J Ind Microbiol Biotechnol (2010) 37:431–435
DOI 10.1007/s10295-009-0687-8

ORIGINAL PAPER

High-yield production of citric acid by *Yarrowia lipolytica* on glycerol in repeated-batch bioreactors

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Received: 3 November 2009 / Accepted: 14 December 2009 / Published online: 3 January 2010

Abstract An acetate negative mutant of *Yarrowia lipolytica* Wratislavia AWG7 was found to be suitable for the production of high amounts of citric acid in long-term repeated-batch cultures. When 40% of fresh replaced medium was fed, this strain produced 154 g l^{-1}, on average, which corresponded to a 0.78 g g^{-1} yield and a productivity of 1.05 g l^{-1} h^{-1}. The activity of the culture remained stable for more than 1,650 h, i.e., 16 cycles of the repeated-batch bioreactors.

Keywords Citric acid · Crude glycerol · Repeated-batch culture · *Yarrowia lipolytica*

Introduction

Citric acid is an important microbial product used in a wide variety of applications. Due to its low toxicity, citric acid is used as an acidulant in pharmaceutical and food industries. Global citric acid production has reached 1.4 million tons, with an annual increase of 3.5–4.0% [1]. Currently, it is most commonly used worldwide for microbial production of citric acid. The strain of *Aspergillus niger* utilizes carbohydrates in submerged fermentations. Because of the ever-increasing demand for citric acid, alternative fermentation processes using high-yield yeast strains of *Yarrowia lipolytica* and several *Candida* species could be preferred for its production [2, 4, 5, 12, 20]. According to Anastassiadis et al. [1], citric acid production by yeasts in semi-continuous and continuous processes reached up to 250 g citric acid l^{-1}. Therefore, the processes utilizing yeasts may replace traditional discontinuous fungi processes. The data in the literature show that citric acid fermentation by yeasts is primarily based on batch cultures, focused mainly on utilization of glucose, ethanol, plant oil, *n*-paraffins and sucrose by wild strains as well as mutants and recombinant constructed strains of *Y. lipolytica* [4, 6, 9]. As compared with traditional batch operations, the repeated-batch mode often makes a fermentation process more efficient. It has been successfully employed for the production of ethanol [18], lactic [21] and itaconic acid [10]. Repeated-batch operations for the production of citric acid by yeasts using glucose [2, 12] and ethanol [3] have increasingly received research interest in the last years; however, the yields achieved were not high enough for a competitive economic industrial operation. Citric acid production by yeasts has been known for many years, whereas the production with the use of glycerol or raw glycerol from biodiesel plants has not yet been studied extensively. The available reports are mainly focused on batch and fed batch processes [8, 11, 13–17]. We have recently reported that the acetate negative mutants of *Y. lipolytica* Wratislavia AWG7 and *Y. lipolytica* Wratislavia 1.31 show the ability to produce large amounts of citric acid (up to 134 g citric acid l^{-1}) in batch cultures containing glycerol [16]. Cost efficiency in citric acid production can be achieved by using low-cost carbon substrates, such as crude glycerol and repeated-batch (RB) cultures.

In the present study, we compared citric acid production by acetate mutants of *Y. lipolytica* grown in crude glycerol medium in RB. The quantitative effects of the replaced

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medium added to the bioreactor on citric acid production, productivity, yield and stability of RB cultures were studied.

