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Lipase Secretion and Citric Acid Production in *Yarrowia lipolytica* Yeast Grown on Animal and Vegetable Fat**

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Summary

The aim of the study was to investigate the potentiality of the utilization of raw agro-industrial fat for the biotechnological production of valuable products (lipase and citric acid) by the yeast *Yarrowia (Candida) lipolytica*. Thirty strains of the aforementioned species were investigated for their capability of lipase secretion and citric acid production on media containing animal fat or rapeseed oil as a sole carbon and energy source. Strain *Y. lipolytica* 704, exhibiting the highest lipase activity on rapeseed oil (2760 U/mL), was selected for the study of biochemical peculiarities of cell growth, and strain *Y. lipolytica* 187/1, exhibiting the maximum citric acid synthesis, was selected for the subsequent studies on citric acid production. A relationship between lipase production and residual rapeseed oil concentration was studied. The essential factor for lipase production was found to be the concentration of rapeseed oil in the medium, which should be no less than 5 g/L. Under optimal conditions of cultivation, citric acid production by rapeseed-oil-grown yeast *Yarrowia lipolytica* 187/1 amounted to 135 g/L; specific rate of citric acid production reached m(CA)/m(cell)=127 mg/(g·h); mass yield (Y_{CA}) and energy yield (η_{CA}) were 1.55 and 0.41, respectively.

Key words: citric acid production, lipase, *Yarrowia lipolytica*, rapeseed oil

Introduction

Citric acid (CA), due to its distinctive properties as an acidulent, flavouring agent and antioxidant, is used mainly in food and beverage industry (70 % of the total CA production). In recent years, the consumption of citric acid and its salt, trisodium citrate, has reached 800 000 t with increase of 5 % per year.

CA is an intermediate of tricarboxylic acid cycle (TCA) and holds a key position in the metabolism of each microbial cell. However, under certain conditions of fermentation, fungi, bacteria, and yeasts produce CA in the excessive amounts. Traditionally, the different strains of fungus, mostly belonging to *Aspergillus niger*,

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have been used in the commercial production of CA from molasses, sucrose or glucose (*1,2*). However, the production of CA with the use of fungi is associated with the accumulation of significant amounts of solid and liquid wastes.

Alternatively, there is a great interest in the possibility of CA production by different strains of yeast. Yeasts belonging to species *Yarrowia (syn. Candida, Saccharomycopsis, Endomycopsis) lipolytica*, *C. guillermondii* and *C. oleophila* are known to be able to produce a wide spectrum of organic acids, including TCA cycle intermediates such as CA and isocitric acid (ICA), from a wider range of carbon sources, than fungi, in particular, *n*-alkanes (*3,4*), glucose (*5–10*), raw glycerol (*11*), ethanol (*12*) and galactose (*13*). These types of transformations have been conducted in batch and continuous cultures and the process has been successfully simulated with the aid of numerical models (*7,10,12,14*). Moreover, yeasts are characterized by greater resistance to high substrate concentrations than fungi, with comparable conversion rates and a greater tolerance to metal ions, thus allowing the use of less refined substrates (*15*).

To date, the biochemical pathways involved in the regulation of CA and ICA production by yeasts have been studied. It is known that the excessive production of CA and ICA has generally been observed under the limitation of yeast growth by nitrogen or other mineral components, such as sulphur, phosphorus, and magnesium (*5,7,16*). It was reported that a ratio between accumulated CA and by-product ICA depended on the carbon source used. In glucose-containing medium, CA was preferably accumulated with insignificant production of ICA, while in the medium with *n*-alkanes, equal amounts of CA and ICA were produced.

At present, crude vegetable oils, animal fat and their industrial derivatives (soap stocks, industrial fatty acids) are of great practical interest for various microbial transformations. Various strains of the yeast *Yarrowia (Candida) lipolytica* have been cultivated on fatty materials (triacylglycerols, soap stocks or industrial fatty acids) and single-cell biomass or reserve lipids have been produced (*17–20*). There is less available information concerning CA production by *Yarrowia lipolytica* grown on fatty materials: *Candida lipolytica* (*21*) cultivated on palm oils, *Saccharomycopsis lipolytica* grown on canola oil (*22*) or *Yarrowia lipolytica* cultivated on mixture of industrial fully saturated fatty acids and raw glycerol and characterized by simultaneous production of CA and intracellular lipids with the composition similar to the cocoa butter (*19*).

To date, the biochemical pathways involved in the regulation of CA and ICA production from vegetable oils by yeast have not been studied in depth. The hydrolysis of oils is believed to be primarily catalysed by lipase with formation of glycerol and fatty acids.

This paper deals with the selection of lipase-active strains of *Yarrowia lipolytica*, the study of physiological parameters of growth and CA-producing ability of the selected strains, and the process of CA production in fermentor.

Materials and Methods

Organisms

Thirty yeast strains used in this study were obtained from the Collection of the Laboratory of Aerobic Metabolism of Microorganisms (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia). The cultures were kept on glucose agar at 4 °C.

Chemicals

Animal (beef) fat and rapeseed oil were commercially available and provided by the Association of the Rape Producers and Refiners, Kazan Seed-Oil-Processing Company (Russia). The fatty acid composition of rapeseed oil was (mass fraction/%): C16:0, 4; C18:0, 1.2; C18:1, 58.8; C18:2, 28.1; C18:3, 5.9; with a total unsaturated fatty acid mass fraction of 93.6 %.

Polyvinyl alcohol and other chemicals and enzymes were of the highest purity, commercially available and provided by Sigma Chemical Company (USA) and Boehringer Mannheim (Germany).

Determination of lipase activity and selection of lipase-active strains

Lipase activity of yeast strains was determined by two methods (A and B). According to method A, yeast cells were grown on Petri dishes with agar medium containing (mass concentration, γ/(g/L)): agar 20, glucose 1, peptone 10 and animal fat 10, at 28 °C; lipase activity was determined by measuring turbid zones developed around colonies within 24–72 h of incubation.

According to method B, lipase activity was measured by titrimetric assay according to the protocol described by Zviagintzeva (*23*) with slight modifications.

