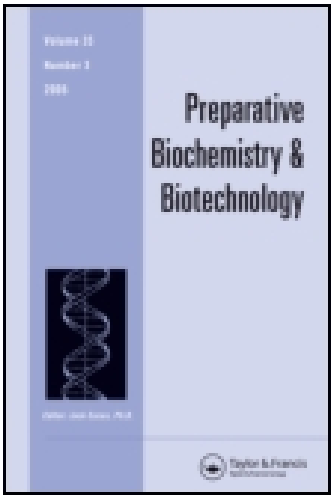

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EFFECT OF AGITATION AND AERATION ON THE CITRIC ACID PRODUCTION BY *Yarrowia lipolytica* GROWN ON GLYCEROL

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□ *The effects of agitation rates from 400 to 900 rpm and aeration rates ranging from 0.18 to 0.6 vvm on biomass and citric acid production on glycerol media by acetate-negative mutants of* Yarrowia lipolytica, *Wratislavia 1.31 and Wratislavia AWG7, in batch culture were studied. The agitation rates of 800 and 900 rpm (at a constant aeration rate of 0.36 vvm) and aeration rates within the range of 0.24–0.48 vvm (at a constant agitation rate of 800 rpm), which generated dissolved oxygen concentration (DO) higher than 40%, were found the best for citric acid biosynthesis from glycerol. An increase in agitation rate (higher than 800 rpm) and aeration rate (higher than 0.36 vvm) had no impact on DO and citric acid production. The highest citric acid concentration (92.8 g/L) and yield (0.63 g/g) were obtained with Wratislavia 1.31 strain at 0.24 vvm. The highest volumetric citric acid production rate (1.15 g/Lh) and specific citric acid production rate (0.071 g/gh) were reached at 0.48 vvm.*

Keywords aeration rate, agitation rate, citric acid, dissolved oxygen concentration, glycerol, *Yarrowia lipolytica*

INTRODUCTION

Global production of citric acid has now reached 1.4 million tons per year and it increases annually by 3.5–5%.[1] Although chemical synthesis of citric acid is possible, it has never been produced commercially. Less than 1% of the total world's citric acid production is still obtained from citrus fruit in Mexico and South America because of their economical availability in those regions.[2] The fermentation process for the production of citric acid is the easiest and economically most successful. While *Aspergillus*

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niger is the primary organism used for the industrial production of citric acid, *Yarrowia lipolytica* yeasts are also used.[3] Citric acid fermentation from raw materials as carbon sources, such as ethanol, vegetable oils, and glucose syrups, has been studied extensively,[2–5] but little information is still available on the impact of operating conditions on citric acid production from glycerol, an important renewable feedstock and a major by-product of biodiesel production processes. Currently, annual production of glycerol by biodiesel industries amounts to approximately 1.9 million tons. The existing world market for pure glycerol for high-quality industrial applications (especially chemical and pharmaceutical) only covers some 0.9–1.0 Mtonnes per year.[6] For this reason, it is justifiable to find alternative ways to convert this substrate into value-added products. The results of our earlier studies on biosynthesis of citric acid from glycerol showed that acetate-negative mutants Wratislavia 1.31 and Wratislavia AWG7 of *Y. lipolytica* strain were the best for this process.[7–9]

The aim of the present study was to determine appropriate culture conditions, especially agitation and aeration rates, for biosynthesis of citric acid from glycerol by acetate negative mutants Wratislavia 1.31 and Wratislavia AWG7 of the *Y. lipolytica* yeast.

MATERIALS AND METHODS

Microorganisms

Yarrowia lipolytica Wratislavia 1.31 and *Y. lipolytica* Wratislavia AWG7, used in this study, were originally obtained from the yeast culture collection belonging to the Department of Biotechnology and Food Microbiology, Wroclaw University of Environmental and Life Sciences, Poland. The strains were isolated after exposure to ultraviolet (UV) irradiation: Wratislavia 1.31 from a wild strain A-101 and Wratislavia AWG7 from a strain Wratislavia 1.31.[10] Both strains are acetate-negative mutants incapable of growing on acetate as the sole carbon and energy source. The colony phenotype of Wratislavia AWG7 is smooth in comparison with Wratislavia 1.31. As compared to the wild A-101 strain, Wratislavia 1.31 and Wratislavia AWG7 strains are characterized by a notably enhanced purity of citrate fermentation on glucose and glycerol medium.[11]

The yeast strains were maintained on YM slants under a layer of paraffin oil at 4°C.

