol was used. The processes were launched as batch cultures in which the initial glycerol concentration was about 80 g·L^{-1}; pulsed additions of glycerol (about 60 g·L^{-1}) were made after 24 and 48 hrs. The process with *Y. lipolytica* Wratislavia 1.31 strain yielded 126 g·L^{-1} of citric acid within 120 hrs (Figure 1a). The strain of *Y. lipolytica* Wratislavia AWG7 produced 113.5 g·L^{-1} of citric acid within 121 hrs (Figure 1b). The biomass concentration of *Y. lipolytica* Wratislavia 1.31 was higher (20 g·L^{-1}) than that of *Y. lipolytica* Wratislavia AWG7 (16.5 g·L^{-1}) yeast.

The yields of citric acid production achieved with *Y. lipolytica* Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG7 strains were comparable and amounted, 0.63 and 0.57 g·g^{-1}, respectively (Table 1).

The selectivity of the fermentation process was high and the concentration of isocitric acid obtained as a by-product did not exceed 6.2 g·L^{-1}, *i.e.* the percentage of this acid in the sum of citric acids did not exceed 4.8% (Table 1). The cultures of the two strains contained similar amounts of mannitol (about 6 g·L^{-1}). Erythritol (5.8 g·L^{-1} within 50 hrs) was found in the process carried out with the use of *Y. lipolytica* Wratislavia AWG7 strain. The amount of erythritol in the process carried out with the use of the other strain was much lower.

Fed-batch culture with 300 g·l^{-1} of glycerol

The processes, in which the total glycerol (SG BODDINS GmbH, Germany) concentration were 300 g·L^{-1}, were also launched as batch cultures. The initial glycerol concentration was 150 g·L^{-1} at a working volume of the reactor of about 1.65 L. After 50 hrs of cultivation, the remaining 150 g·L^{-1} glycerol was added at a constant feeding rate of 1.4 g h^{-1}, which resulted in citric acid concentrations as high as 155.2 g·L^{-1} with the strain of *Y. lipolytica* Wratislavia 1.31 (Figure 2a) and 157.5 g·L^{-1} with *Y. lipolytica* Wratislavia AWG7 (Figure 2a), and it is worth nothing that the medium still contained residue glycerol. The biomass concentration decreased from 25-20 g·L^{-1} within 50 hrs to ca. 9 g·L^{-1} at the end of the processes, which was due to continuous dilution of the culture by glycerol and NaOH solution, pulsed automatically in order to neutralize the medium. In the fed-batch with *Y. lipolytica* Wratislavia 1.31 strain, the quantity of the base added to the bioreactor was 0.48 L. The volume of NaOH used with *Y. lipolytica* Wratislavia AWG7 was 0.58 L.

The concentrations of erythritol and mannitol at the end of the process carried out with the use of Y. lipolytica Wratislavia 1.31 strain were 39.4 and 7.6 g·L-1, respectively, and 44.1 and 11.2 g·L-1 when Y. lipolytica Wratislavia AWG7 strain was used (Figure 3a and b). The percentages of isocitric acid were slightly higher (5.5 with Y. lipolytica Wratislavia 1.31 and 5.7% with Y. *lipolytica* Wratislavia AWG7) as compared to thepreviouscultures (Table 2).

The yield of citric acid was higher with *Y. lipolytica* Wratislavia 1.31, similarly as in the previous cultures. In the two processes, a significant decrease in both the specific and volumetric citric production rates was observed, especially in the cultures with higher substrate concentrations (Figure 3). These two parameters were very low at the end of these processes. As a consequence, the volumetric citric acid production rate throughout the entire process was as low as 0.6 g·L^{-1} h^{-1}, as compared to 1.05 g·L^{-1} h^{-1} observed with the strain of *Y. lipolytica* Wratislavia

Table 1. Fermentation parameters for citric acid production by acetate negative mutants of *Y. lipolytica* depend on total glycerol concentration in fed-batch system.

Parameter	*Y. lipolytica* Wratislavia 1.31		*Y. lipolytica* Wratislavia AWG7	
	Glycerol concentrations ($g \cdot L^{-1}$)			
	200	300	200	300
$Y_{CA/S}$ ($g \cdot g^{-1}$)	0.63 ± 0.015	0.58 ± 0.012	0.57 ± 0.011	0.55 ± 0.01
Q ($g \cdot L^{-1} h^{-1}$)	1.05 ± 0.06	0.6 ± 0.03	0.94 ± 0.04	0.6 ± 0.03
ICA (%)	4.7 ± 0.3	5.5 ± 0.4	4.8 ± 0.3	5.7 ± 0.4

1.31 and 0.94 $g \cdot L^{-1} h^{-1}$ with *Y. lipolytica* Wratislavia AWG7 in the processes carried out with 200 $g \cdot L^{-1}$ of the substrate.