In order to optimise the lipase assay for *Y. lipolytica* yeasts, the effects of carbon substrate (olive oil, rapeseed oil or sunflower oil), emulsificators (gum arabic and polyvinyl alcohol), temperature and pH on the level of lipase activity were studied (Table 1). *Y. lipolytica* H181 obtained from the Collection of Department of Environmental Biotechnology, UFZ Centre for Environmental Research Leipzig-Halle was used in these experiments.

The substrate emulsion was prepared with carbon source (olive oil or sunflower oil (m/V=40 %) or rapeseed oil (m/V=40 %)) and emulsificator (polyvinyl alcohol (2 %) or gum arabic (50 %)). Variations of the substrate emulsion are presented in Table 1. The solution was emulsified in a Warring blender in order to obtain the dispersion of fat into the aqueous phase. The sample (100–200 μL, pure or diluted) containing cells and cultural medium was added to the substrate emulsion (5 mL) and 50 mM phosphate buffer (4.5 mL) and incubated for 1 h on a shaker (150 rpm) at 30 °C. The effect of pH on lipase activity was studied at a broad range of pH (from 4.5 to 9). The optimal pH for lipase activity was found to be 8. Control sample was not incubated. The reaction was terminated by adding 4 mL of an acetone-ethanol (volume ratio=50:50) mixture.

Lipase activity was determined by titration of the released fatty acids with 50 mM sodium hydroxide (up to final pH=9). The amount of enzyme that catalysed the

release of 1 μmol of fatty acids per hour at 30 °C was taken as the unit of lipase activity. All the data presented are the means of four measurements; standard deviations were calculated.

To select strains exhibiting high lipase activity, yeast strains (listed in Table 2) were grown during 15–18 h in 750-mL flasks with 50 mL of the medium containing (mass concentration, γ/(g/L)): rapeseed oil 20, $(NH_4)_2SO_4$ 5, KH_2PO4 1, K_2HPO_4 0.1, $MgSO_4{\cdot}7H_2O$ 0.7, $Ca(NO_3)_2$ 0.4, NaCl 0.5, thiamine-HCl 0.2 mg/L and trace elements according to Burkholder *et al.* (*24*). Yeast cultivation was performed on a shaker (220 rpm) at 28 °C, while pH was maintained at 4.5–5.5 with 5–10 % NaOH.

Screening of yeast strains for citric acid production

Yeast strains exhibiting high lipase activity (listed in Table 2) were grown under nitrogen-limiting conditions in 750-mL flasks with 50 mL of the medium containing (mass concentration, γ/(g/L)): rapeseed oil 20, $(NH_4)_2SO_4$ 0.3, KH_2PO4 1, K_2HPO_4 0.1, $MgSO_4{\cdot}7H_2O$ 0.7, $Ca(NO_3)_2$ 0.4, NaCl 0.5, thiamine-HCl 0.2 mg/L and trace elements according to Burkholder *et al.* (*24*). Yeasts were cultivated on a shaker (220 rpm) at 28 °C for 6 days, and pH was maintained at 4.5–5.5 with 5–10 % NaOH.

Batch cultivation of Y. lipolytica in a fermentor

In order to investigate the growth characteristics, the selected strain *Y. lipolytica* 704, which exhibited the highest lipase activity, was cultivated in a 3-L ANKUM-2 M fermentor (Russia) with an operating volume of 1.5 L in the medium containing (mass concentration, γ/(g/L)): $(NH_4)_2SO_4$ 5, KH_2PO_4 2, K_2HPO_4 0.2, $MgSO_4{\cdot}7H_2O$ 1.4, $Ca(NO_3)_2$ 0.8, NaCl 1, thiamine 0.5 mg/L and trace elements as described by Burkholder *et al.* (*24*). Rapeseed oil (20 g/L) was used as the sole carbon and energy source. Fermentation parameters were maintained automatically at the constant level: pH=(5.0±0.1) was adjusted with 15 % NaOH, dissolved oxygen concentration was 50 % of air saturation, and temperature was T=(28±0.1) °C. Silicone antifoam 0.5 g/L was added to the medium at the beginning of the incubation.

In order to investigate CA production, the selected strain *Y. lipolytica* 187/1 was cultivated in a 10-L ANKUM-2 M fermentor (Russia) with an operating volume of 6 L in the medium containing (mass concentration, γ/(g/L)): $(NH_4)_2SO_4$ 3, KH_2PO_4 2, K_2HPO_4 0.2, $MgSO_4{\cdot}7H_2O$ 1.4, $Ca(NO_3)_2$ 0.8, NaCl 1, thiamine 0.5 mg/L and trace elements as described by Burkholder *et al.* (*24*). Fermentation parameters were maintained automatically at the constant level: pH=(4.5±0.1) was adjusted with 15–25 % NaOH, dissolved oxygen concentration was 50 % of air saturation during the phase of growth and 25–30 % during the phase of acid-formation, and temperature was T=(28±0.1) °C. Silicone antifoam 0.5 g/L was added into the medium at the beginning of the incubation. Cultivation was performed for 6 days.

Biomass determination and extraction of rapeseed oil from a culture liquid

For the extraction of extracellular fat from the culture liquid, the modified method of Kates was performed (*25*). Biomass determination: 10–50 mL of the culture liquid were filtered through membrane filters and yeast cells were washed with *n*-hexane. Cells were controlled by the absence of lipids. Also, in order to verify that no losses of cell components occurred during washing with *n*-hexane, yeast cells produced on glucose were washed twice with distilled water or as previously described (*n*-hexane). In both cases, yeast cells were dried at 110 °C to constant mass.

The filtrate was used for the oil content analysis; it was washed twice with *n*-hexane, the mixture was divided into two layers, of which the upper phase was composed of *n*-hexane and contained lipids, while the lower phase contained a water layer. Hexane extract was collected into a glass flask very accurately. Lipids from a water layer were extracted by chloroform and combined with the *n*-hexane extract. The combined lipid extract was dried by passing it through a glass filter with anhydrous sodium sulphate; solvents were evaporated to constant mass.

In order to determine individual lipid fractions, thin layer chromatography was carried out on 60G silica gel plates (Merck) by using a solvent system *n*-hexane-diethyl ether-acetic acid in volume ratio=85:15:1. Lipid fractions were visualized by iodine vapour.