Media and Culture Conditions

The growing medium for inoculum preparation contained 50 g pure glycerol (98% w/w), 3 g yeast extract, 3 g malt extract, and 5 g bactopepton

per liter of distilled water. The cultures were grown in 300-mL flasks containing 50 mL of the growing medium on a shaker at 30°C for 3 days. An inoculum of 200 mL was introduced into a bioreactor containing 1800 mL of the production medium. Citric acid production was conducted on a production medium containing 150 g pure glycerol, 3 g NH_4Cl, 1 g $MgSO_4 \cdot 7H_2O$, 0.2 g KH_2PO_4, and 1 g yeast extract per liter of tap water.

The cultivations were carried out in a 5-L stirred-tank reactor (Biostat B-Plus Sartorius) with a working volume of 2 L at 30°C. The pH was maintained automatically at 5.5 by adding 40% (w/v) NaOH solution. The agitation rates were 400, 500, 600, 800, and 900 rpm. The aeration rates were fixed at 0.18, 0.24, 0.36, 0.48, and 0.6 vvm (volume per volume per minute). Sterile air filters with 0.2-μm pores were used for air transfer into bioreactor. The dissolved oxygen concentration (DO) in the culture broth was measured using a pO_2 electrode (Oxyferm FDA 325).

All the cultures were cultivated until the complete consumption of glycerol was reached, excluding the cultures carried out at 400 rpm. All cultures were carried out in three replications.

Analytical Methods

Biomass was determined by the dry weight method, 10-mL samples were filtered on Millipore HA filters, and the biomass was washed in distilled water and dried at 105°C to a constant weight. Isocitric acid was determined using an enzymatic method as described by Goldberg and Ellis.[12] The concentrations of citric acid and glycerol were determined by high-performance liquid chromatography (HPLC; Beckman Gold System, USA) on an Aminex HPX87H organic acid column coupled to a UV detector at 210 nm and a refractive index (RI) detector. The column was eluted with 20 m*M* of H_2SO_4 at room temperature at a flow rate of 0.6 mL/min.

Microscopic Observations

Yeast cells morphology was checked using an optical microscope (Axio Scope A1 Zeiss) with 400× magnification, coupled with camera (Canon PowerShot G9), linked to a personal computer by a frame grabber.

Calculation of Fermentation Parameters

The yield of citric acid production (Y_{CA}), expressed in grams per gram, from glycerol was calculated from:

$$Y_{CA} = \mathrm{CA/S}$$

The volumetric citric acid production rate (Q_{CA}), expressed in grams per liter per hour was calculated from:

$$Q_{CA} = \mathrm{CA/t}$$

The specific citric acid production rate (q_{CA}), expressed in grams per gram cells per hour was calculated from:

$$q_{CA} = \mathrm{Q_{CA}/X}$$

In these equations. CA is citric acid concentration in the culture liquid at the end of cultivation (g/L); S, total amount of glycerol consumed (g/L); t, the fermentation duration (h); and X, maximum biomass concentration (g/L).

RESULTS AND DISCUSSION

Effects of Agitation Rates on Biomass and Citric Acid Production

Five agitation rates (400, 500, 600, 800, and 900 rpm) were investigated in this study, at a constant aeration rate of 0.36 vvm. Figure 1 shows the influence of agitation on *Y. lipolytica* Wraislavia 1.31 and *Y. lipolytica* Wratislavia AWG7 biomass production and glycerol consumption during batch processes. The final concentration of the biomass ranged from 12.5 to 18 g/L and from 9.0 to 20.2 g/L for Wratislavia 1.31 and Wratislavia AWG7 strain, respectively. However, at low agitation rates (400 and 500 rpm), the duration of growth phase was prolonged and lower biomass concentrations were reached compared with higher agitation rates of 800 and 900 rpm. The results reported by Crolla and Kennedy[13] show similar biomass concentration of *C. lipolytica* NRRL-Y-1095 on *n*-paraffin at various agitation rates (from 400 to 1200 rpm), but in their experiments the aeration rate was high (1.0 vvm).

