ISSN 1330-9862
(FTB-2010)

original scientific paper

Biosynthesis of Citric Acid from Glycerol by Acetate Mutants of *Yarrowia lipolytica* in Fed-Batch Fermentation

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Received: November 23, 2007
Accepted: May 7, 2008

Summary

Pure and crude glycerol from biodiesel production have been used as substrates for citric acid production by acetate-negative mutants of *Yarrowia lipolytica* in fed-batch fermentation. Both the final concentration and the yield of the product were the highest when *Y. lipolytica* Wratislavia AWG7 strain was used in the culture with pure or crude glycerol. With a medium containing 200 g/L of glycerol, production reached a maximum of citric acid of 139 g/L after 120 h. This high yield of the product (up to 0.69 g of citric acid per gram of glycerol consumed) was achieved with both pure and crude glycerol. Lower yield of citric acid in the culture with *Y. lipolytica* Wratislavia K1 strain (about 0.45 g/g) resulted from increased erythritol concentrations (up to 40 g/L), accumulated simultaneously with the citric acid. The concentration of isocitric acid, a by-product in this fermentation, was very low, in the range from 2.6 to 4.6 g/L.

Key words: citric acid, crude glycerol, erythritol, fed-batch process, *Yarrowia lipolytica*

Introduction

Various aspects of citric acid (CA) production, especially from glucose, by *Yarrowia lipolytica* yeast have been studied during the past four decades (*1–4*). Although CA fermentation in glucose, hydrocarbon, ethanol, fatty acid and plant oil media has been studied extensively (*2,5–11*), little information is still available on the production of this compound from glycerol (*12–17*). Since the number of biodiesel producing units is steadily growing, glycerol waste from that production is becoming easily available on a large commercial scale. The overcapacity of more than 600 000 t of residual glycerol was expected due to the development of biodiesel industry in Europe until the end of 2007 (*18*). The very low cost (or even none) of crude glycerol makes it very attractive raw material for microbial industry. The use of agroindustrial residues in submerged fermentations is important economically, and besides, they minimize environmental problems (*12–19*). Raw glycerol could be utilized in microbial bioconversions for the production of a range of high value-added products that could be used either as end-products or as precursors for the production of other chemicals. The microbial production of 1,3-propanediol by *Citrobacter, Klebsiella* and *Clostridia* has recently attracted much attention because of its large potential in commercial applications, especially as a monomer of polyesters, polyethers or polyurethanes (*18, 20,21*). An alternative way of value addition refers to its biotransformation in the production of citric acid by *Y. lipolytica* (*16–18*), succinic acid by *Anaerobiospirillum succiniciproducens* (*22*), single cell oil by *Cryptococcus curvatus* (*23*) and *Mortierella isabellina* (*18*), hydrogen and ethanol by *Enterobacter aerogenes* (*24*) and 3-hydroxypropionaldehyde by *Lactobacillus reuteri* (*25*). For economic reasons, crude glycerol should be used as a carbon source, without prior purification. However, depending on the biodiesel production process and the rapeseed used as parent

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feedstock, crude glycerol can contain significant quantities of impurities (*e.g.* methanol, catalysts, potassium and sodium salts, heavy metals and soaps) that, in several cases, can seriously provoke inhibition of microbial cells (*26*). In this paper, the use of pure and unpurified crude glycerol from methyl ester production is described for CA production by the yeast *Y. lipolytica*. One disadvantage of yeasts is that they produce substantial amounts of isocitric acid (ICA). The results of our earlier studies have shown that acetate-negative mutant strains of *Y. lipolytica* (strains Wratislavia 1.31, Wratislavia AWG7 and Wratislavia K1) cultivated in the medium containing glycerol produced low quantities of ICA, *i.e.* 3.0, 4.6 and 5.0 %, respectively (*16*). In this study, two of them (Wratislavia AWG7 and Wratislavia K1) are compared with regard to the citric acid production rates and yields in fed-batch fermentations with pure and crude glycerol.