Methyl esters of fatty acids were obtained by the method of Sultanovich *et al.* (*26*) and analysed by gas-liquid chromatography on a Chrom-5 chromatograph (Czech Republic) with a flame-ionisation detector. The column (2 m × 3 mm) was packed with 15 % Reoplex-400 applied to Chromaton N-AW (0.16–0.20 mm). The temperature of the column was 200 °C. The lipid content in the biomass was determined from the total fatty acid content with *n*-docosane ($C_{22}H_{46}$) or heptadecanoic acid as internal standards.

Other biochemical assays

Filtered aliquots of the culture liquid were used for the analysis of nitrogen, CA, isocitric acid (ICA), and glycerol.

Ammonium concentration was assayed with an Orion ionometer (USA). Concentration of organic acids was determined using HPLC on an HPX-87H column (7.8 × 300 mm, Biorad) at 210 nm. Organic acids from Boehringer were used as standards. CA and ICA were not resolved into separate peaks, so they were determined as the sum of acids. Moreover, diagnostic kits (Roche Diagnostics GmbH) were used for the assay of CA and ICA. The determination of CA was based on the measurement of the NADH produced during conversion of CA to oxaloacetate and its decarboxylation product pyruvate, and following conversion to L-malate and L-lactate. Reactions are catalysed by citrate lyase, malate dehydrogenase and L-lactate dehydrogenase. The determination of ICA was based on the measurement of the NADPH produced during conversion of ICA to α-ketoglutarate, a reaction catalysed by isocitrate dehydrogenase.

Glycerol was analysed enzymatically using biochemical kit (Boehringer Mannheim/R-Biopharm). The determination of glycerol was based on the measurement of the NADH produced during conversion of glycerol to L-lactate in coupled reactions, the reactions are catalysed

by glycerol kinase, pyruvate kinase and L-lactate dehydrogenase.

Characteristics of cell growth and citric acid production

To take into account the medium dilution due to the addition of NaOH solution for maintaining the constant pH value, the total amounts of CA and biomass in the culture liquid were used for calculations.

The specific growth rate (μ) was calculated using the following equation: $\mu=2.3(\log X_2-\log X_1)/(t_2-t_1)$, where X_2 and X_1 are the biomass at the moment of time t_2 and t_1.

The biomass yield was calculated as follows: $Y_{x/s}= X/S$, where X is the total amount of biomass in the culture liquid at the end of fermentation (g/L) and S is the amount of oil consumed (g/L).

The specific rate of CA production (q_{CA}) was calculated using the following equation: $q_{CA}=(P/X)\cdot t$; where P is the total amount of CA in the culture liquid at the end of fermentation (g), X is biomass content in a fermentor at the end of fermentation (g), and t is fermentation time (h).

The mass yield coefficient of CA production was calculated as follows: $Y_{CA}= P/S$, where P is the total amount of CA in the culture liquid at the end of fermentation (g/L) and S is the amount of oil consumed (g/L).

The energy yield coefficient of CA production (η_{CA}) characterises the fraction of chemical energy of the consumed substrate, which is converted into CA. Using the mass-energy balance method (*27,28*), we calculated η_{CA} on the basis of the balance equation: $\eta_{CA} = (\gamma_{CA}\cdot\delta_{CA}/\gamma_S\cdot\delta_S)\cdot Y_{CA}$, where γ_{CA} and γ_S are the reductance degrees of CA and substrate, respectively, δ_{CA} and δ_S are the mass fractions of carbon in CA and substrate molecule, respectively, and Y_{CA} is a mass yield of CA. The reductance degree designates the number of equivalents of available electrons (or redoxons) required for oxidation of the compound to CO_2 and H_2O. The reductance degree of the compound of the composition CH_pO_n is calculated by equation (*27,28*): $\gamma =4+p-2n$.

Therefore, the $\gamma_{CA}\cdot\delta_{CA}$ and $\gamma_S\cdot\delta_S$ were 1.125 and 4.28, respectively, while the energy efficiency coefficient of CA production from rapeseed oil may be calculated by the formula $1.125/4.28\cdot Y_{CA.}$

Results and Discussion

Screening of oil-growing yeasts and determination of citric acid production by various yeast strains

Thirty yeast strains of the genera *Candida* and *Yarrowia* were tested for their ability to excrete lipase when grown either on agar medium containing animal fat (method A) or in liquid medium containing rapeseed oil (method B) as a source of carbon and energy.

Up to date, pathways involved in the utilization of triglycerides by microbial cells are still unclear. An extracellular and two cell-bound types of lipase activity corresponding to lipase I and lipase II were described by Ota *et al*. (*29*). However, recently it has been found that deletion of *LIP 2* gene, which encodes the extracellular lipase in *Y. lipolytica*, resulted in the loss of extracellular lipase activity, while at the same time, the mutant was still able to grow on triglycerides (*30*), which suggests the operation of an alternative pathway for triglyceride utilization in *Y. lipolytica*. In our experiments, all the known forms of lipase activity, such as extracellular, cell-bound, membrane-bound, and oil-bound lipases, were revealed in crude extract of *Y. lipolytica* H181 containing both cells and culture liquid.

The effects of carbon substrate (olive oil, rapeseed oil or sunflower oil), emulsificators (gum arabic, polyvinyl alcohol), temperature, and pH on the level of lipase activity produced by *Y. lipolytica* H181 were studied (Table 1).

It was revealed that variations in carbon substrates, type of emulsificator and temperature showed no significant effect on the level of lipase activity. The following conditions were chosen for further measurements of lipase activity in crude extracts: the substrate solution containing sunflower oil emulsified with polyvinyl alcohol and temperature of 30 °C. The effect of pH on lipase

Table 1. Effect of different parameters on the lipase activity in yeast *Y. lipolytica* H181

Effect of carbon source and emulsificator[a]				
Type of emulsion	Olive oil – polyvinyl alcohol	Olive oil – gum arabic	Rapeseed oil – polyvinyl alcohol	Sunflower oil – polyvinyl alcohol
Relative lipase activity/%	100	80.2	105	114.5

Effect of temperature[b]				
T/°C	25	28	30	37
Relative lipase activity/%	87	92	100	90

Effect of pH[c]								
pH	4.5	5	6	7	7.4	8	8.5	9
Relative lipase activity/%	0	0	0	29.7	63	100	90	40.3

[a]experiments were performed at pH=8 and 30 °C
[b]experiments were performed with rapeseed oil – polyvinyl alcohol emulsion at pH=8
[c]experiments were performed with rapeseed oil – polyvinyl alcohol emulsion at 30 °C

activity was studied at a broad range of pH (from 4.5 to 9). Surprisingly, the optimal pH for lipase activity was found to be 8; this is rather unexpected result, since the optimum pH for growth of *Y. lipolytica* ranged from 4.5 to 6. It is difficult to imagine physiological explanations for such a high optimal pH value for the performance of lipase assay in this yeast strain.