Citric acid production by yeast and fungi is an aerobic process, highly dependent on oxygen supply in bioreactor. Air saturation (percent air) and dissolved oxygen concentrations (DO) are the most accurate controllable parameters in terms of oxygen influence on citric acid production and secretion in aerobic fermentations.[2] Figures 2A and 2C show the DO profile versus time for the two strains. The DO decreased rapidly at the beginning of all the processes. According to Wentworth and Cooper,[14] when the nitrogen source was completely exhausted, the organism's respiration rate decreased, resulting in increasing DO. However, the DO was very low (0%) at low agitation rate 400 rpm (Figures 2A and 2C), which resulted

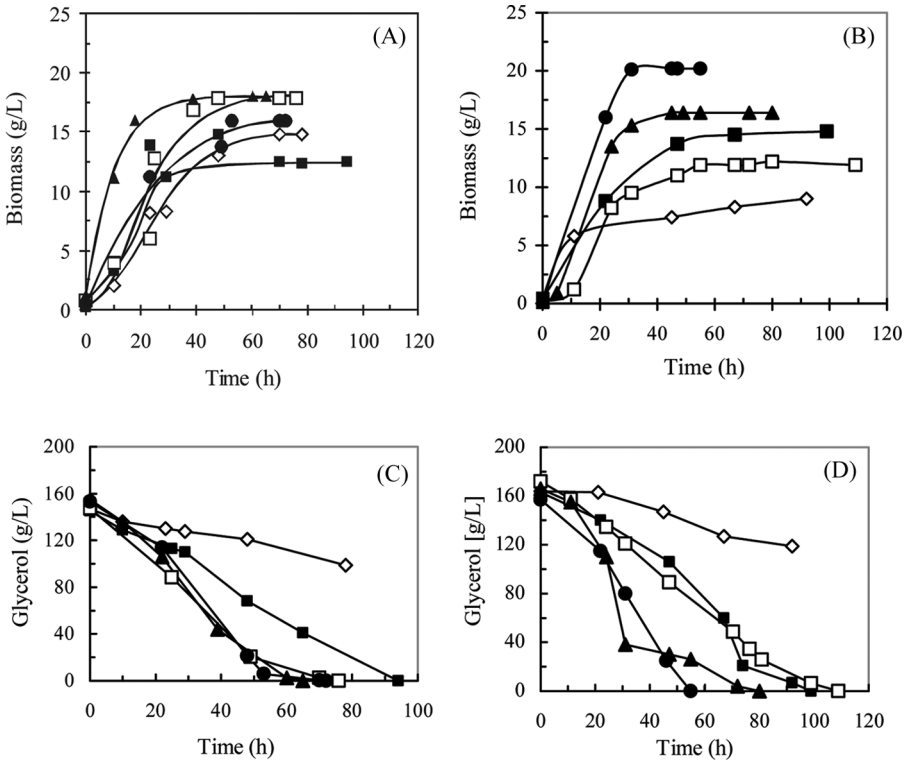

FIGURE 1 Biomass production and glycerol consumption by *Y. lipolytica* Wratislavia 1.31 (A, C) and *Y. lipolytica* Wratislavia AWG7 (B, D) at various agitation rates.: 400 (◇), 500 (■), 600 (□), 800 (▲), and 900 rpm (●). Culture conditions: aeration rate 0.36 vvm.

in low citric acid production (Figures 2B and 2D). The concentration of unconsumed glycerol at the end of the process carried out at 400 rpm with Wratislavia 1.31 and Wratislavia AWG7 strain was 98.9 and 119 g/L, respectively (Figures 1C and 1D); a small amount of citric acid was produced by these strains under this condition. Glycerol was used primarily for production of biomass, but small amounts of erythritol and mannitol, up to 7.4 g/L (data not presented), were also synthesized. It is likely that under these adverse conditions the accumulation of intracellular lipids occurred (date not determined).

