APPLIED MICROBIAL AND CELL PHYSIOLOGY

Optimization of medium composition and fermentation conditions for α-ketoglutaric acid production from biodiesel waste by *Yarrowia lipolytica*

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Abstract

This work demonstrates the ability of the yeast *Yarrowia lipolytica* cultivated on biodiesel waste to synthesize α-ketoglutaric acid with a minimal content of pyruvic acid as the main byproduct. The key factor promoting the microbial production of α-ketoglutaric acid from the waste is a strong deficiency of thiamine in the cultivation medium. The production of α-ketoglutaric acid by the yeast can be regulated by changing the concentration of nitrogen, iron, zinc, copper, and manganese in the medium, as well as by pH medium and the aeration rate. The optimization of these parameters in flask experiments allowed us to increase the concentration of α-ketoglutaric acid in the medium by 2.6 times and to shift the α-ketoglutaric acid/pyruvic acid ratio from 5:1 to 30:1. During cultivation in a fermentor under optimized conditions, *Y. lipolytica* produced 80.4 g/L α-ketoglutaric acid with a process selectivity of 96.7% and the product yield (Y_{KGA}) equal to 1.01 g/g.

Key points

• *α-Ketoglutaric acid is commercially important biotechnological product.*
• *Biosynthesis of α-ketoglutaric acid from biodiesel waste.*
• *Optimization of cultivation medium and nutrition medium.*

Keywords α-Ketoglutaric acid production · *Yarrowia lipolytica* · Waste from biodiesel production · Optimization

Introduction

Now the annual world production of biodiesel is 41 million tons and increases by 4.5% each year. The production of one ton of biodiesel gives rise to 100–150 kg of waste byproduct, representing a mixture of glycerol (40–60%); free fatty acids, mono-, di-, and triglycerides; methanol; methyl ester; water; and traces of catalysts. The most promising method of waste removal is its conversion by microorganisms to valuable organic compounds, such as proteins, lipids, polyols, polyhydroxybutyrate, and citric and succinic acids (Thompson and He 2006; Debabov 2008; Chatzifragkou et al. 2011; Rywińska et al. 2013; Magdouli et al. 2017; Papanikolaou et al. 2017; Vivek et al. 2017; Do et al. 2019; Kumar et al. 2019).

Recently, there is increasing interest to the production of α-ketoglutaric acid (KGA) from the glycerol-containing biodiesel waste with the aid of the yeast *Yarrowia lipolytica* (Zhou et al. 2010, 2012; Otto et al. 2012; Yu et al. 2012; Yin et al. 2012; Guo et al. 2014, 2015, 2016; Zeng et al. 2017; Cybulski et al. 2018; Fickers et al. 2020).

The microbial production of KGA has an advantage over chemical synthesis, primarily due to the low toxicity of the microbially produced product. Such KGA can safely be used in medicine for antitumor therapy, treatment of schizophrenia, alcoholism, and other disorders, as well for diagnostics of a wide range of diseases (hepatitises, myocardium infections, muscle dystrophy, dermatitises, and others) (Finogenova et al. 2005; Stottmeister et al. 2005; Aurich et al. 2012; Otto et al. 2011, 2013; Guo et al. 2016; Song et al. 2016; Fickers

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et al. 2020). Besides, the chemically produced KGA has a high cost of $15–20 per kg which presumably can be reduced tenfold, as is evident from the low cost of other organic acids produced microbially on an industrial scale. Indeed, the cost of thus produced citric, lactic, and itaconic acids is as low as 0.6, 1.5, and $2.0/kg, respectively (Zeng et al. 2017).

The conversion of biodiesel waste into KGA by the yeast *Y. lipolytica* has a great disadvantage related to the simultaneous formation of pyruvic acid (PA) (Zhou et al. 2010, 2012; Otto et al. 2012; Yu et al. 2012; Yin et al. 2012; Guo et al. 2014, 2015, 2016; Zeng et al. 2017; Cybulski et al. 2018; Fickers et al. 2020). This significantly reduces the product yield (Y_{KGA}) and complicates the process of KGA isolation.

The aim of this work was to study the main peculiarities of KGA synthesis from biodiesel waste by yeast *Y. lipolytica* and to determine conditions favoring the production of KGA with a minimal content of PA.

Materials and methods

Yeast strain

Experiments were carried out with the yeast strain *Y. lipolytica* VKM Y-2412, which was selected earlier and showed its ability to synthesize KGA from ethanol and rapeseed oil in sufficient amounts (Kamzolova et al. 2012, Kamzolova and Morgunov 2013).