Materials and methods

Microorganisms and media

Two acetate negative mutants of *Y. lipolytica*, Wratislavia AWG7 and Wratislavia 1.31, were used in this study. They belonged to the Wroclaw University of Environmental and Life Sciences, Poland. The growth medium for inoculum preparation contained: 50 g glycerol l^{-1}, 3 g yeast extract l^{-1}, 3 g malt extract l^{-1} and 5 g bactopepton l^{-1}. For the first 132 h, citric acid production was conducted in a medium consisting of 100 g glycerol l^{-1}, 3 g NH_4Cl l^{-1}, 1 g $MgSO_4 \times 7H_2O$ l^{-1}, 0.2 g KH_2PO_4 l^{-1} and 1 g yeast extract l^{-1}. After 48 h of cultivation, crude glycerol solution (92% wt/wt) was added (at a constant feeding rate of 1.4 g h^{-1}) until a total concentration of 200 g glycerol l^{-1} was obtained. After utilization of the glycerol by yeasts in a fed-batch culture, a portion of the culture liquid (1, 0.8, 0.6 or 0.4 l) was withdrawn, and the same volume of the replaced production medium was added. This procedure was repeated four times for each volume. The replaced medium contained: 200, 250, 333.3 or 500 g glycerol l^{-1} (when 1, 0.8, 0.6 or 0.4 l of the replaced medium added), 4 g NH_4Cl l^{-1}, 1 g $MgSO_4 \times 7H_2O$ l^{-1}, 0.2 g KH_2PO_4 l^{-1} and 1 g yeast extract l^{-1}. The volume of culture broth at the start of each cycle of repeated-batch culture was 2 l, and the concentration of glycerol was 100 g l^{-1}. In repeated-batch culture, the end of each cycle was determined when the concentration of glycerol was below 0.5 g l^{-1} and an appropriate volume of replaced medium was added.

The carbon source used was a raw glycerol from a biodiesel (fatty acid methyl esters) production unit [BDK Biodiesel GmbH, Kyritz, Germany; glycerol content, 92% (wt/wt)]. The impurities in the industrial 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)].

Culture conditions

A seed culture was grown in a 300-ml flask (containing 50 ml of growth medium) on a shaker at 30°C for 3 days. An inoculum of 200 ml was introduced into the fermenter containing 1.8 l of the production medium. Fed-batch and all repeated-batch cultures were performed in a 5-l jar fermenter (Biostat B Plus, Sartorius, Germany) with a working volume of 2 l at 30°C. The aeration rate was fixed at 0.6 l min^{-1}. The stirrer speed was adjusted to 800 rpm, and the dissolved oxygen concentration was maintained at 25 ± 5% saturation. The pH was maintained automatically at 5.5 by the addition of NaOH (20% w/v). To account for dilution, due to NaOH solution addition for maintaining the stable pH of the medium, total amounts of citric acid, biomass and polyols in the culture liquid were used for calculations.

Analytical methods

The biomass was determined gravimetrically after drying in a drier at 105°C. The concentrations of citric acid, glycerol, erythritol and mannitol were measured by HPLC using an Aminex HPX87H Organic Acid column coupled to a UV detector at 210 nm and a CORONA detector. The column was eluted with 15 mM of trifluoroacetic acid at 65°C and a flow rate of 0.6 ml min^{-1}. Protein content was determined by the method of Stewart [19], using bovine serum albumin as standard. Isocitric acid was identified using enzymatic methods as described by Goldberg and Ellis [7]. The stability of ace^- mutants (reversion frequency of phenotype ace^-) was determined at the end of each RBC cycle using a plate method on YNB agar containing sodium acetate as the sole carbon source.

Results and discussion

The results obtained in our recent investigations show that acetate negative mutants of *Y. lipolytica* Wratislavia AWG7 and *Y. lipolytica* Wratislavia 1.31 are suitable for the production of high amounts of citric acid from crude glycerol in batch and fed-batch cultivation [16, 17]. In the present study, these strains were examined for citric acid production from crude glycerol in repeated-batch cultures. In these experiments, the quantitative effects of fresh medium added during citric acid production by these strains were examined. The RB portions, which accounted for 50, 40, 30 and 20% of the culture broth, were withdrawn, and the same volume of the fresh medium was added. Subsequent batch cultures were conducted in a similar manner. Each RB culture was repeated four times.