Furthermore, as can be seen in Figure 3, low agitation rates caused yeast–mycelium transition in the two strains (Figures 3A, 3B, 3F, and 3G), although the colony phenotype of Wratislavia AWG7 is smooth. These findings were in concurrence with the results reported by Lopez et al.[15] and Tabuchi,[16] who found decreasing citric acid production and the occurrence of true septa and long cells under oxygen deficiency. *Y. lipolytica* is natural dimorphic fungus, which forms yeast cells, pseudohyphae, and

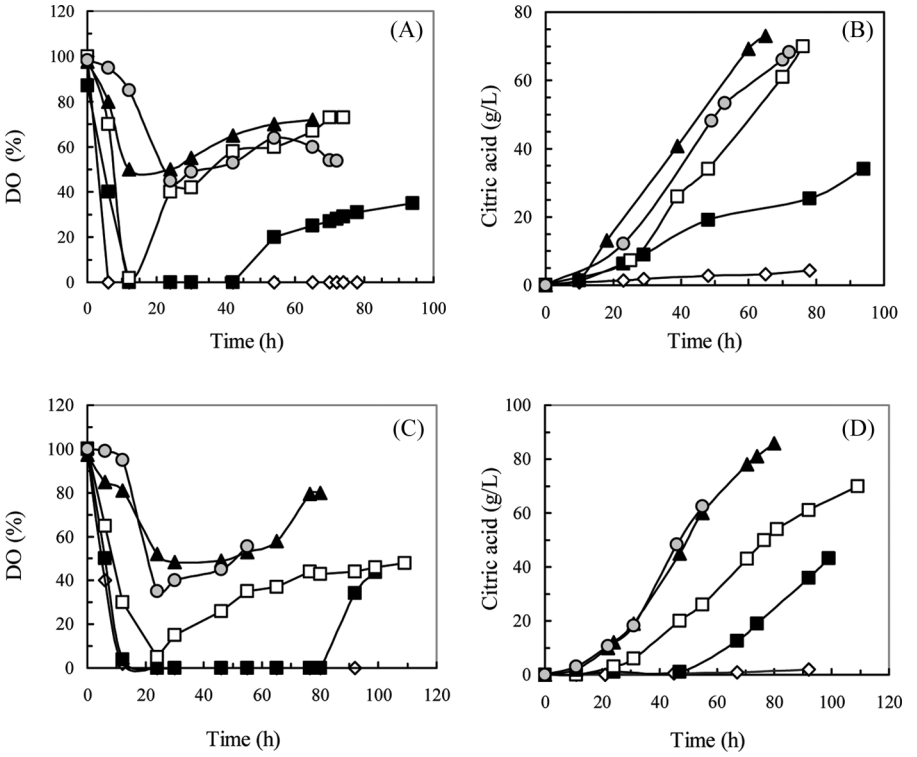

FIGURE 2 Dissolved oxygen concentration (DO) and citric acid production from glycerol during batch process with *Y. lipolytica* Wratislavia 1.31 (A, B) and *Y. lipolytica* Wratislavia AWG7 (C, D) strain at various agitation rates. 400 (◇), 500 (■), 600 (□), 800 (▲), and 900 rpm (●). Culture conditions: aeration rate 0.36 vvm.

septate hyphae. According to Zinjarde[17] and O'Shea and Walsh,[18] low DO is the main factor influencing the morphology of *Y. lipolytica* yeast. This yeast changed its morphology from yeast cells to mycelium forms under this condition. Other factors like presence or absence of fatty materials, the growth stage, and the pH of the culture influenced the morphology of *Y. lipolytica* also. One the other hand, mycelial development is inhibited by a deficiency of metal ions.[19–22] According to Makri et al.,[19] short true mycelia and pseudo-mycelia were predominant during the exponential growth phase only, but in their experiments DO was maintained above 30% saturation by varying agitation rate (200–250 rpm).

