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Citric Acid Production by *Yarrowia lipolytica* Yeast on Different Renewable Raw Materials

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Abstract: The world market of citric acid (CA) is one of the largest and fastest growing markets in the biotechnological industry. Microbiological processes for CA production have usually used the mycelial fungi *Aspergillus niger* as a producer and molasses as a carbon source. In this paper, we propose methods for CA production from renewable carbon substrates (rapeseed oil, glucose, glycerol, ethanol, glycerol-containing waste of biodiesel industry and glucose-containing aspen waste) by the mutant strain *Yarrowia lipolytica* NG40/UV5. It was revealed that *Y. lipolytica* grew and synthesized CA using all tested raw materials. The obtained results are sufficient for industrial use of most of the raw materials studied for CA production. Using rapeseed oil, ethanol and raw glycerol (which is an important feedstock of biodiesel production), a high CA production (100–140 g L^{-1}) was achieved.

Keywords: yeast *Yarrowia lipolytica*; citric acid (CA) production; raw materials

1. Introduction

Citric acid (CA) and its salts are widely used as an acidulate, flavoring agent and antioxidant in the production of beverages and confectionery, in infant formula, as well as in the chemical, pharmaceutical, electronic, defense, and other industries. The volume of citric acid globally exceeds two million tons per year and its production is annually increased by 5% [1].

Modern technologies of citric acid (CA) production are based on using various mutant strains of the mycelial fungi *Aspergillus niger* as a producer and molasses as a raw material. CA production by fungi is a complicated and environmentally unsafe process; as a result of its implementation, a large number of both liquid effluents containing mineral acids, ballast organic substances, cyanides, and solid wastes, primarily gypsum, are accumulated. Moreover, *A. niger* is an opportunistic pathogenic fungi and can cause allergic diseases and aspergillosis [2,3].

Over the past 40 years, the interest of researchers has focused on yeast as a producer of CA; the yeast *Yarrowia lipolytica* has been the most used CA producer [1–3]. Initially, this kind of yeast attracted the attention of researchers due to its ability to grow and synthesize CA in media with n alkanes—an available and cheap substrate [2]. However, due to changes in the world oil market, the use of this substrate has become economically unprofitable. In this regard, it is of interest to use other types of raw materials.

The choice of raw materials for developing CA biotechnology is determined by factors such as renewability, ability of the producer to assimilate the substrate with a high conversion rate, consumption value and cost price of the target product. To carry out fermentation processes, in addition to very expensive food raw materials, such as glucose [4–7] and plant oils [8–10], much cheaper substrates which are waste products of various industries, such as glycerol-containing waste of the biodiesel industry [9–17], glucose-containing wood hydrolysates [18,19], olive mill waste-water [20],

and inulin [21] are used. Ethanol, a water-soluble individual compound which ensures the formation of a pure product and facilitates the isolation process, is also of great importance [22,23]. In the works of the above-mentioned researchers it was shown that all these substrates are promising for CA production and the use of glycerol-containing waste of biodiesel industry and glucose-containing wood hydrolyzates can increase the profitability of CA production process.

The aim of this work was a comparative study of CA production by the yeast *Y. lipolytica* on different types of renewable raw materials.

2. Materials and Methods

2.1. Microorganisms and Chemicals

The mutant strain *Y. lipolytica* NG40/UV5 was obtained as described previously [9,24].

Chemicals, the manufacturer of the carbon sources, and their characteristics were presented in our published articles [7,9,15,17].

2.2. Media and Cultivation Conditions

All experiments were done using the same equipment, nutrient medium and cultivation conditions. Strain *Y. lipolytica* NG40/UV5 was cultivated in a 10-L ANKUM-2M fermenter (Pushchino, Russia) with an initial volume of 5 L. The medium contained (g L^{-1}): carbon source: $(NH_4)_2SO_4$, 6; $MgSO_4 \cdot 7H_2O$, 1.4; NaCl, 0.5; $Ca(NO_3)_2$, 0.8; KH_2PO_4, 2.0; K_2HPO_4, 0.2; Difco yeast extract (BD Diagnostic Systems, Sparks, MD, USA), 1.0; trace elements (mg L^{-1}): $FeSO_4 \times 7H_2O$, 14.9; $MnSO_4 \times 4H_2O$, 0.2; $ZnSO_4 \times 7H_2O$, 8.1; $CuSO_4 \times 5H_2O$, 3.9. The fermentation conditions were maintained automatically at a constant level: Temperature 28−0.5 °C; pH 4.5.0−0.1; pO_2 50% (of air saturation); agitation rate of 800 rpm. Pulsed addition of carbon source (by 2–20 g L^{-1}) depending on the carbon source used was performed as the pO_2 value changed by 10%. Cultivation was continued for 6 days.