Results of the detection of lipase activity in various yeast strains are presented in Table 2. By using method A, it was found that *Candida catenulata*, *C. rugosa* and *C. brupmtii* were incapable of lipase secretion. All of the *Yarrowia lipolytica* strains tested produced lipase. We observed variations in the sizes of turbid zones formed around the colonies that indicated the heterogeneity of the *Y. lipolytica* strains with respect to lipase production. Consequently, the strains were divided into a few types: 5 strains exhibiting a small turbid zone around the colonies and hence possessing low lipase activity (+ or ++); 11 strains possess high lipase activity (+++) and 11 strains possess a super high lipase activity (++++). This assumption was also confirmed in experiments in which method B was applied for the detection of lipase activity. Volumetric activity varied from 120 to 2760 U/mL for strains of *Y. lipolytica*: 6 strains possessed low lipase activity (<300 U/mL), 17 strains exhibited high lipase activity (300–1200 U/mL) and 4 strains possessed a super high lipase activity (>1200 U/mL).

Table 2. Lipolytic activity and CA production of *Candida* strains grown on animal fat as the sole substrate (method A) and in liquid medium containing rapeseed oil (method B)

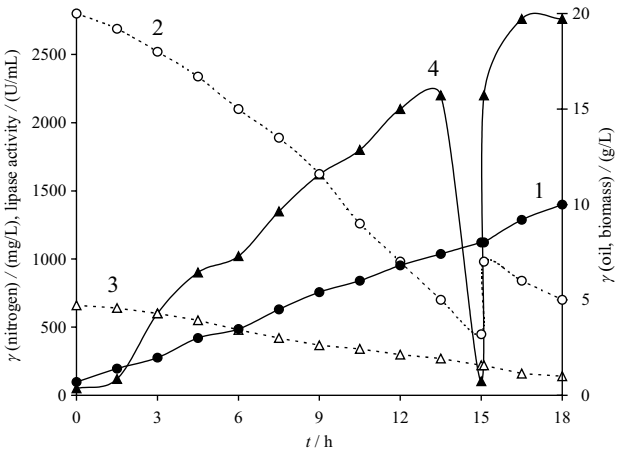
Strains	Lipase activity/(U/mL)		γ(biomass)	γ(CA)	γ(ICA)
	Method A	Method B	g/L	g/L	g/L
Candida brumptii	0	0	0	n.d.	n.d.
Candida catenulata	0	0	0	n.d.	n.d.
Candida rugosa – 67	0	0	0	n.d.	n.d.
Yarrowia lipolytica 47	+ + + +	1956	3.1	11.2	2.5
Y. lipolytica 187/1	+ + + +	1836	3.0	18.0	1.25
Y. lipolytica 212	+ + +	600	2.7	12.4	4.2
Y. lipolytica 217	+ + +	600	2.9	9.2	0.52
Y. lipolytica 374	+++	396	2.5	7.2	1.5
Y. lipolytica 607	+ + +	900	3.8	11.0	5.1
Y. lipolytica 645	+ + + +	756	2.9	15.6	4.9
Y. lipolytica 646	+	264	n.d.	n.d.	n.d.
Y. lipolytica 655	+ +	288	n.d.	n.d.	n.d.
Y. lipolytica 666	+	222	n.d.	n.d.	n.d.
Y. lipolytica 667	+ + +	516	2.7	15.4	4.6
Y. lipolytica 668	+ ++	798	4.0	15.6	4.7
Y. lipolytica 672	+ + + +	120	n.d.	n.d.	n.d.
Y. lipolytica 674	+ + + +	1680	3.2	10.7	4.6
Y. lipolytica 681	+ +	180	n.d.	n.d.	n.d.
Y. lipolytica 682	+ + + +	840	5.1	13.8	2.1
Y. lipolytica 683	+ + + +	1200	4.0	12.6	1.3
Y. lipolytica 694	+ + +	1200	2.8	13.0	4.3
Y. lipolytica 695	+ + +	396	1.7	6.8	2.6
Y. lipolytica 696	+ +	264	n.d.	n.d.	n.d.
Y. lipolytica 702	+ + +	396	3.8	10.8	4.6
Y. lipolytica 704	+ + + +	2760	2.7	13.4	5.2
Y. lipolytica 706	+ + + +	798	4.1	7.0	3.7
Y. lipolytica 709	+ + + +	420	4.1	13.4	3.4
Y. lipolytica 710	+ + + +	840	5.3	14.8	4.8
Y. lipolytica 711	+ + +	600	5.1	12.4	0.9
Y. lipolytica 716	+ + +	810	4.4	9.6	3.9

Method A – lipase activity was determined from the sizes of the turbid zones formed around the colonies. Conditions: cultures were grown on Petri dishes with agar medium containing animal fat as a carbon substrate; 28 °C; 48–72 h. (+), (++), (+++), (++++) stand for turbid zone diameter of ≤0.2 mm, 0.2–0.6 mm, 0.6–0.9 mm, 0.9–1.2 and ≥1.3 mm, respectively. Method B – cultures were grown in liquid medium with rapeseed oil (20 g/L) as sole carbon and energy source at 220 rpm (pH=4.5–5.5)
n.d. – not determined

Twenty-one yeast strains exhibiting high levels of lipase activity (more than 300 U/mL) were tested for their ability to produce CA from rapeseed oil. As seen from Table 2, the yeast strains studied differed considerably in the level of lipase and CA production. The strains were divided into 2 groups: 9 strains exhibiting high CA production (<12 g/L) and 12 strains possessing super high CA production (>12 g/L). Of the 12 strains possessing super high CA production, strain *Y. lipolytica* 704, which exhibited the highest lipase activity (2760 U/mL), was selected for the study of biochemical peculiarities of cell growth, whereas strain *Y. lipolytica* 187/1, which exhibited the maximum CA synthesis and low content of undesirable by-product, ICA, was selected for the subsequent studies on CA production.