As can be seen in Fig. 1a and b, the RB mode was run for 1,350 h (56 days) and 1,680 h (69 days) with the Wratislavia 1.31 and Wratislavia AWG7 strains, respectively. We found that 16 repeated-batch experiments with the two strains were carried out with no technological and microbiological stability problems. This proves stability and feasibility of the new fermentation processes for citric acid production. Arzumanov et al. [3] reported that a mutant of *Y.lipolytica* VKM Y-2373 was active for more than 700 h during citric acid production from ethanol in

RB cultivation. A 50% feed every 3-day mode was selected as it had the highest citric acid concentration (105 g citric acid l^{-1}) and product yield (88.3%). As can be seen in Figs. 1 and 2 and Tables 1 and 2, the average final citric acid concentration, the productivity and the yield of citric acid as well as the concentration of by-products were dependent on both the strain used and the amount of the replaced medium fed. A decrease in the amount of replaced medium from 50 to 20% resulted in a significant increase in citric acid concentration and an increased content of erythritol (undesired product in this process). The Wratislavia AWG7 strain seemed to be the most suitable producer of high-yield citric acid production in long-term RB systems. The average concentration of citric acid achieved with this strain ranged from 119 to 197 g l^{-1}, when the amount of the replaced medium was decreased from 50 to 20%. In these conditions, the Wratislavia 1.31 strain produced from 119 to 176 g citric acid l^{-1}. RB cultivations of the two strains gave the highest citric acid production at 197 and 176 g l^{-1} when 20% of the replaced medium was fed. These values were higher compared to those reported for batch, fed-batch and repeated-batch fermentations by various yeasts using glycerol and ethanol [3, 14, 16, 17] and comparable when glucose was used as the sole substrate [2, 12]. Today, on an industrial scale, 150–180 g citric acid l^{-1} is produced in traditional batch or fed-batch fermentations, with the use of *A. niger* for over 6–10 days of cultivation [5]. The fermentation characteristics of the two mutants of *Y. lipolytica* grown in RB cultures are summarized in Table 2. The key fermentation parameters, the final average volumetric citric acid production rate (Q_{CA}) and citric acid yields (Y_{CA}) during each cycle of RB fermentation processes depended on the yeast strains used and the amount of replaced medium added, and ranged from 0.44 to 1.05 g l^{-1} h^{-1} and from 0.62 to 0.78 g g^{-1} for the Wratislavia AWG7 and from 0.62 to 0.85 g l^{-1} h^{-1} and from 0.56 to 0.64 g g^{-1} for the Wratislavia 1.31 strain, respectively. The highest average volumetric citric acid production rate (1.05 g l^{-1} h^{-1}) and the yield (0.78 g g^{-1}) were determined for the Wratislavia AWG7 strain in an RB system, where 40% (0.8 l) of the culture broth was withdrawn and the same volume of fresh medium was fed (Table 2). In the present study, no control experiment with pure glycerol was carried out. However, the results of our earlier studies showed that the Wratislavia AWG7 strain in the medium containing pure or crude glycerol produced similar amounts of citric acid in fed-batch fermentations (139 and 131.5 g l^{-1}, respectively). The citric acid productivity (1.05 g l^{-1}) and yield (0.66 g g^{-1}) obtained with crude glycerol were slightly lower than those observed with pure glycerol (1.16 and 0.66 g g^{-1}) [17]. For any fermentation processes based on waste substrates, it is advantageous when the producing microorganism shows little sensitivity to impurities in the substrate.