2.3. Assays

Biomass, concentration of CA, isocitric acid (ICA) and other organic acids were determined as described previously [7].

2.4. Calculations

Earlier, it was found that the mass yield of CA production (Y_{CA}), expressed in g of CA per g of carbon source, and the fermenter productivity, expressed in g $(L \cdot h)^{-1}$ were influenced by the medium dilution due to the addition of NaOH solution for maintaining a constant pH value [9,17]. In this regard, the total amount of CA in the culture broth at the end of the fermentation was used to calculate Y_{CA} and fermenter productivity. Formulas for calculation of Y_{CA} value and fermenter productivity were described earlier [9,17].

All the data presented are the mean values of three experiments and two measurements for each experiment; standard deviations were calculated (S.D. < 10%).

3. Results and Discussion

The dynamics of nitrogen consumption and the accumulation of biomass and CA by *Y. lipolytica* NG40/UV5 grown on rapeseed oil are shown in Figure 1a, while the logarithmic growth curve (μ) and the specific rate of biosynthesis of CA (q_p) are shown in Figure 1b. As it can be seen in the latter figure, the growth curve had an exponential phase (phase I) lasting for 12 h, growth retardation phase (phase II) lasting from 12 to 36 h of cultivation, and stationary phase (phase III) lasting from 36 h to the end of cultivation. The retardation of growth coincided with the exhaustion of nitrogen from the medium. The specific growth rate attained a maximum (μ_{max} = 0.360 h^{-1}) in the exponential growth phase (12 h of cultivation). This value of μmax was more than two times higher than that of the other CA-producing strain *Y. lipolytica* (0.17–0.22 h^{-1}) [4,8]. After 12 h of cultivation, μ gradually decreased

to zero after 48 h of cultivation. The excretion of CA did not occur in the exponential growth phase but became active in the growth retardation and stationary phases. Within this cultivation period, the specific rate of CA production (q_p) was between 0.065 and 0.104 g CA/g·h. At the end of cultivation (144 h), the strain produced 140 g L^{-1} CA and 5.3 g L^{-1} ICA (data not shown) with CA:ICA ratio of 26.4:1. The CA production yield Y_{CA} was 1.5 g g^{-1}; the fermenter productivity was calculated to be 1.46 g $(L{\cdot}h)^{-1}$ with account for the dilution factor.