Growth of Y. lipolytica 704 on rapeseed oil and synthesis of citric acid

A typical curve of the exponential growth phase of *Y. lipolytica* 704 in a medium containing rapeseed oil is shown in Fig. 1. The maximum specific growth rate (μ_{max}) obtained from the linear part of the growth curve amounted to 0.281 h^{-1}, which was lower than those reported previously for various *Y. lipolytica* strains cultivated on fatty materials (*e.g.* methyl-ricinoleate, mixture of industrial fatty acids, triacylglycerols) (*18,31–33*). The biomass yield from rapeseed oil consumed ($Y_{x/s}$) presents the value of 0.638 g/g, which is relatively lower magnitude compared with various *Y. lipolytica* strains on fatty materials (values around (0.9±0.2) g/g) (*17,18,31,33*) due to the additional energy expenditure for lipid hydrolysis.



1– biomass (●), 2 – oil (○), 3 – nitrogen (△), 4 – lipase (▲)

Fig. 1. The time course of exponential growth of *Y. lipolytica* 704 on rapeseed oil.
Culture conditions: yeasts were cultivated in a 3-L fermentor with an operating volume of 1.5 L, pH=5, dissolved oxygen concentration was 50 % of air saturation, T=(28±0.1) °C. When oil concentration decreased to less than 5 g/L (at 15th hour), pulsed addition of rapeseed oil into the medium (3.8 g/L) was performed

As seen from Fig. 1, during the exponential growth, *Y. lipolytica* 704 produced significant quantities of lipase (up to 2760 U/mL). This value is comparable with that

Table 3. Glycerol level and fatty acid composition of the extracellular unconsumed fat during growth of *Y. lipolytica* 704 on rapeseed oil in bioreactor

Index	*t*/h				
	3	6	9	15	18
γ(glycerol) mg/L	0.039±0.01	0.035±0.01	0.056±0.01	0.043±0.01	0.028±0.01
Acids percentage/%					
C_{14}	traces	n.d.	traces	n.d.	traces
C_{15}	traces	n.d.	traces	n.d.	traces
C_{16}	4.0±0.1	n.d.	4.2±0.1	n.d.	4.6±0.1
$C_{16:1}$	traces	n.d.	traces	n.d.	traces
C_{17}	traces	n.d.	traces	n.d.	traces
$C_{17:1}$	traces	n.d.	traces	n.d.	traces
C_{18}	1.2±0.1	n.d.	1.4±0.02	n.d.	1.3±0.02
$^{\Delta 9}$C18:1	58.8±0.1	n.d.	61.0±0.7	n.d.	61.3±0.8
$^{\Delta 9,12}$C18:2	28.1±0.1	n.d.	26.8±0.7	n.d.	26.5±0.7
$^{\Delta 9,12,15}$C18:3	5.9±0.05	n.d.	4.8±0.3	n.d.	4.6±0.3
C_{20}	0.3±0.02	n.d.	0.2±0.02	n.d.	traces
$C_{20:1}$	0.8±0.02	n.d.	0.6±0.08	n.d.	0.6±0.08
$C_{20:2}$	traces	n.d.	traces	n.d.	traces
C_{22}	0.1±0.02	n.d.	traces	n.d.	traces
C_{23}	0.5±0.01	n.d.	0.6±0.1	n.d.	0.6±0.1

Culture conditions: exponential growth, 28 °C, pH=5, dissolved oxygen concentration was 50 % of air saturation.
Values are means of four measurements±standard deviation.
Traces – acid percentage less than 0.1 %
n.d. – not determined

reported by Pereira-Meirelles (*34*) but it is lower than those reported for other strains (*e.g.* various genetically and non-genetically modified mutants of *Y. lipolytica*) (*30,35,36*).

As seen from Fig. 1, the lowering of residual oil content to less than 5 g/L resulted in dramatical decrease in lipase activity, while biomass level did not differ considerably. Introduction of the additional amount of rapeseed oil (3.8 g/L) into the medium caused an immediate restoring of the lipase activity (27-folds). It should be noted that only additional amount of oil promoted this effect; residual nitrogen level was kept at a high level.

Thus, the essential factor for lipase secretion by strain *Y. lipolytica* 704 was found to be the content of rapeseed oil in the medium, and it should be no less than 5 g/L.

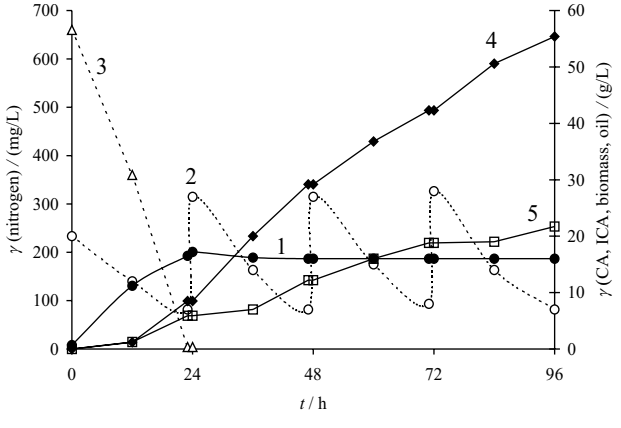
In the course of cell cultivation, the pool of glycerol and free fatty acids formed as a result of hydrolysis of rapeseed oil were measured. As seen from Table 3, glycerol content remained constant during cultivation. The qualitative analysis of lipid fractions in the culture liquid revealed no essential changes in the level of triacylglycerides, sterols, and free fatty acids in the course of cultivation (data not shown). Thus, the pool of free fatty acids remained constant during yeast cultivation. It seems that during cell growth, the consumption of glycerol and fatty acids occurred simultaneously. These results are in agreement with the data reported by Papanikolaou *et al.* for *Yarrowia lipolytica* utilizing technical glycerol and stearin simultaneously (*19*).