DISCUSSION

Fed-batch cultures of *Y. lipolytica* Wratislavia AWG7 and *Y. lipolytica* Wratislavia 1.31 were accompanied by high yield production of citric acid (from 113.5 to 157.5 g L^{-1}), a quantity comparable or higher with literature values batch fermentations of *Y. lipolytica* cultured on glycerol as sole substrate. According to Papanikolaou et al. 2002 and Papanikolaou et al. 2008, *Y. lipolytica* LGAM S(7)1 and *Y. lipolytica* ACA-DC 50109 strains in the medium containing raw glycerol in shake flask cultures produced 33.6 and 62.5 g L^{-1} of citric acid, respectively. Levinson et al. 2007 reported, that the highest yielding strain, *Y. lipolytica* NRRL YB-423, produced 21.6 g L^{-1} citric acid from 40 g L^{-1} glycerol (54% yield). The other strain, *Y. lipolytica* NCIM 3589 produced 77.4 g L^{-1} citric acid from raw glycerol (Imandi et al. 2007).

Table 2 compares the very impressive results achieved in the present study with the data derived from literature. As shown in Table 2, the final concentration of citric acid, specific citric acid production rate as well as citric acid yield during several different fermentation configurations depended on both the yeast strains and substrates used.

As can be seen in Table 2, maximum citric acid concentrations (157.5 and 155.2 $g \cdot L^{-1}$), obtained from 300 $g \cdot L^{-1}$ of glycerol, were significantly higher than those reported in the literature for citric acid production in fed-batch cultures (Anastassiadis et al. 2002; Crolla and Kennedy, 2004; Kamzolova et al. 2005; Förster et al. 2007). Only Aurich et al. (2003) obtained higher citric acid concentration, *i.e.* 198 $g \cdot L^{-1}$. The yield of citric acid production in the two fed-batch systems under investigation was similar (0.55-0.63 $g \cdot g^{-1}$). However, in the culture with higher substrate concentration, the greater was the reduction in the volumetric of citric acid production rate, which proves that a periodic feeding with 200 $g \cdot L^{-1}$ of the substrate was more efficient. It seems quite likely that accumulation of large amounts of citric acid along with the presence of glycerol resulted in an increase in osmotic pressure (data not presented). A similar reduction in the dynamics of citric acid production was also observed during its biosynthesis from glucose hydrol by a wild strain of *Y. lipolytica* A-101 (Wojtatowicz and Rymowicz, 1991) and from glucose by the strain of *Candida olephila* ATCC 20177 (Anastassiadis et al. 2002).

The volumetric citric acid productivity of the process with 200 $g \cdot L^{-1}$ of glycerol is comparable to or higher than that reported by other authors who used such carbon substrates as: sunflower oil, n-paraffins, rapeseed oil, sucrose and glucose (Table 2). Other investigations showed that the volumetric citric acid productivities achieved in shake flask experiments, containing raw glycerol as a carbon sole sources, were lower and ranged from 0.09 to 0.14 g $L^{-1} h^{-1}$ (Papanikolaou et al. 2002; Levinson et al. 2007; Papanikolaou et al. 2008).

The yields of the citric acid production obtained with the two strains under investigation in fed-batch processes with 200 $g \cdot L^{-1}$ of the substrate, were higher than those obtained in batch cultures, with the same concentration of the substrate, 0.62 and 0.46 $g \cdot g^{-1}$, with *Y. lipolytica* Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG7 strain, respectively (Rymowicz et al. 2006).

It is interesting to note that high amount of erythritol and mannitol were produced in the culture broth by employed acetate negative mutants of Y. lipolytica. The productions of these sugar alcohols from glycerol by Y. lipolytica are not very common. On the other hand, a high initialconcentration of glucose and fructose, up to 450 g L-1, favors erythritol and mannitol production by osmophilic microorganisms, mainly by yeasts (Park et al. 2005, Lee et al. 2007). The biosynthetic mechanism of erythritol in C. magnoliae suggests that glucose is converted to erythrose-4-phosphate via the pentose-phosphate pathway, and then erythrose-4-phosphate is dephosphorylated into erythrose which is reduced to erythritol by erythrose reductase (Park et al. 2005). Results obtained by Sawada et al. 2009, suggest that erythritol may be produced mainly through the pentose phosphate pathway and the high activity of transketolase is correlated with high erythritol productivity

Table 2. Comparison between previous and present results with literature values regarding citric acid production in fed-batch cultures.