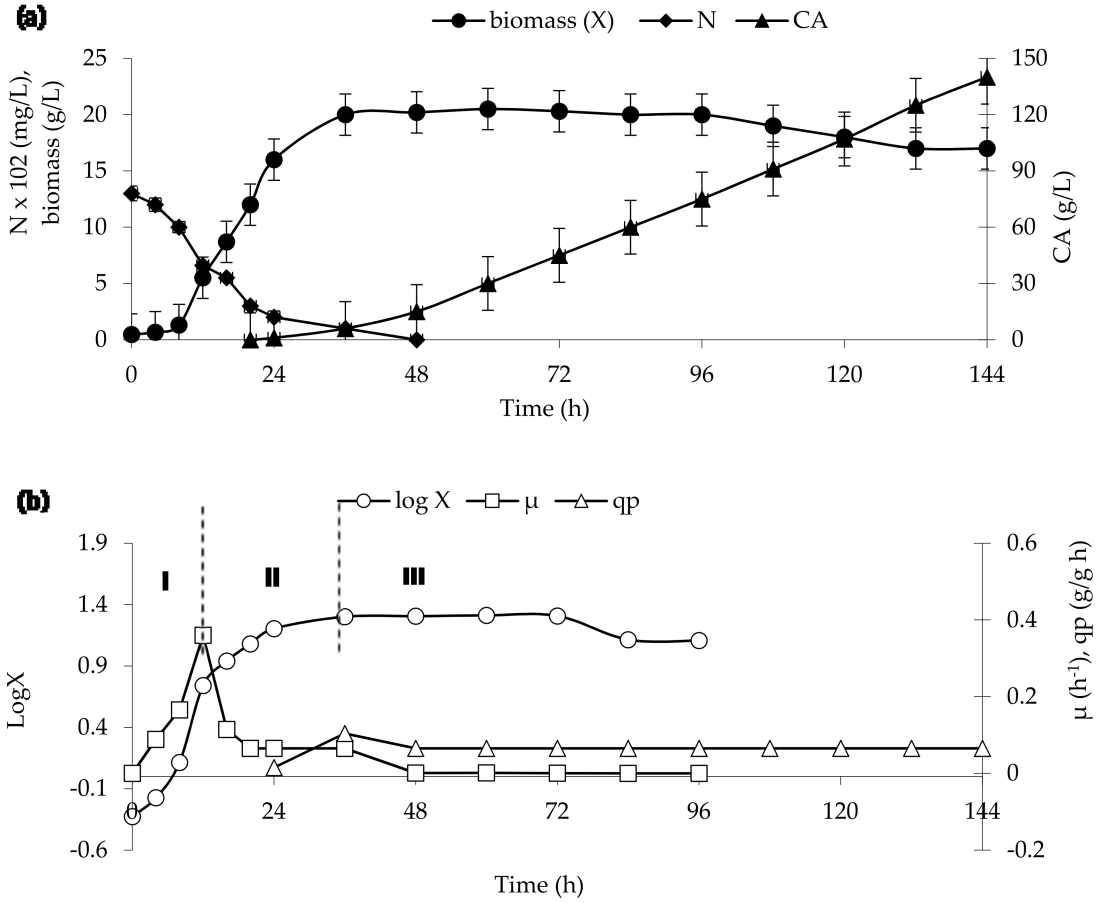

Figure 1. Time courses of nitrogen consumption, biomass accumulation, and citric acid production in *Y. lipolytica* grown on rapeseed oil (**a**) and calculated parameters of the process (**b**): I—the exponential cell growth; II—the cell growth retardation; III—the stationary phase.

The data of the accumulation of biomass and CA by *Y. lipolytica* NG40/UV5 grown on other substrates compared to rapeseed oil are shown in Table 1.

Table 1. Citric acid production by *Y. lipolytica* on various carbon sources.

Substrates	Biomass (g L^{-1})	CA (g L^{-1})	ICA (g L^{-1})	CA:ICA	Productivity (g $(L{\cdot}h)^{-1}$)	Y_{CA} (g g^{-1})
Rapeseed oil	17.0 ± 1.1	140.0 ± 5.0	5.3 ± 0.8	26.2:1	1.46	1.50
Glucose	18.7 ± 1.3	100.8 ± 9.2	4.9 ± 0.9	20.6:1	1.05	0.80
Glucose-containing aspen waste	5.6 ± 0.8	31.2 ± 2.1	7.84 ± 0.9	4:1	0.325	0.50
Glycerol	16.8 ± 1.1	87 ± 6.4	13 ± 1.1	6.7:1	0.906	0.64
Glycerol waste of biodiesel industry	20.0 ± 1.8	100 ± 3.4	15 ± 1.2	7.7:1	1.04	0.90
Ethanol	15.3 ± 1.4	106.7 ± 2.7	15 ± 1.4	7.1:1	1.32	0.87

As seen in Table 1, at the end of cultivation (144 h), *Y. lipolytica* NG40/UV5 produced 100.8 g L^{-1} CA and 4.9 g L^{-1} ICA with CA:ICA ratio of 20.6:1 in the medium containing glucose. The CA production yield Y_{CA} was 0.80 g g^{-1}; the fermenter productivity was calculated to be 1.05 g $(L{\cdot}h)^{-1}$.

As seen in Table 1, *Y. lipolytica* NG40/UV5 only produced 31.2 g L^{-1} CA and 7.84 g L^{-1} ICA with CA:ICA ratio of 4:1 in the medium containing glucose-containing aspen waste. The CA production yield Y_{CA} was 0.50 g g^{-1}; the fermenter productivity was calculated to be 0.325 g $(L{\cdot}h)^{-1}$.