The data on the fatty acid composition of total lipids in extracellular fat indicated that oleic ($^{\Delta 9}$C18:1, 58.8–61.3 %), and linoleic ($^{\Delta 9,12}$C18:2, 26.6–28.1 %) acid prevailed (Table 3). It can be seen that the amount of $^{\Delta 9,12}$C18:2 and $^{\Delta 9,12,15}$C18:3 in oil slightly decreases during growth, while the amount of $^{\Delta 9}$C18:1 slightly increases. In agreement with the strain under investigation, *Candida lipolytica* 1094 cultivated on corn oil did not remarkably modify the composition of the medium oil, while the additionally accumulated lipid did not present notable composition differences with the initial substrate oil (*17*). Similar results were reported for other *C. lipolytica* strains grown on olive oil (*31*). However, another *Y. lipolytica* LGAM S(7) strain grown on the mixture of industrial saturated and unsaturated fatty acids preferentially consumed oligounsaturated and polyunsaturated fatty acids from the medium, which at the end of fermentation presented radical differences in its fatty acid composition compared with the initial medium oil (*20*). In that case, and in spite of the rapid uptake of unsaturated fatty acids, the microorganism accumulated a globally saturated lipid with the composition similar to the cocoa butter (*19*).

Fig. 2 presents experimental data from fed-batch fermentation of *Y. lipolytica* 704. Pulsed addition of rapeseed oil into the medium in the course of cultivation of *Y. lipolytica* 704 was performed; its amount in the medium was maintained at the level of no less than 5 g/L. The nitrogen supply was exhausted after 24 h of cultivation, which coincided with the maximum biomass accumulation and the beginning of CA and ICA production. Thus, CA and ICA were produced principally during the stationary growth phase; the total CA and ICA concentration reached maximum of 76.8 g/L by the 96th hour. It should be noted that the major disadvantage of *Y. lipolytica* 704 lies in the high production of ICA as a by-product of fermentation (a ratio CA:ICA was equal to 2.55:1).

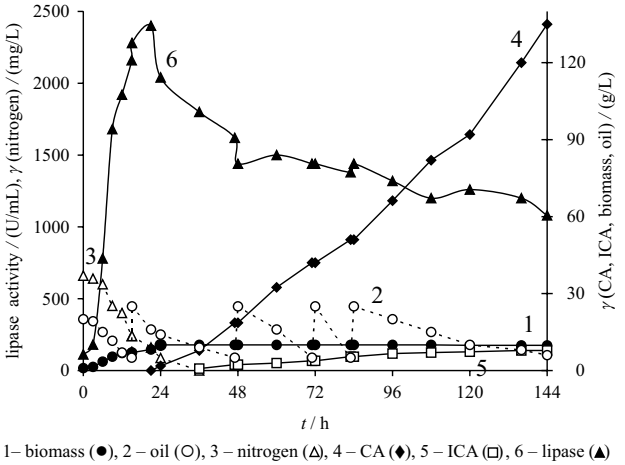
Citric acid production by strain Y. lipolytica 187/1

In order to improve the process of CA production by a decrease in ICA production, *Y. lipolytica* 187/1, selected from the shake-flask experiments, was studied. During growth of *Y. lipolytica* 187/1 the concentration of oil was maintained at the level of no less than 5 g/L (Fig. 3). As seen from Fig. 3, the maximum CA concentration (135 g/L) was obtained at the end of cultivation



1– biomass (●), 2 – oil (○), 3 – nitrogen (△), 4 – CA (♦), 5 – ICA (□)

Fig. 2. The time course of growth of *Y. lipolytica* 704 and citric acid production.
Culture conditions: yeasts were cultivated in a 10-L fermentor with an operating volume of 6 L, pH=5, dissolved oxygen concentration was 50 % of air saturation, T=(28±0.1) °C. When oil concentration decreased to less than 5 g/L, pulsed addition of rapeseed oil into the medium was performed



1– biomass (●), 2 – oil (○), 3 – nitrogen (△), 4 – CA (♦), 5 – ICA (□), 6 – lipase (▲)

Fig. 3. The time course of growth of *Y. lipolytica* 187/1 and citric acid production.
Culture conditions: yeasts were cultivated in a 10-L fermentor with an operating volume of 6 L, pH=5, dissolved oxygen concentration was 50 % of air saturation, T=(28±0.1) °C. When oil concentration decreased to less than 5 g/L, pulsed addition of rapeseed oil into the medium was performed

(144 h). This value was significantly higher than those reported in the literature for CA-producing yeasts (*7–9*). Moreover, the major undesired metabolite, ICA, comprised only 7.8 g/L. Specific rate of CA synthesis q_{CA} reached m(CA)/m(cell)=127 mg/(g·h), which was considerably higher than those reported previously by other researches for CA-producing yeasts grown on glucose (m(CA)/m(cell)=30– 55 mg/(g·h)) (*7–9*), and was comparable with the values obtained with ethanol-grown *Y. lipolytica* (m(CA)/m(cell)=120 mg/(g·h)) (*37*) and *Y. lipolytica,* grown on soybean oil (m(CA)/m(cell)=158 mg/(g·h)) (*38*). The volumetric productivity of the process was rather high (m(CA)/V(cell)=1.25 g/(L·h)) compared to the values reported previously (from 0.2 to 1.25 g/(L·h)) (*7–9,35*). The CA yield obtained in this study (1.55 g/g) was considerably higher than those reported previously. Thus, Rane and Sims (*9*) reported the maximum CA yield of 0.77 g/g when studying the effect of nitrogen and biomass concentrations on CA production by glucose-grown yeast *Y. lipolytica.* Wojtatowicz *et al.* (*8*) revealed the yield of total acids (citric and isocitric) equal to 0.93 g/g in experiments with yeast *Y. lipolytica* grown on hydrolysed potato starch containing 15 % glucose. We reported the CA yield of 0.87 g/g for mutant strain of *Y. lipolytica* grown on ethanol (*37*). However, since equal masses of different organic substrates have different energy capacities, it is incorrect to compare the values of Y_{CA} obtained during the cultivation of microorganisms on different substrates. Therefore, in order to evaluate the efficiency of CA production, the energy yield coefficient for CA production (η_{CA}) was calculated. In our experiments with rapeseed oil-grown yeast *Y. lipolytica* 187/1, the maximum value of η_{CA} was equal to 0.41, whereas the maximum η_{CA} value calculated on the basis of data published in literature comprised 0.44 for glucose-grown yeasts (*8*) and 0.57 for ethanol-grown yeasts (*37*). The lower energy efficiency of CA production from plant oil than from the other carbon substrates is probably due to the additional energy expenditure for lipid hydrolysis.