Strain	Substrate	CA_{max} ($g \cdot L^{-1}$)	$Y_{CA/S}$ ($g \cdot g^{-1}$)	Q ($g \cdot L^{-1}h^{-1}$)	q_{CA} ($g \cdot g^{-1}h^{-1}$)	References
Candida oleophila ATCC 20177	glucose	79.1	-	0.41	-	(Anastassiadis et al. 2002)
Y. lipolytica H181	sunflower oil	198	1,16	-	-	(Aurich et al. 2003)
Candida lipolytica	*n*-paraffins	42	0.8-1.0	0,1	-	(Crolla and Kennedy, 2004)
Y. lipolytica H222-S4(p67ICL1)T5	sucrose	140	0,82	0,73	0,091	(Förster et al. 2007)
Y. lipolytica 187/1	rapeseed oil	135	1.55	0,94	0.127	(Kamzolova et al. 2005)
Y. lipolytica N15	sunflower oil	150	1,32	1,04	-	(Kamzolova et al. 2008)
Y. lipolytica 1.31	crude glicerol (25% wt/wt)	144,5	0,72	0,69	0,045	(Rymowicz et al. 2005)
Y. lipolytica Wratislavia AWG7	pure glycerol	139	0.69	1.16	0.06	(Rywińska et al. 2009)
Y. lipolytica Wratislavia AWG7	crudeglycerol (86% wt/wt)	131.5	0.66	1.05	0.06	(Rywińska et al. 2009)
Y. lipolytica Wratislavia K1	pure glycerol	89	0.45	1.0	0.05	(Rywińska et al. 2009)
Y. lipolytica Wratislavia K1	crude glycerol (86% wt/wt)	86.8	0.43	0.99	0.045	(Rywińska et al. 2009)
Y. lipolytica Wratislavia 1.31	crude glicerol (35% wt/wt) (56% wt/wt) (86% wt/wt)	 146 137 130	 0.73 0.69 0.65	 0.99 1.00 1.11	 0.07 0.068 0.069	(Rywińska and Rymowicz, 2009)
Y. lipolytica Wratislavia 1.31	crude glycerol (91.5% wt/wt)*	126	0.63	1.05	0.053	this work
Y. lipolytica Wratislavia AWG7	crude glycerol (91.5% wt/wt)*	113.5	0.57	0.94	0.057	this work
Y. lipolytica Wratislavia 1.31	crude glycerol (86% wt/wt)**	157.5	0.58	0.6	-	this work
Y. lipolytica Wratislavia 1.31	crude glycerol (86% wt/wt)**	155.2	0.55	0.6	-	this work

* 200 $g \cdot L^{-1}$ of glycerol
** 300 $g \cdot L^{-1}$ of glycerol

in Trichosporonoides megachiliensis SN-G42. Additionally, in the cultures in which erythritol was produced after completion of cell growth, the enzyme activities of the pentose phosphate pathway were higher than those of the Krebs cycle.

To date, biochemical pathways involved in the regulation of erythritol production from glycerol by *Y. lipolytica* have not been studied in depth. It is know that glycerol catabolism in respiratory-sufficient yeasts occurs only via the Embden-Meyerhoff-Parnas (EMP) glycolysis. The glycerol kinase pathway is responsible for glycerol degradation. The glycerol catabolic pathway includes phosphorylation by glycerol kinase and a subsequent oxidation by a flavin adenine dinucleotide (FAD)-dependent glycerol 3- phosphate dehydrogenase, located on the outer surface of the mitochondrial inner membrane. The dihydroxyacetone phosphate formed enters the glycolytic pathway. Glycerol, a by-product of methyl esters production, has been used for aerobic process such as citric acid biosynthesis by yeast of *Y. lipolytica* since 2002 (Papanikolaou et al. 2002). There are only a few reports

demonstrating that the yield of citric acid production in shaking cultures was found within the range from 0.44 to 0.56 $g \cdot g^{-1}$ (Papanikolaou et al. 2002; Papanikolaou and Aggelis, 2003; Imandi et al. 2007; Levinson et al. 2007; Papanikolaou et al. 2008). In our earlier studies, we used both crude glycerol from methyl (Rywińska et al. 2009), ethyl esters production (Rymowicz et al. 2005) and pure glycerol (Rywińska et al. 2009). The yields of citric acid production by the strains of *Y. lipolytica* Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG7 were comparable, irrespective of the type of glycerol used for the study, which proves their suitability for citric acid biosynthesis.

CONCLUDING REMARKS

Although the concentration of citric acid in the culture with 300 $g \cdot L^{-1}$ of glycerol was high, the system with pulsed additions of glycerol to the total concentration of 200 $g \cdot L^{-1}$ proved to be more effective because of the volumetric citric acid production rate. Citric acid production by acetate-negative mutants of *Y. lipolytica* Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG7 obtained in the present study and the results obtained in our earlier investigations confirm that the two strains can be suitable for citric acid production from crude glycerol on an industrial scale.

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