An increase in agitation to 600 rpm increased both the DO (to 40–70%) and citric acid production (Figure 2). The results of our study show that the highest concentration of citric acid (Figures 2B and 2D) and the highest yield and specific rate of citric acid production were obtained at 800 rpm (Table 1), when the DO was higher than 50% (Figure 2A and C). Similar

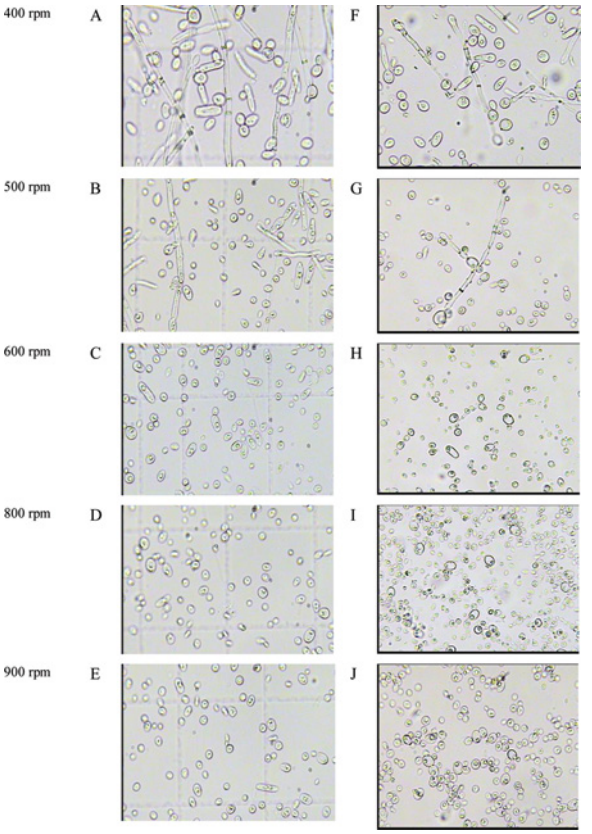

FIGURE 3 Effect of agitation rate on the morphology of *Y. lipolytica* Wratislavia 1.31 (A–E) and *Y. lipolytica* Wratislavia AWG7 (F–J) cells. Magnification 400× (color figure available online).

results for citric acid biosynthesis by yeast have been reported by other authors.[23] Agitation rates between 500 and 1000 rpm have been reported to be optimum for maximum citric acid production.[2,13,24–26] Agitation is a

TABLE 1 Effect of Agitation Rate on Citric Acid Yield (Y_{CA}), Volumetric Citric Acid Production Rate (Q_{CA}), and Specific Citric Acid Production Rate (q_{CA}) during Batch Process with *Y. lipolytica* Strains

Agitation Rate (rpm)	*Y. lipolytica* Wratislavia 1.31			*Y. lipolytica* Wratislavia AWG7		
	Y_{CA} (g/g)	Q_{CA} (g/L-h)	q_{CA} (g/g-h)	Y_{CA} (g/g)	Q_{CA} (g/L-h)	q_{CA} (g/g-h)
400	0.09	0.056	0.0038	0.027	0.02	0.0024
500	0.23	0.36	0.029	0.28	0.46	0.029
600	0.47	0.92	0.052	0.47	0.64	0.054
800	0.47	1.12	0.062	0.52	1.07	0.065
900	0.45	0.95	0.060	0.40	1.13	0.056

Note. Culture conditions: aeration rate 0.36 vvm.