Chemicals

All chemicals were of analytical grade (Mosreactiv, Russia). Biodiesel waste contained 70.8% glycerol and 23.9% fatty acids. Composition of fatty acids (% of the sum): tetradecanoic, 0.2; palmitic, 12.7; stearic, 18.0; oleic, 11.3; linoleic, 49.2; α-linolenic, 8.3; eicosanoic, 0.3.

Main medium

Initially, we used Reader medium containing (g/L): $(NH_4)_2SO_4$, 3.0; $MgSO_4 \cdot 7H_2O$, 0.7; $Ca(NO_3)_2 \cdot 4H_2O$, 0.4; NaCl, 0.5; KH_2PO_4, 1.0; K_2HPO_4, 0.2. This medium was supplemented with the microelement solution containing (mg/L): KJ, 0.1; $Na_2B_4O_7 \cdot 7H_2O$, 0.08; $MnSO_4 \cdot 5H_2O$ 0.05; $ZnSO_4 \cdot 7H_2O$ 0.04; $CuSO_4$, 0.04; Na_2MoO_4, 0.03; $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 0.35 (Burkholder et al. 1944) and the essential vitamin thiamine. The concentrations of thiamine and biodiesel waste were varied as described in the text.

Cultivation conditions

The effects of thiamine, biotin, and nitrogen source were studied using 750-mL Erlenmeyer flasks with 50 mL of Reader medium supplemented with 40 g/L biodiesel waste. The flasks were shaken at 180 rpm. The cultivation temperature was 29 °C. The pH of the medium was maintained during cultivation at a value between 3.5 and 6.0 by the daily addition of a sterile 10% solution of KOH. Thiamine and nitrogen source were added as indicated in the text. The cultivation time was 8 days.

The effect of pH on acid formation by growing cells was studied using the cultivation medium with the increased concentrations of the following salts (mg/L): $ZnSO_4 \cdot 6H_2O$, 25.1; $MnSO_4 \cdot 5H_2O$, 1.3; $CuSO_4 \cdot 5H_2O$, 5.5; and $FeSO_4 \cdot 7H_2O$, 14.9, while thiamine was in shortage (0.15 μg/L). To facilitate the maintenance of pH on the desired level (between 2.0 and 8.0), 1 M phosphate buffer was added to each cultivation flask in a volume of 10 mL. During cultivation, the pH of the medium was determined every 12 h and, if necessary, corrected by adding a certain volume of 10% solution of either KOH or H_2SO_4.

In experiments on the effect of pH on acid formation by resting cells, the inoculum was grown for 3 days in a 10-l ANKUM-2 M fermentor (IBP RAN, Russia) with 5 L of a modified Reader medium supplemented with 20 g/L biodiesel waste and a small amount of thiamine (0.15 μg/L). The cultivation temperature, pH, and the concentration of dissolved oxygen (pO_2) were automatically maintained at levels of 29 °C, 5.0 units, and 20% oxygen saturation, respectively. After 3 days of cultivation, the yeast cells were separated from the medium, washed, and placed in amounts of 3 g cells/L in the flasks with the modified Reader medium without thiamine and nitrogen source. The pH values of media in different flasks were between 2.0 and 8.0. The flasks were shaken under the same conditions as during the cultivation of growing cells (180 rpm and 29 °C) for 36 h. During the experiment, the pH of the buffered medium decreased insignificantly (by 0.3–0.5 units) and did not require correction.

The effect of aeration rate on acid formation was studied using the same fermentor and the same fermentation conditions except that the concentration of biodiesel waste was increased to 40 g/L and that of thiamine to 0.75 μg/L in order to reach the cell biomass of 10–15 g/L. During the experiment, oxygen was supplied in two regimes. In the first regime (high aeration), the aeration rate was maintained for the first 2 days at 20% oxygen saturation, and with 48 h the aeration rate was elevated to 60% oxygen saturation and maintained at this level to the end of the experiment. In the second regime (low aeration), the aeration rate was maintained at a level of 20% oxygen saturation during the entire experiment. It should be noted that the desired level of aeration (20 and 60% oxygen saturation) was reached by changing the rate of air supply from 0.25 to 10 L air/total amount of medium in liters per min and the agitation rate from 600 to 1000 rpm. As for pH, it was maintained at pH 5 for first 3 days of cultivation, and then allowed to spontaneously drop to 3.0–4.0, and maintained at this level

until the end of cultivation in both types of experiments. In both regimes of aeration, after 3 days of cultivation, the medium was additionally supplemented daily with ammonium sulfate in 1.2 g/L portions. If the concentration of dissolved oxygen (pO_2) exceeded the preset saturation value by 10%, the biodiesel waste was added to the cultivation medium in 40 g/L portions.