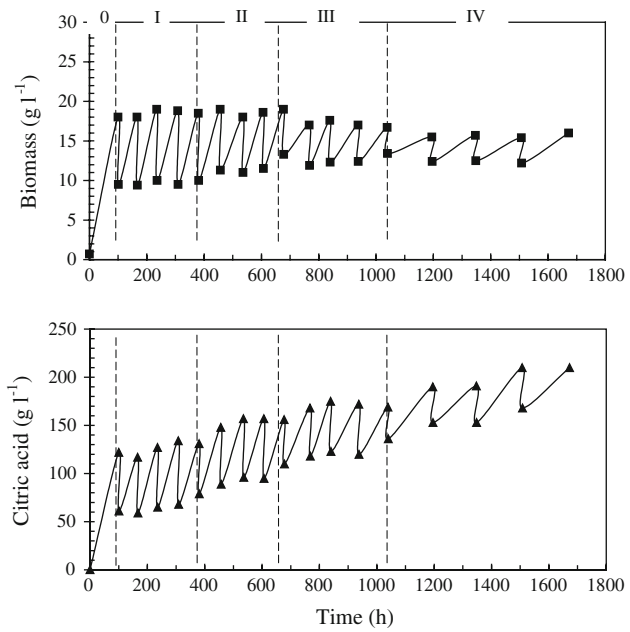

Fig. 1 Effect of the amount of replaced medium on the biomass and citric acid production from crude glycerol by the *Y. lipolytica* Wratislavia AWG7 strain during RBC mode. 0, batch culture; I, 50%; II, 40%; III, 30%; IV, 20% medium was replaced. Each variant of RB cultures was repeated four times

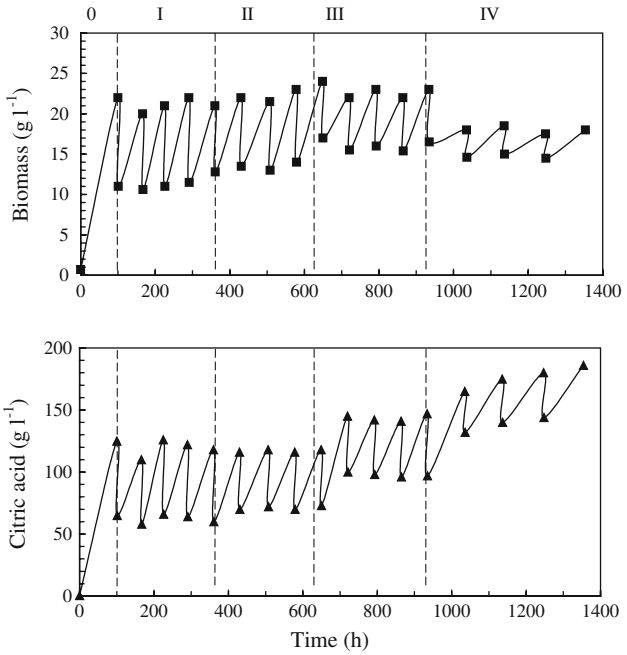

Fig. 2 Effect of the amount of replaced medium on the biomass and citric acid production from crude glycerol by the *Y. lipolytica* Wratislavia 1.31 strain during RBC mode. 0, batch culture; I, 50%; II, 40%; III, 30%; IV, 20% medium was replaced. Each variant of RB cultures was repeated four times

Table 1 Effect of the amount of replaced medium on biomass, citric acid, products and intracellular protein concentrations, in repeated-batch cultures by various *Yarrowia lipolytica* strains grown in a medium containing 100 g glycerol l^{-1} at pH 5.5

RBC cultivation (l) (%)		Biomass (g l^{-1})	Citric acid (g l^{-1})	Mannitol (g l^{-1})	Erythritol (g l^{-1})	Isocitric acid (g l^{-1})	Intracellular protein (%)
Yarrowia lipolytica Wratislavia AWG7							
1.0	50	18.4 ± 0.62	119 ± 15.1	3.5 ± 1.55	2.8 ± 4.4	5.2 ± 0.6	28 ± 1.5
0.8	40	18.5 ± 0.71	154 ± 4.4	0.9 ± 0.26	6.4 ± 2.9	6.5 ± 0.7	25 ± 0.5
0.6	30	16.9 ± 0.66	170 ± 3.1	1.2 ± 0.39	6.8 ± 2.6	6.4 ± 0.5	23 ± 0.8
0.4	20	15.3 ± 0.54	197 ± 15	2.1 ± 0.57	15.5 ± 2.3	5.5 ± 0.4	22 ± 0.7
Yarrowia lipolytica Wratislavia 1.31							
1.0	50	22.0 ± 0.82	119 ± 6.8	1.5 ± 0.66	0.5 ± 0.66	5.9 ± 0.6	27 ± 0.5
0.8	40	22.6 ± 1.1	125 ± 1.2	1.6 ± 0.67	0.8 ± 0.75	5.4 ± 0.4	28 ± 0.3
0.6	30	22.4 ± 0.75	136 ± 2.7	1.1 ± 0.59	1.2 ± 0.95	6.1 ± 0.3	29 ± 1.1
0.4	20	17.6 ± 0.48	176 ± 9.0	1.7 ± 0.74	18.7 ± 5.4	5.2 ± 0.6	31 ± 0.8