critical parameter for citric acid production by yeast and *A. niger* in stirred fermenters, influencing dispersion of pellets, the DO, and enzyme activities.[2] The effect of pO_2 on citric acid synthesis has also been studied in several yeasts. According to many authors, an increase in the DO resulted in citric acid production. An increase in pO_2 from 20 to 80% saturation during the phase of active citric acid production by *Candida lipolytica* Y 1095 growing on glucose resulted in increasing specific citric acid production rate (from 0.017 to 0.029 g/g-h) and volumetric productivity (from 0.52 to 0.9 g/L-h).[27] Similar effects were observed with this yeast strain in cell-recycle and fed-batch fermenters,[28] as well as in citric acid fermentation by *A. niger*.[29] Higher productivity was obtained in batch and repeated batch cultures at 80% than at 20% air saturation.[24] However, according to Kamzolova et al.,[30] citric acid production by *Y. lipolytica* N-1 strain on ethanol was performed at a relatively low pO_2 (20% saturation) and high iron concentration (3.5 mg/L). The citric acid concentration was high and reached 120 g/L (0.87 g/g), the specific rate of citric acid synthesis reached 0.12 g/g-h, and the volumetric productivity was 1.15 g/L-h. The highest citric acid production in chemostat cultures of *Candida oleophila* ATCC 20177 was found at 20% air saturation as compared to 50, 80, and 133%, but in this particular case, the authors suggested that a kind of a Crabtree effect was attained, simulating an anaerobic glycolytic pathway under aerobic conditions.[24,31]

Effects of Aeration on Biomass and Citric Acid Production

The effects of aeration rates (0.18, 0.24, 0.36, 0.48, and 0.6 vvm) on yeast growth and citric acid production were studied in batch cultivation at a constant agitation rate of 800 rpm. No significant differences in yeast growth and biomass concentration at the end of the process were observed (Figure 4). Biomass level reached from 16.1 to 19.4 g/L for Wratislavia 1.31 strain and from 16.4 to 19.3 g/L for the Wratislavia AWG7 strain, depending on the aeration rate. The highest citrate concentrations of 92.8 g/L and 85.9 g/L were achieved at the aeration rates of 0.24 and 0.36 vvm for the Wratislavia 1.31 and Wratislavia AWG7 strain, respectively (Figure 4).

The yield of citric acid production (Y_{CA}), ranged from 0.45 to 0.63 g/g for the Wratislavia 1.31 strain and from 0.44 to 0.52 g/g for Wratislavia AWG7, depending on aeration rate. The best results were obtained at 0.24 and 0.36 vvm, respectively (Table 2). The yields of citric acid obtained in this study were similar or higher than reported in literature, 0.42–0.56 g/g for biosynthesis of citric acid on glycerol media.[32–35] Higher yields of citric acid production by different strains of yeasts are obtained from such substrates as ethanol, *n*-paraffins, rapeseed oil, glucose, or sucrose.[5,13,24,25,30,36]

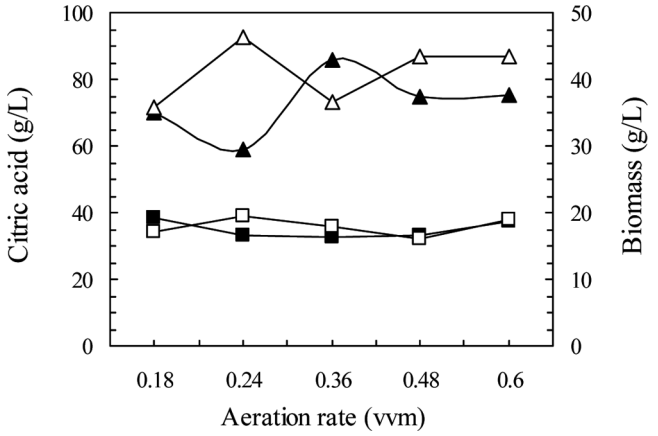

FIGURE 4 Effect of aeration rate on the production of citric acid (△, ■) and biomass (□, ■) by *Y. lipolytica* Wratislavia 1.31 (open symbols) and *Y. lipolytica* Wratislavia AWG7 (closed symbols). Culture conditions: agitation rate 800 rpm.