Analyses

Biomass, KGA, PA, and other organic acids in the cultural medium were analyzed as described earlier (Kamzolova et al. 2012; Kamzolova and Morgunov 2013).

The content of lipids and the fatty acid composition was determined as described earlier (Dedyukhina et al. 2014). The content of microelements in cell biomass was determined using a 5100 PCAAS-ZeeMan (Perkin-Elmer) atomic absorption spectrometer (Khavezov and Tsalev 1983).

Calculations

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

The specific rate of KGA synthesis (q_{KGA}) was calculated as the amount of KGA in mg synthesized by 1 g of yeast cells per hour. The product yield (Y_{KGA}) was calculated as the amount of KGA in gram found in the culture liquid by the end of the experiment and divided by the amount (in g) of glycerol and fatty acids consumed from the utilized biodiesel waste.

Results

Effect of thiamine

The effect of thiamine on the growth of the yeast strain *Y. lipolytica* VKM Y-2412 and the production of ketoacids was studied using this vitamin at concentrations between 0 and 10 μg/L. As seen from Table 1, the yeast can grow even in the absence of added thiamine. The active production of KGA (12.6–13.6 g/L) was observed when thiamine was in a strong shortage (0.15–0.3 μg/L). When the concentration of thiamine in the medium was increased from 0.3 to 5 μg/L, the production of KGA fell by 13 times. The concentration of thiamine equal to 10 μg/L totally suppressed KGA synthesis but provides for the maximum growth of biomass (9.3 g/L). It should be noted that at all thiamine concentrations tested, the culture liquid contained not only the target product KGA but also some amount of PA (from 0.3 to 2.6 g/L) as the main byproduct. The total content of other detected organic acids (citric, isocitric, succinic, fumaric, and malic) was as low as 0.5 g/L. The maximum value of the KGA:PA ratio (5:1) was also observed under the strong deficiency of thiamine (0.15–0.3 μg/L). Similarly, the maximum values of the specific rate of KGA synthesis (q_{KGA} = 35 mg/g h) and the product yield Y_{KGA} (0.48 g/g) were attained at the concentration of thiamine equal to 0.15 μg/L. The increase in the thiamine concentration to 0.6 μg/L diminished q_{KGA} and Y_{KGA} by 2.5 and 1.7 times, respectively. For this reason, further experiments were carried out at the thiamine concentration equal to 0.15 μg/L.

Effect of nitrogen source

It might be assumed that nitrogen deficiency stops the amination of KGA into glutamate, resulting in the excretion of KGA from the cell into the medium. In fact, however, the decrease in the concentration of nitrogen source not only did not enhance KGA excretion from the cells but even suppressed it (Table 2). When the concentration of the nitrogen source decreased from 0.636 g/L typical of Reader medium to a small value of 0.021 g/L, the yeast biomass declined only by 17%, while KGA biosynthesis by 44 times. As seen from the data of Table 2, the high rate of KGA synthesis (12.6–13.6 g/L) was provided by high concentrations of nitrogen source in the cultivation medium (no less than 0.424 g/L) and the C/N ratio no less than 43:1. The maximum values of the specific rate of KGA synthesis (q_{KGA} = 26–35 mg/g h) and the product yield Y_{KGA} (0.43–0.48 g/g) were attained at the concentrations of $(NH_4)_2SO_4$ between 2 and 10 g/L.

Further experiments were carried out using cultivation media with 3 g/L ammonium sulfate.

Effect of cofactors of key enzymes

The optimal content of Mg, Ca, Na, Zn, Mn, Cu, and Fe was calculated on the basis of the elemental composition of the yeast biomass using the formula (Dudina and Eroshin 1979):

$$S = a \times X$$

where S is the amount of an element in 1 L of the medium; a is the amount of this element in 1 g of yeast biomass; and X is the planned amount of biomass in 1 L of the medium.

Comparative data on the content of Mg, Ca, Na, Zn, Mn, Cu, and Fe in the optimized and ordinary Reader media are summarized in Table 3. Optimization was based on the elemental composition of the biomass grown in the regime of pH-auxostat at pH = 5 and the cultivation temperature of 29 °C. The planned amount of biomass in calculations was 10 g/L. As seen from Table 3, the ordinary Reader medium contains Na in sufficient amount, Mg and Ca are in excess (2.8- and 7.5-fold, respectively), while Zn, Fe, Cu, and Mn are in shortage (610-, 60-, 140- and 30-fold, respectively).