As seen from Fig. 3, in the course of fermentation, cells exhibited high activity of lipase. The maximum specific activity of lipase was observed for cells of exponential growth phase, and the transition of yeast cells from the exponential growth phase to the growth retardation caused by the exhaustion of nitrogen in the medium was accompanied by a decrease in specific activity of lipase. During stationary phase, when active CA production occurred, a significant decrease in the specific activity of lipase was observed; which is apparently due to a decrease in the rate of synthesis of lipase under conditions of nitrogen limitation.

Conclusions

Results of this study show that plant oils appear to be promising substrates for *Y. lipolytica* 187/1 yeast growth and CA production. Under conditions of nitrogen limitation, CA production was as high as 135 g/L, the specific rate of citric acid synthesis reached m(CA)/m(cell)=127 mg/(g·h), the mass yield was 1.55 and the energy yield was 0.41. Moreover, citrate-producing strains *Y. lipolytica* 187/1 possess a high lipase activity (1200–2040 U/mL) during stationary phase when active CA production occurred and can find additional applications in food industry and as additives for synthetic detergents.

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References

1. B. Kristiansen, C.G. Sinclair, Production of citric acid in continuous culture, *Biotechnol. Bioeng. 21* (1979) 297–315.
2. J.L. Meers, P.E. Milsom: Organic Acids and Amino Acids. In: *Basic Biotechnology*, J. Bu'Lock, B. Kristiansen (Eds.), Academic Press, Orlando (1987) pp. 359–383.
3. S. Aiba, M. Matsouka, Citrate production from *n*-alcane by *Candida lipolytica* in reference to carbon fluxes in vivo, *Eur. J. Appl. Microbiol. Biotechnol. 5* (1978) 247–261.
4. U. Behrens, A. Thiersch, E. Weissbrodt, U. Stottmeister, Particularities in the kinetics of growth and citric-acid accumulation by *Saccharomycopsis lipolytica*, *Acta Biotechnol. 7* (1987) 179–183.
5. S. Aiba, M. Matsouka, Identification of metabolic model: Citrate production from glucose by *Candida lipolytica*, *Biotechnol. Bioeng. 21* (1978) 1373–1386.
6. T.V. Finogenova, N.V. Shishkanova, I.T. Ermakova, I.A. Kataeva, Properties of *Candida lipolytica* mutant with modified glyoxylate cycle and their ability to produce citric and isocitric acid. II. Synthesis of citric and isocitric acid by *C.lipolytica* mutants and peculiarities of their enzyme systems, *Appl. Microbiol. Biotechnol. 23* (1986) 378–383.
7. T.K. Klasson, E.C. Clausen, J.L. Gaddy, Continuous fermentation for the production of citric acid from glucose, *Appl. Biochem. Biotechnol. 20* (1989) 491–509.
8. M. Wojtatowicz, W. Rymowicz, H. Kautola, Comparison of different strains of the yeast *Yarrowia lipolytica* for citric acid production from glucose hydrol, *Appl. Biochem. Biotechnol. 31* (1991) 165–174.
9. K.D. Rane, K.A. Sims, Citric acid production by *Yarrowia lipolytica*: Effect of nitrogen and biomass concentration on yield and productivity, *Biotechnol. Lett. 18* (1996) 1139–1144.
10. S.G. Anastassiadis, A. Aivasidis, C. Wandrey, Citric acid production by *Candida* strains under intracellular nitrogen limitation, *Appl. Microbiol. Biotechnol. 60* (2002) 81–87.
11. S. Papanikolaou, L. Muniglia, I. Chevalot, G. Aggelis, I. Marc, *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol, *J. Appl. Microbiol. 92* (2002) 737–744.
12. T.V. Finogenova, S.V. Kamzolova, E.G. Dedyukhina, N.V. Shishkanova, A.P. Il'chenko, I.G. Morgunov, O.G. Chernyavskaya, A.P. Sokolov, Biosynthesis of citric and isocitric acids from ethanol by mutant *Yarrowia lipolytica* N 1 under continuous cultivation, *Appl. Microbiol. Biotechnol. 59* (2002) 493–500.
13. D. Tisnadjaja, N. Gutierrez, I. Maddox, Citric acid production in a bubble-column reactor using cells of the yeast *Candida guilliermondii* immobilized by absorption into sawdust, *Enzyme Microb. Technol. 19* (1996) 343–347.