Volumetric citric acid production rate (Q_{CA}) was in a narrow range from 0.93 (0.6 vvm) for the Wratislavia 1.31 strain to 1.15 g/L-h (0.48 vvm), and for the Wratislavia AWG7 strain was from 0.76 (0.18 vvm) to 1.07 g/L-h (0.36 vvm) (Table 2). In comparison, in the studies carried out by Papanikolaou et al.,[34] the values of this parameter were from 0.07 to 0.13 g/L-h, depending on the initial concentration of industrial glycerol. Similarly, a high volumetric citric acid production rate of 0.8–1.15 g/L-h was obtained in batch cultures with sunflower oil,[3] glucose,[37] ethanol,[36] and glycerol.[26] The results of Q_{CA} obtained in our study were significantly higher than those obtained by Förster et al.,[25] who obtained 0.4–0.73 g/L-h in cultures of *Y. lipolytica* H222-S4 (p67ICL1) T5 grown in the media containing sucrose and 0.04–0.09 g/L-h in fed-batch cultures of *C. lipolytica* NRRL-Y-1095 strains with n-paraffins.[13]

TABLE 2 Effect of Aeration Rate on Citric Acid Yield (Y_{CA}), Volumetric Citric Acid Production Rate (Q_{CA}) and Specific Citric Acid Production Rate (q_{CA}) during Batch Process with *Y. lipolytica* Strains

Aeration Rate (vvm)	*Y. lipolytica* Wratislavia 1.31			*Y. lipolytica* Wratislavia AWG7		
	Y_{CA} (g/g)	Q_{CA} (g/L-h)	q_{CA} (g/g-h)	Y_{CA} (g/g)	Q_{CA} (g/L-h)	q_{CA} (g/g-h)
0.18	0.45	0.95	0.055	0.44	0.76	0.040
0.24	0.63	1.00	0.052	0.39	0.82	0.050
0.36	0.47	1.12	0.062	0.52	1.07	0.065
0.48	0.53	1.15	0.071	0.48	1.02	0.062
0.60	0.60	0.93	0.049	0.49	1.03	0.055

Note. Culture conditions: agitation rate 800 rpm.

Specific production rate of citric acid (q_{CA}), depending on aeration rate, was from 0.049 (0.6 vvm) to 0.071 g/g-h (0.48 vvm) for the Wratislavia 1.31 strain, and from 0.04 (0.18 vvm) to 0.065 g/g-h (0.36 vvm) for the Wratislavia AWG7 strain (Table 2). These values were comparable to those reported by Kamzolova et al.[26] for *Y. lipolytica* N15 grown on glycerol media, but lower than those obtained with other carbon sources.[5,25]

As can be seen in Figure 5, the oxygen requirement is higher during the growth phase. The DO initially decreased rapidly, but with elapsed time, it started to rise. When the Wratislavia 1.31 strain was used, the concentration of oxygen in the media started to grow after 20 h, while the time at which an increase in the DO was observed with Wratislavia AWG7 varied. At aeration rates from 0.36 to 0.6 vvm, the DO in the media was found within a range from 50 to 80%. However, as can be seen in Figure 5, at a constant agitation rate (800 rpm), an increase in the aeration rate higher than 0.36 vvm had no effect either on the DO or citric acid yield (Table 2). At the highest aeration rate (0.6 vvm) in spite of a high concentration of dissolved oxygen in the media, volumetric and specific citric acid production rate was decreasing. Moresi et al.[38] reported that at high agitation (1000 rpm) and aeration (0.43 vvm) rates, citric acid production by *Candida lipolytica* Cooper and *C. lipolytica* ATCC 8661 strains was inhibited. Different optimum aeration rates from 0.6 to 1.3 vvm have been reported to enhance citric acid production. Optimum air saturation higher than 50% were reported in literature for discontinuous citric acid production by yeasts.[23,31,39]

Perhaps the good results of citric acid biosynthesis could be achieved using a lower (than 0.36 vvm) aeration but higher (than 800 rpm) agitation rate. It could also be interesting to conduct the culture at a constant