Material and Methods

Microorganisms and growth

Y. lipolytica Wratislavia AWG7 and *Y. lipolytica* Wratislavia K1 were originally obtained from the yeast culture collection belonging to the Department of Biotechnology and Food Microbiology, Wroclaw University of Environmental and Life Sciences in Poland. Both strains are acetate negative (ace$^-$) mutants incapable of growth on acetate as the sole carbon and energy source and additionally are bearing mutation leading to smooth colony phenotype. They were isolated from a Wratislavia 1.31 ace$^-$ mutant strain (formerly named A-101-1.31) after exposure to UV irradiation (AWG7 strain) or in the course of continuous citric acid production from glucose in nitrogen-limited chemostat (K1 strain) at dilution rate D=0.016^{-1}. Cells of Wratislavia AWG7 strain are smaller and they also form smaller colonies compared to Wratislavia K1. The yeast strains were maintained on yeast maltose agar (YM) slants at 4 °C. The growth medium for inoculum preparation contained (in g/L of tap water): glycerol 50, yeast extract 3 (Difco), malt extract 3 (Difco) and bactopepton 5 (Difco). Citric acid production was conducted in the production medium, which at the initial stage of fermentation contained (in g/L of tap water) glycerol 80–100, NH_4Cl 3, $MgSO_4{\cdot}7H_2O$ 1, KH_2PO_4 0.2, and yeast extract 1. The culture was fed with two portions of glycerol after 24 and 48 h of cultivation (see Fig. 1) in order to obtain a final glycerol concentration of 200 g/L.

The carbon sources were pure glycerol (Merck) containing 980 g/L of glycerol (A), and unpurified crude glycerol from methyl ester production (from SG BOD-DINS GmbH, Germany) containing 860 g/L of glycerol, 65 g/L of NaCl and methanol below 2 g/L (B). A preculture was carried out in 300-mL flasks containing 50 mL of growth medium on an Elpan (Poland) rotary shaker at 30 °C for 3 days. An inoculum of 100 mL was introduced into the bioreactor containing 1.2 L of the production medium. Cultivations were carried out in a 3.5-litre stirred-tank reactor (Bioflo III, New Brunswick Scientific Co., USA) with a working volume of 1.3 L at 30 °C. The aeration rate was fixed at 0.2 L/min. The stirrer speed was adjusted to 600 rpm and the pH was maintained automatically at 5.5 by the addition of 40 % (by mass per volume) NaOH solution. All cultures were carried out in two replications.

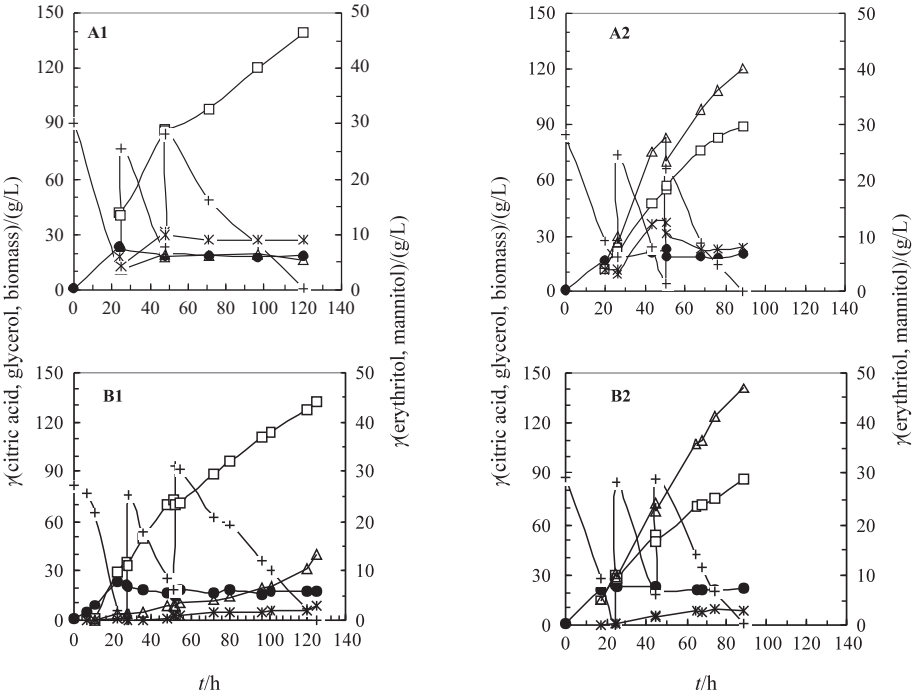

Fig. 1. Time course of citric acid, biomass, glycerol, mannitol and erthritol during fed-batch fermentations by *Y. lipolytica* Wratislavia AWG7 (A1, B1) and *Y. lipolytica* Wratislavia K1 (A2, B2) strains on pure glycerol (A1, A2) and crude glycerol (B1, B2). Symbols: citric acid (□), glycerol (+), biomass (●), erythritol (△), mannitol (∗). Cultivation conditions: culture broth medium was fed with two portions of glycerol after 24 and 48 h of cultivation in order to obtain a final glycerol concentration of 200 and 3.0 g/L of NH_4Cl; pH=5.5

Analytical methods

Biomass was determined gravimetrically after drying in a drier at 105 °C. Isocitric acid was determined using an enzymatic method as described by Goldberg and Ellis (*27*). 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 refractive index (RI) detector. The column was eluted with 20 mM H_2SO_4 at room temperature at a flow rate of 0.6 mL/min. The retention times for citric acid, mannitol, erythritol and glycerol were 7.9, 9.8, 11.3 and 12.9 min, respectively.