As it can be seen from the data in Table 1, the mutant grows perfectly and synthesizes CA both in a medium with pure glycerol and in a medium with biodiesel-derived glycerol. *Y. lipolytica* NG40/UV5 produced 87 g L^{-1} CA with a ratio of CA to ICA of 6.7:1. The application of waste glycerol for *Y. lipolytica* NG40/UV5 cultivation increased CA production by 15% (up to 100 g L^{-1}) compared to that obtained from pure glycerol; the CA to ICA ratio was 7.7:1. The fermenter productivity was high and reached 0.906 and 1.04 (g $(L{\cdot}h)^{-1}$) in the media with pure- and biodiesel-derived glycerol, respectively. The mass yield (Y_{CA}) reached 0.64 and 0.9 g/g in the media with pure- and biodiesel-derived glycerol, respectively.

As seen in Table 1, *Y. lipolytica* NG40/UV5 produced 106.7 g L^{-1} CA and 15 g L^{-1} ICA with CA:ICA ratio of 7.1:1 in the medium containing ethanol. The CA production yield Y_{CA} was 0.87 g g^{-1}; the fermenter productivity was calculated to be 1.32 g/L·h.

Comparative data on the most efficient processes of CA production by yeasts *Y. lipolytica* from various carbohydrate-containing substrates are given in Table 2. As seen in this table, in the experiments with wild and mutant strains, *Y. lipolytica* produced CA in industrially sufficient amounts. For instance, in the mutant strain *Saccharomycopsis lipolytica* NTG9 grown on rapeseed oil, the CA concentration reached 152.3 g L^{-1} with the yield (Y_{CA}) of 1.5 g g^{-1} [25]. Aurich et al. [26] obtained a CA concentration of 198 g L^{-1} with the yield (Y_{CA}) of 1.16 g g^{-1}, which was achieved after a 300 h fed-batch cultivation of the wild strain *Y. lipolytica* H181. The wild strains *Y. lipolytica* H222 and *Y. lipolytica* W29, grown on glucose, produced 41 g L^{-1} [4] and 49 g L^{-1} of CA, respectively [27]. Recently, we found that the wild strain *Y. lipolytica* VKM Y 2373, cultivated in a medium with glucose under cell growth limitation using nitrogen, phosphorus and sulfur, produced CA at a level of 80–85 g L^{-1} with a yield of 0.70–0.75 g g^{-1} [7]. The overexpression of gene *PYR* encoding pyruvate carboxylase in *Y. lipolytica*, resulted in the production of CA at a level of 95–111.1 g L^{-1} with the yield (Y_{CA}) of 0.75–0.93 g g^{-1} [5,6]. Strain *Y. lipolytica* ACA-DS 50109 cultivated on glucose and olive mill wastewaters produced CA (28.9 g L^{-1}) with the product yield (Y_{CA}) of 0.53 g g^{-1} [28]. Later, the authors of the last article improved the process of CA production up to 52.0 g L^{-1} with the product yield (Y_{CA}) of 0.64 g g^{-1} using strain *Y. lipolytica* ACA-YC 5033, which was also able to remove harmful phenolic compounds from olive mill wastewaters [29]. The glycerol-grown yeast *Y. lipolytica* NRRL YB-423 produced 21.6 g L^{-1} of CA with mass yield of 0.54 g g^{-1} [12]; strain *Y. lipolytica* ACA-DC 50109 synthesized 62.5 g L^{-1} of CA with mass yield of 0.56 g g^{-1} from raw glycerol [20], while the recombinant strain *Y. lipolytica* NCYC3825 was able to produce 58 g L^{-1} of CA [30]. Earlier, we indicated that the other mutant *Y. lipolytica* NG40/UV7 synthesized, CA (122.2 g L^{-1}) with the yield of 0.95 g g^{-1}. The high CA production (up to 140 g L^{-1}) has also been reported for acetate-negative mutant *Y. lipolytica* Wratislavia AWG7, grown on crude glycerol [13] and recombinant strain *Y. lipolytica* H222-S4 (p67ICL1), harboring the invertase encoding *ScSUC2* gene of *Saccharomyces cerevisiae* under inducible XPR2 promoter control and multiple ICL1 copies, cultivated on sucrose [31].

Table 2. Comparative data of the processes of CA production from various substrates using *Y. lipolytica* strains.