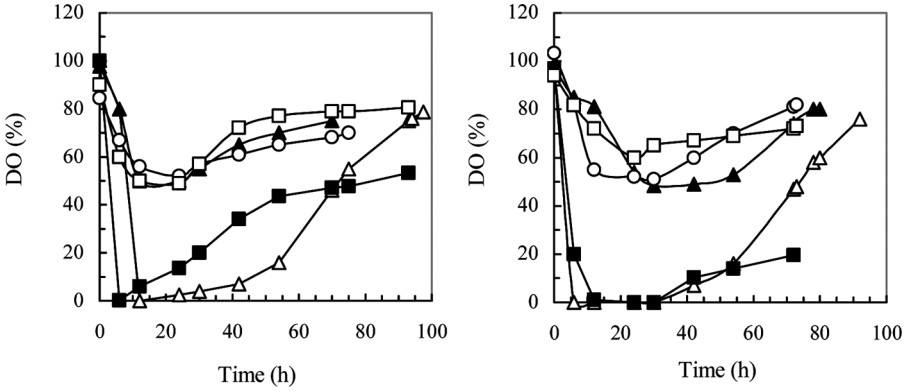

FIGURE 5 Dissolved oxygen concentration (DO) during batch process with *Y. lipolytica* Wratislavia 1.31 (A) and *Y. lipolytica* Wratislavia AWG7 (B) strain at various aeration rates: 0.18 (Δ), 0.24 (■), 0.36 (▲), 0.48 (○), and 0.6 vvm (□). Culture conditions: agitation rate 800 rpm.

TABLE 3 Percentage of Isocitric Acid in Total Amount of Citric Acids, Depending on Agitation Rate and Aeration Rate in Batch Culture on Glycerol

Isocitric acid (%)			Isocitric acid (%)		
Agitation Rate (rpm)	*Y. lipolytica* Wratislavia 1.31	*Y. lipolytica* Wratislavia AWG7	Aeration Rate (vvm)	*Y. lipolytica* Wratislavia 1.31	*Y. lipolytica* Wratislavia AWG7
400	—	—	0.18	6.05%	10%
500	10.76%	12%	0.24	3.93%	10.6%
600	5.66%	3.6%	0.36	5.8%	3.5%
800	5.8%	3.5%	0.48	2.6%	6.56%
900	5.19%	5.7%	0.6	2.27%	5.8%

concentration of DO in the media (50–80%), by applying an alternating aeration and variable agitation rate.

The strains under investigation produced isocitric acid in small amounts (from 0.9 to 7.3 g/L) (Table 3). The ratio of citric acid to isocitric acid with *Y. lipolytica* mainly depended on the substrate and the strain used;[40] moreover, the product selectivity is also strongly influenced by the air saturation.[24] The amount of isocitric acid produced by the two strains used in the present study was the highest when the aeration and agitation rates were the lowest. The lowest percentage of isocitric acid in the total amount of acids (3.48% for Wratislavia AWG7 and 2.27% for Wratislavia 1.31) was obtained at high aeration rates of 0.36 and 0.6 vvm, respectively (Table 3). Based on the results of our studies as well as the data in literature, it can be stated that the amount of citric acid increased with an increasing DO, whereas the isocitric acid concentration decreased at the same time.[23] The ability of *Y. lipolytica* yeast to produce citric and isocitric acid is associated with very high activity of citrate synthase compared with relatively low activities of other enzymes of the tricarboxylic (TCA) cycle.[41] During yeast growth on substrates such as *n*-alkanes, ethanol, and glycerol the glyoxylate pathway is also active. Probably, low DO in the medium leads to an increase in the activity of aconitate hydratase with a simultaneous decrease in the activity of isocitrate lyase and isocitrate dehydrogenase, which is associated with the excretion of isocitrate.[41]

CONCLUSIONS

The operating conditions for the conversion of glycerol to citric acid by the acetate-negative mutants of *Y. lipolytica* Wratislavia 1.31 and Wratislavia AWG7 were as follows: agitation rates of 800–900 rpm and aeration rates of 0.24–0.36 vvm. In these conditions the DO was maintained within the range from 50 to 80%; it can be concluded that the air saturation seems to be the real determining factor, rather than varying agitation or aeration rate. The

results obtained in the present study confirm the importance of DO on biomass, citric acid production, and the ratio of citric acid to isocitric acid on glycerol media.

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