Results and Discussion

Two strains of *Y. lipolytica* (Wratislavia AWG7 and Wratislavia K1) were compared for citric acid (CA) production from pure and crude glycerol in fed-batch fermentations. Both of them were obtained from the parental strain of *Y. lipolytica* Wratislavia 1.31 in two ways and varied in morphology of cells and colonies (see Materials and Methods). Like the parental strain, they did not utilise *n*-alkanes, oils and short chain carboxylic acids and showed significantly improved purity of citrate fermentation in glucose media, as compared to wild type parental A-101 strain. Mutation to smooth colony phenotype did not affect improved CA:ICA ratio. In contrast to the rough parental Wratislavia 1.31 strain, which was extremely unstable during continuous fermentations (*28*), both smooth mutants revealed high stability and good efficiency of CA production.

Fig. 1 shows time courses for growth, metabolite production, and glycerol consumption. The final biomass was similar in the cultures with pure and crude glycerol containing Wratislavia AWG7 strain (19 and 18.1 g/L, respectively). The differences in biomass level were higher with Wratislavia K1 strain, but it was interesting to note that in the culture with crude glycerol, the biomass was higher (22.1 g/L) than in the process with pure glycerol (20.4 g/L). Due to the addition of substrate during fed-batch processes and NaOH solution as well (maintenance of pH at 5.5) into the fermentor, the volume of fermentation broth increased and the concentration of biomass in the late phases of the culture decreased (Fig. 1). In the batch mode of our previous investigation, the maximum biomass level with Wratislavia K1 strain, with 200 g/L of crude glycerol (containing 550 g/L of glycerol) was significantly higher (26.5 g/L) than that observed with Wratislavia AWG7 strain (22.1 g/L) (*16*). The results suggest that the impurities in crude glycerol did not affect biomass production by Wratislavia AWG7 strain, while Wratislavia K1 strain cells utilized crude glycerol ingredients. For any fermentation process based on waste substrates, it is advantageous when the producing microorganism shows little sensitivity to impurities in the substrate. The biomass yield on glycerol consumed ($Y_{x/s}$) for all trials was in the range from 0.43 to 0.47 g/g (Table 1). This also applies to citric acid production. The CA concentration obtained from crude glycerol was slightly lower than that observed in pure glycerol; Wratislavia AWG7 strain produced 139 g/L in process A1 and 131.5 g/L in process B1 (Fig. 1, Table 1). The final CA concentration was significantly lower with Wratislavia K1 strain (about 89 g/L in each process). Moreover, the concentration of isocitric acid produced by these two strains was very low, from 2.6 to 4.6 g/L in comparison with wild parent strain A-101, which produced higher concentrations of ICA, in the range from 12 to 15 g/L, in media containing glycerol (data not published). During the fed-batch culture, the pH was adjusted to 5.5 by the automatic addition of 40 % NaOH. In the fed batch-culture of Wratislavia AWG7 strain with pure and crude glycerol, the quantity of the base added to the bioreactor was about 195 mL.

Table 1. Citric acid production from pure and crude glycerol by *Y. lipolytica* strains

Strain	*Y. lipolytica* Wratislavia AWG7		*Y. lipolytica* Wratislavia K1	
Parameters	A1	B1	A2	B2
Cultivation time/h	120	125	89	88
X_{max}/(g/L)	19	18.1	20.4	22.1
$Y_{x/s}$/(g/L)	0.45	0.47	0.43	0.46
CA_{max}/(g/L)	139	131.5	89.0	86.8
ICA_{max}/(g/L)	3.7	4.6	2.7	2.6
ERY_{max}/(g/L)	5.4	13.4	40.2	46.9
MAN_{max}/(g/L)	9.0	2.8	7.8	2.7
Q_{CA}/(g/(L·h))	1.16	1.05	1.0	0.99
q_{CA}/(g/(g·h))	0.06	0.06	0.05	0.045
$Y_{CA/S}$/(g/g)	0.69	0.66	0.45	0.43

X_{max}=maximum biomass concentration, CA_{max}=maximum citric acid concentration, ICA_{max}=maximum isocitric acid concentration, ERY_{max}=maximum erythrytol concentration, MAN_{max}=maximum mannitol concentration, Q_{CA}=citric acid volumetric productivity, q_{CA}=specific citric acid production rate, $Y_{x/s}$=biomass yield per glycerol consumed, and $Y_{CA/S}$=total conversion yield of citric acid produced per glycerol consumed. Q_{CA}, q_{CA} and $Y_{CA/S}$ values are presented when the maximum concentration of citric acid had been achieved. A – pure glycerol, B – crude glycerol; for culture conditions see Fig. 1.
All values are expressed as means derived from duplicate experiments

The volume of NaOH used with Wratislavia K1 was about 125 mL.