Table 2 Effect of the amount of replaced medium on volumetric citric acid production rate (Q_{CA}) and citric acid yield (Y_{CA}) in repeated-batch cultures by various *Yarrowia lipolytica* strains grown in a medium containing 100 g glycerol l^{-1} at pH 5.5

Y. lipolytica strain	Parameters	RBC cultivation mode (%)			
		20	30	40	50
Wratislavia AWG7	Q_{CA} (g l^{-1} h^{-1})	0.44 ± 0.04	0.74 ± 0.07	1.05 ± 0.06	0.89 ± 0.03
	Y_{CA} (g g^{-1})	0.68 ± 0.05	0.71 ± 0.08	0.78 ± 0.03	0.62 ± 0.06
Wratislavia 1.31	Q_{CA} (g l^{-1} h^{-1})	0.62 ± 0.08	0.81 ± 0.09	0.79 ± 0.06	0.85 ± 0.06
	Y_{CA} (g g^{-1})	0.64 ± 0.07	0.57 ± 0.05	0.56 ± 0.05	0.56 ± 0.07

In the case of the Wratislavia 1.31 strain, the maximum average volumetric citric acid production rate (0.79–0.85 g dm^{-3} h^{-1}) and citric acid yield (0.56–0.57 g g^{-1}) were achieved within a wider range of the replaced medium (from 30 to 50%).

In the medium containing glycerol, both strains produced various by-products such as isocitric acid, erythritol and mannitol. Their concentrations depended on culture conditions. The highest amounts of erythritol (15.5 and 18.7 g l^{-1}, respectively) were determined in RB cultures with the Wratislavia AWG7 and Wratislavia 1.31 strains when 20% culture broth was replaced. The concentration of isocitric acid produced by the two strains ranged from 5.2 to 6.5 g l^{-1}. The citric acid to isocitric acid ratio produced by Wratislavia AWG7 ranged from 20.2 to 35.8 and was comparable with that obtained with the Wratislavia 1.31 strain.

As can be seen in Table 1, the amount of replaced medium strongly influenced the concentration of biomass. A decrease in the amount of the replaced medium decreased the biomass level by about 17% with Wratislavia AWG7 and 22% with Wratislavia 1.31. Table 1 shows that the intracellular protein content in the biomass of Wratislavia AWG7 is a little lower as compared to that of Wratislavia 1.31. Average concentrations of intracellular protein in the biomass ranged from 22 to 31%, and a slightly smaller concentration was found in Wartislavia AWG7, which proved to be a better producer of citric acid in the system under investigation. During cultivation of this strain, the intracellular protein concentration decreased from 28 to 22%. According to Moresi et al. [12], one of the indicators of intense overproduction of citric acid by yeast was a decrease in cellular protein content to 17–24%.

The results obtained in this study clearly indicate that the production of citric acid from raw glycerol by the newly selected strain of *Y. lipolytica* Wratislavia AWG7 in RB bioreactors is superior to citric acid production by other strains in several aspects. Firstly, we achieved a high citric acid concentration (154 g citric acid l^{-1}) and yield (0.78 g g^{-1}) when 40% of the culture broth was replaced. Secondly, under these conditions, our strain synthesized isocitric acid, mannitol and erythritol in small quantities (the compounds being by-products in this process). Additionally, long-term stability of the activity of the culture was observed.

In conclusion, the results obtained in the proposed RB cultivation with the use of crude glycerol confirm that it can be used as a carbon source for citric acid production in industrial applications.

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