14. S. Papanikolaou, G. Aggelis, Modelling aspects of the biotechnological valorization of raw glycerol: Production of citric acid by *Yarrowia lipolytica* and 1,3-propanediol by *Clostridium butyricum*, *J. Chem. Technol. Biotechnol. 78* (2003) 542–547.
15. K. Rane, K. Sims, Production of citric acid by *Candida lipolytica* Y 1095: Effect of glucose concentration on yield and productivity, *Enzyme Microb. Technol. 15* (1993) 646–651.
16. A.B. Lozinov, T.V. Finogenova, L.M. Glazunova, V.I. Illarionova, Limitation of *Candida lipolytica* growth and overproduction of some metabolites, *Mikrobiologiya, 43* (1974) 786–790.
17. N. Bati, E.G. Hammond, B.A. Glatz, Biomodification of fats and oils: Trials with *Candida lipolytica, J. Am. Oil Chem. Soc. 61* (1984) 1743–1746.
18. D. Montet, R. Ratomahenina, P. Galzy, M. Pina, J. Graille, A study of the influence of the growth media on the fatty acid composition in *Candida lipolytica* DIDDENS and LODDER, *Biotechnol. Lett. 7* (1985) 733–736.
19. S. Papanikolaou, L. Muniglia, I. Chevalot, G. Aggelis, I. Marc. Accumulation of a cocoa-butter-like lipid by *Yarrowia lipolytica* cultivated on agro-industrial residues, *Curr. Microbiol. 46* (2003) 124–130.
20. S. Papanikolaou, G. Aggelis, Selective uptake of fatty acids by yeast *Yarrowia lipolytica, Eur. J. Lipid Sci. Technol. 105* (2003) 651–655.
21. Y. Ikeno, M. Masuda, K. Tanno, I. Oomori, N. Takahashi, Citric acid production from various raw materials by yeast, *J. Ferment. Technol. 53* (1975) 752–756.
22. D.W. Good, R. Droniuk, R.G. Lawford, J.E. Fein, Isolation and characterization of a *Saccharomycopsis lipolytica* mutant showing increased production of citric acid from canola oil, *Can. J. Microbiol. 31* (1985) 436–440.
23. I.S. Zviagintzeva, Lipase activity of some yeasts, *Mikrobiologiya, 41* (1972) 24–28.
24. P.R. Burkholder, J. McVeigh, D. Moyer, Studies on some growth factors of yeasts, *J. Bacteriol. 48* (1944) 385–391.
25. M. Kates: Separation of Lipid Mixtures. In: *Techniques of Lipidology*, M. Kates (Ed.), Mir, Moscow (1975) pp. 150–167.
26. Yu.A. Sultanovich, A.P. Nechaev, I.A. Barsukova, *Method of lipid determination. USSR Inventor's Certificate No. 968072, Byull. Izobret. 39* (1982) p. 136.
27. I.G. Minkevich, V.K. Eroshin, Productivity and heat generation of fermentation under oxygen limitation, *Folia Microbiol. 18* (1973) 376–385.
28. L.E. Erickson, I.G. Minkevich, V.K. Eroshin, Application of mass and energy balance reguliarities in fermentation, *Biotechnol. Bioeng. 67* (2000) 748–773.
29. Y. Ota, S. Oikawa, Y. Morimoto, Y. Minoda, Purification and some properties of cell-bound lipase from *Saccharomycopsis lipolytica*, *Agric. Biol. Chem. 46* (1982) 2885–2893.
30. G. Pignede, H. Wang, F. Fidalej, G. Gaillardin, M. Seman, J.M. Nicaud, Characterization of an extracellular lipase encoded by LIP2 in *Yarrowia lipolytica, J. Bacteriol. 182* (2000) 2802–2810.
31. B.A. Glatz, E.G. Hammomd, K.H. Hsu, L. Baehman, N. Bati, W. Bednarski, D. Brown, M. Floetenmeyer: Production and Modification of Fats and Oils by Yeast Fermentation. In: *Biotechnology for the Oils and Fats Industry*, C. Ratledge, J.B.M. Rattray, P.S.S. Dawson (Eds.), American Oil Chemists Society, Champaign (1984) pp. 163–176.
32. Y. Pagot, A. Endrizzi, J.M. Nicaud, J.M. Belin, Utilization of an auxotrophic strain of the yeast *Yarrowia lipolytica* to improve γ-decalactone production yields, *Lett. Appl. Microbiol. 25* (1997) 113–116.
33. S. Papanikolaou, I. Chevalot, M. Komaitis, G. Aggelis, Kinetic profile of the cellular lipid composition in an oleaginous *Yarrowia lipolytica* capable of producing a cocoa-butter substitute from industrial fats, *Antonie Van Leeuwenhoek, 80* (2001) 215–224.
34. F.V. Pereira-Meirelles, M.H.M. Rocha-Leao, A stable lipase from *Candida lipolytica*, *Appl. Biochem. Biotechnol. 63–65* (1997) 73–85.
35. P. Fickers, J.M. Nicaud, J. Destrain, P. Thonard, Overproduction of lipase by *Yarrowia lipolytica* mutants, *Appl. Microbiol. Biotechnol. 63* (2003) 136–142.
36. P. Fickers, J.M. Nicaud, C. Gaillardin, J. Destrain, P. Thonard, Carbon and nitrogen sources modulate lipase production in the yeast *Yarrowia lipolytica, J. Appl. Microbiol. 96* (2004) 742–749.
37. S.V. Kamzolova, N.V. Shishkanova, I.G. Morgunov, T.V. Finogenova, Oxygen requirements for growth and citric acid production of *Yarrowia lipolytica, FEMS Yeast Res. 3* (2003) 217–222.
38. M. Wojtatowicz, W. Rymowicz, J. Sobieszczanski, Citric acid production from various substrates by *Saccharomycopsis lipolytica* A-101, *Abstracts of the 8th International Biotechnology Symposium,* Paris (1988) p. 166.

Sekrecija lipaze i proizvodnja limunske kiseline s pomoću kvasca *Yarrowia lipolytica* uzgojenog na životinjskim i biljnim masnoćama

Sažetak

Svrha je rada bila istražiti mogućnosti korištenja sirovih agroindustrijskih masnoća za biotehnološku proizvodnju korisnih proizvoda (lipaze i limunske kiseline) s pomoću kvasca *Yarrowia* (*Candida*) *lipolytica*. Ispitana je sposobnost sekrecije lipaze i proizvodnja limunske kiseline 30 sojeva navedenog kvasca u podlogama koje su sadržavale masnoće životinjskog podrijetla ili repičino ulje kao jedini izvor ugljika i energije. Soj *Y. lipolytica* 704 koji je pokazao najveću lipaznu aktivnost (2760 U/mL) na repičinom ulju odabran je za istraživanje biokemijskih pokazatelja rasta stanica. Budući da soj *Y. lipolytica* 187/1 proizvodi najveću količinu limunske kiseline, odabran je za daljnje istraživanje. Ispitan je odnos između proizvodnje lipaze i preostale koncentracije repičinog ulja. Nađeno je da je bitan faktor za proizvodnju lipaze koncentracija repičinog ulja u podlozi, koja ne smije biti ma-

nja od 5 g/L. Pod optimalnim uvjetima uzgoja kvasca *Y. lipolytica* 187/1 na repičinom ulju, proizvodnja limunske kiseline iznosila je 135 g/L; specifična brzina proizvodnje limunske kiseline m(limunska kiselina)/m(stanica)=127 mg/(g·h); iskorištenje mase Y_{CA}=1,55, a energije η_{CA}=0,41.