It was very interesting to observe that the cultivation time of Wratislavia K1 strain needed for total glycerol consumption was shorter than that needed when Wratislavia AWG7 was used (Fig. 1, Table 1). Wratislavia K1 strain reached the maximum concentration of CA in about 75 % of cultivation time of the cultures with Wratislavia AWG7 strain. This was due to the fact that apart from CA accumulation, the by-products such as erythritol and mannitol were produced simultaneously in the culture broth. The amount of the accumulated erythritol by the two strains varied significantly. The highest concentrations of this sugar alcohol were obtained with Wratislavia K1 strain and reached 40.2 g/L in the culture with pure glycerol and 46.9 g/L in the culture with crude glycerol. Wratislavia AWG7 strain produced less erythritol (5.6 and 13.4 g/L, from pure and crude glycerol, respectively). The final concentrations of mannitol were similar with the two strains, but they were the highest (about 8–9 g/L) in the cultures with pure glycerol, as compared to about 3 g/L in the culture with crude glycerol. Production of these sugar alcohols from glycerol by *Y. lipolytica* yeast is not very common.

As has been observed, sugar alcohols, including mannitol and erythritol, protect plants, fungi, yeasts and bacteria during stress conditions, *e.g.* osmotic stress (*29*). It is quite likely that the production of polyols in this study resulted from the exposure of the strain to high concentrations of CA and glycerol. Moreover, our recent investigation has shown that a high initial concentration of glycerol up to 150 g/L and the total glycerol concentration of 250 g/L favour erythritol production by Wratislavia K1 strain (*17*). Probably in response to a high external osmotic environment, this strain accumulates a very high amount of erythritol (up to 83 g/L), which compensates for differences between the extracellular and intracellular water potential. To date, biochemical pathways involved in the regulation of erythritol and mannitol production from glycerol by *Y. lipolytica* have not been studied in depth.

As can be seen in Table 1, the yield of CA was the highest using Wratislavia AWG7 strain in the culture with pure and crude glycerol (0.69 and 0.66 g/g, respectively). In contrast, when Wratislavia K1 was used, the yield of CA was lower, but comparable when pure glycerol and crude glycerol were used (0.45 and 0.43 g/g, respectively). The results of CA production from glycerol by yeast obtained in our study with submerged processes are worth attention because the data reported in international literature describe mainly the experiments carried out in shake flasks (*11–15*). Papanikolaou *et al.* (*14*) cultivated *Y. lipolytica* LGAM S(7)1 on crude glycerol and found a CA concentration of 35 g/L with the yield of 0.42–0.44 g/g. However, when glycerol concentration increased, the respective values of CA and conversion yield were 62.5 g/L and 0.56 g/g (*18*). Additionally, the strain LGAM S(7)1 gave a yield of around 0.5 g/g of CA produced from glycerol when it was cultivated in mixtures containing crude glycerol and industrial derivative of tallow (*15*). As reported by Levinson *et al.* (*13*), the NRRL YB-423 strain of *Y. lipolytica* produced 21.6 g/L of citric acid from 40 g/L of glycerol (54 % yield). Very high citric acid concentrations, ranging from 60 to 75.5 g/L, were reported by Imandi *et al.* (*12*). However, although they used a raw glycerol by-product from biodiesel production (30–80 g/L), the percentage of glycerol in this by-product was not indicated. Recently, Förster *et al.* (*10*) have demonstrated CA and ICA production of 40–80 g/L from 10 % pure glycerol in shake flasks with engineered strains exhibiting low ICA formation of 5 %, compared to 10–12 % ICA when using wild type strains.