Strain	Substrate	Characteristics of Strain	CA ($g\ L^{-1}$)	Y_{CA} ($g\ g^{-1}$)	References
S. lipolytica NTG9	canola oil	mutant/nitrosoguanidine	152.3	1.50	[25]
Y. lipolytica H181	sunflower oil	wild type	198.0	1.16	[26]
Y. lipolytica H222		wild type	41.0	0.55	[4]
Y. lipolytica W29l		wild type	49.0	0.85	[27]
Y. lipolytica VKM Y 2373	glucose	wild type	80–85	0.70–0.75	[7]
Y. lipolytica PG86		*PYC* gene expression	95.0	0.75	[5]
Y. lipolytica PR32		*PYC* gene expression	111.1	0.93	[6]
Y. lipolytica ACA-DS 50109	glucose + olive mill wastewaters	wild type	28.9	0.53	[28]
Y. lipolytica ACA-YC 5033		wild type	52.0	0.64	[29]
Y. lipolytica NRRL YB-423	glycerol	wild type	21.6	0.54	[12]
Y. lipolytica ACA-DC 50109	raw glycerol	wild type	62.5	0.56	[20]
Y. lipolytica NCYC 3825	raw glycerol	multigene expression	58.8	0.17	[30]
Y. lipolytica NG40/UV7	raw glycerol	mutant/nitrosoguanidine/UV	122.2	0.95	[17]
Y. lipolytica Wratislavia AWG7	raw glycerol	mutant/acetate^{-}	139.0	0.70	[13]
Y.lipolytica H222-S4 (p67ICL1)	sucrose	ScSUC2/ICL1	127–140	0.75–0.82	[31]
Y. lipolytica XYL+	xylose	*XYL* gene expression	80.0	0.53	[19]
Y. lipolytica Wratislavia K1	inulin	*INU1* gene expression	105.2	0.53	[21]
Y. lipolytica NG40/UV5	rapeseed oil	mutant/nitrosoguanidine/UV	140.0	1.5	Present study

It should be noted that plant raw materials, such as wood, straw, and agricultural products processing waste, are inexpensive, accessible, renewable, and environmentally friendly substrates for microbiological synthesis of practically valuable compounds. However, the effective conversion of these substrates into easily assimilable carbohydrates (glucose, xylose, and higher glucose-containing polymers) is a difficult task. The traditional technologies of hydrolysis of plant raw materials with the use of strong acids and alkalis are associated with the formation of by-products that inhibit the growth of microorganisms and biosynthesis of target substances. Therefore, recombinant producers, effectively assimilating plant raw materials, were developed. For instance, Ledesmo-Amaro et al. (2016) engineered *Y. lipolytica* able to metabolize xylose to produce CA and lipids. The overexpression of xylose reductase, xylitol dehydrogenase and xylulokinase resulted in a production of 80 $g\ L^{-1}$ of CA from xylose by mutant strain *Y. lipolytica* [19].

Rakicka et al. (2016) reported the production from inulin by engineered strain *Y. lipolytica* Wratislavia K1. The overexpression of the *INU1* gene from *Kluyveromyces marxianus* coding inulinase resulted in the effective hydrolysis of inulin by mutant and the production of a high amount of CA (105.2 $g\ L^{-1}$ from 200 $g\ L^{-1}$ inulin) [21].

4. Conclusions

The results of the experiments indicated that the mutant *Y. lipolytica* NG40/UV5 was able to grow and synthesize CA on media containing all types of the investigated renewable raw materials. However, it should be noted that the accumulation of the by-product of fermentation—ICA—was even very high in four of the six substrates investigated (glucose-containing aspen waste, glycerol, glycerol waste of biodiesel industry and ethanol). The best results were obtained using rapeseed oil (140 $g\ L^{-1}$ CA; CA:ICA ratio of 26.2:1; mass yield of CA production (Y_{CA}) of 1.5 $g\ g^{-1}$, and fermenter productivity of 1.46 $g\ (L{\cdot}h)^{-1}$). However, economic considerations may lead to the fact that using a cheaper and less pure substrate (glycerol waste of biodiesel industry; 100 $g\ L^{-1}$ CA; CA:ICA ratio of 7.7:1; mass yield of CA production (Y_{CA}) of 0.9 $g\ g^{-1}$, and fermenter productivity of 1.04 $g\ (L{\cdot}h)^{-1}$) would be preferable on a production scale.

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