From economic point of view, integral Q_{CA} (the volumetric citric acid productivity) and q_{CA} (the specific citric acid production rate) are the most important specific fermentation parameters, determining the efficiency of a fermentation process. As can be seen from Table 2, the highest values of these kinetic parameters were observed in each culture at the beginning of the production phase, during a short period of 14–24 h, and then they gradually decreased during the cultivation. Markedly lower, but constant rates were achieved between 65 and 125 h of cultivation. In addition, the specific and volumetric rates were primarily dependent on the strain. The highest volumetric production rate (2.45 g/(L·h)) and specific CA production rate (0.13 g/(g cells·h)) were achieved with Wratislavia AWG7 strain, whereas the parameters obtained with Wratislavia K1 strain were markedly lower, especially in the medium with pure glycerol. No significant differences in the values of kinetic parameters were found between pure and crude glycerol with

Table 2. Dynamics of volumetric citric acid productivity (Q_{CA}) and specific citric acid production rate (q_{CA}) in fed-batch cultures of *Y. lipolytica* strains grown in pure and crude glycerol

Strain	*Y. lipolytica* Wratislavia AWG7		*Y. lipolytica* Wratislavia K1	
		Pure glycerol (A)		
Cultivation time/h	24–48	96–120	26–43	68–89
Q_{CA}/(g/(L·h))	2.45	0.83	1.77	0.52
q_{CA}/(g/(g·h))	0.13	0.044	0.08	0.033
		Crude glycerol (B)		
Cultivation time/h	22–36	72–125	24–45	65–88
Q_{CA}/(g/(L·h))	2.45	0.84	2.36	0.74
q_{CA}/(g/(g·h))	0.13	0.047	0.105	0.034

All values are expressed as means derived from duplicate experiments

Table 3. Comparison between the present results and literature values regarding citric acid production by yeasts

Strain	Substrate	CA_{max} g/L	$Y_{CA/S}$ g/g	q_{CA} g/(g·h)	Configuration	Ref.
Candida oleophila ATCC 20177 (var.)	glucose	142–150	0.75	–	continuous bioreactor	*1*
Y. lipolytica 187/1	rapeseed oil	135	1.55	0.127	batch bioreactor	*6*
Y. lipolytica H222-4(p67ICL1)T5	sucrose	140	0.82	0.091	batch bioreactor	*11*
Y. lipolytica Wratislavia 1.31	raw glycerol	125.5	0.62	0.05	batch bioreactor	*16*
Y. lipolytica ACA-DC 50109	raw glycerol	62.5	0.56	0.014	flask	*18*
Y. lipolytica VKM Y-2373	ethanol	105.4	0.883	0.138	repeat-batch bioreactor	*30*
Y. lipolytica N-1	petrolatum	217	1.47	–	batch bioreactor	*31*
Y. lipolytica N-1	ethanol	120	0.87	0.116	continuous bioreactor	*32*
Candida lipolytica	*n*-parrafins	42	0.8–1.0	–	fed-batch bioreactor	*33*
Y. lipolytica Wratislavia K1	crude glycerol	86.8	0.43	0.045	fed-batch bioreactor	this work
Y. lipolytica Wratislavia AWG7	crude glicerol	131.5	0.66	0.06	fed-batch bioreactor	this work

either of the strains. However, at the second, slower production phase, higher values were achieved in the culture with crude glycerol. Similar changes in the dynamics of CA production from glucose were reported in earlier studies (*4*).

The presented results of CA production from glycerol by ace$^-$ mutants of *Y. lipolytica* are significant and comparable with the highest values obtained in the literature. Table 3 compares the very impressive results achieved in the present study with the data derived from literature.

As shown in Table 3, 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 (*1*,*6*,*11*,*16*,*18*,*30*–*33*). Our results show that Wratislavia AWG7 strain produced similar amount of citric acid from unpurified crude glycerol compared to other processes containing glucose (*1*), ethanol (*30*), rapeseed oil (*6*) and sucrose (*11*) as a carbon source, but lower than that achieved with petrolatum (*31*).

Conclusions

Bioconversion of glycerol to CA by *Y. lipolytica* Wratislavia AWG7 and *Y. lipolytica* Wratislavia K1 was studied in order to determine if the fermentation process is possible when crude glycerol is used as the sole carbon source. Crude glycerol from the biodiesel production process can be used in fed-batch cultures of acetate negative (ace$^-$) mutants of *Y. lipolytica* giving the results similar to those obtained with pure glycerol. The final concentration of CA (139 g/L) obtained from pure glycerol was comparable to that obtained from crude glycerol (131.5 g/L) with Wratislavia AWG7 strain. Significantly lower concentrations of CA with Wratislavia K1 strain (about 87–89 g/L) resulted from high concentration of erythritol (up to 47 g/L) in the fermentation broth.

Acknowledgement

This work was financed by the Ministry of Sciences and Higher Education of Poland under Project No. 2P06T 044 30.

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