Table 1 Effect of thiamine on the growth of *Y. lipolytica* VKM Y-2412 and production of KGA

Thiamine (µg/L)	Biomass (g/L)	KGA (g/L)	PA (g/L)	KGA:PA	q_{KGA} (mg/g h)	Y_{KGA} (g/g)
0	1.2 ± 0.2	5.4 ± 1.2	1.4 ± 0.2	4:1	23	0.19
0.15	2.0 ± 0.2	13.6 ± 1.5	2.6 ± 0.4	5:1	35	0.48
0.3	2.5 ± 0.2	12.6 ± 1.6	2.6 ± 0.4	5:1	26	0.44
0.6	2.8 ± 0.3	7.8 ± 1.2	1.8 ± 0.5	4:1	15	0.27
1.0	3.0 ± 0.3	4.6 ± 0.5	1.1 ± 0.2	4:1	8	0.16
1.5	3.3 ± 0.1	4.3 ± 0.5	1.2 ± 0.2	4:1	7	0.15
2.0	4.3 ± 0.8	3.8 ± 0.4	1.0 ± 0.2	4:1	5	0.13
3.0	4.8 ± 1.1	2.7 ± 0.5	1.0 ± 0.1	3:1	3	0.10
4.0	5.3 ± 0.8	1.7 ± 0.2	1.0 ± 0.2	2:1	2	0.10
5.0	5.3 ± 1.1	1.0 ± 0.2	1.0 ± 0.1	1:1	1	0.03
10.0	9.3 ± 2.1	0.1 ± 0.1	0.3 ± 0.1	1:3	n.d.	n.d.

Initially, the medium was supplemented with 3.0 g/L $(NH_4)_2SO_4$ and 40 g/L biodiesel waste containing glycerol (28.3 g/L) and fatty acids (9.56 g/L). *n.d,* not determined

Further experiments were carried out with the optimized Reader medium which contained Zn, Fe, Cu, and Mn elements in increased amounts (6.1, 3, 1.4, and 0.3 mg/L, respectively).

The effect of biotin on the growth of *Y. lipolytica* VKM Y-2412 and production of ketoacids in the optimized medium are shown in Table 4. As seen from Table 4, biotin does not influence yeast growth and KGA production. At the same time, the comparison of data in Tables 2 and 4 clearly shows that the optimization of the Reader medium has doubled the production of KGA.

Effect of pH

The effect of pH on the formation of ketoacids by *Y. lipolytica* VKM Y-2412 was studied using two types of yeast cells, growing and resting. The incubation of resting cells in the medium lacking nitrogen source and thiamine did not allow cell reproduction, so the initial concentration of the

cells (3 g/L) did not change within the experiment. The results of experiments with these two types of cells are shown in Table 5. As seen from this table, the yeast strain can grow in a wide range of pH values from 2 to 8; however, the intensity of acid formation strongly depends on pH. At the pH values 2 and 8, the concentration of KGA in the culture liquid did not exceed 6.1–6.3 g/L, the growth substrate was consumed only partially, and the KGA yield Y_{KGA} was low (0.15–0.16 g/g). In contrast, at pH values from 3 to 7, the concentration of KGA in the culture liquid increased to 22.3–35.3 g/L, while the concentration of PA remained low (0.7–1.2 g/L). The maximum values of KGA production (35.3 g/L) and product yield Y_{KGA} (0.88 g/g) were observed at pH = 4. As also seen from Table 5, at the pH values most favorable for growth (from 5 to 7), the specific rate of KGA synthesis (q_{KGA}) is 1.7 times lower than at the pH values relatively unfavorable for growth (3.0–4.5).

As for the resting yeast cells, they can produce KGA at all the pH values studied (Table 5). The specific rate of KGA

Table 2 Effect of nitrogen source on the growth of *Y. lipolytica* VKM Y-2412 and parameters of KGA production

Nitrogen concentration in the medium			Biomass (g/L)	KGA (g/L)	PA (g/L)	KGA:PA	q_{KGA} (mg/g h)	Y_{KGA} (g/g)
$(NH_4)_2SO_4$ (g/L)	N (g/L)	C*:N						
0.1	0.021	876:1	1.7 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	3:1	1	n.d.
0.3	0.064	174:1	1.9 ± 0.1	6.1 ± 0.6	1.9 ± 0.3	3:1	17	0.20
1.0	0.212	87:1	2.1 ± 0.1	7.6 ± 1.0	1.8 ± 0.3	4:1	19	0.25
2.0	0.424	43:1	2.1 ± 0.2	13.2 ± 1.2	2.5 ± 0.5	5:1	33	0.43
3.0	0.636	29:1	2.0 ± 0.2	13.6 ± 1.5	2.6 ± 0.4	5:1	35	0.48
6.0	1.272	14:1	2.5 ± 0.1	12.6 ± 1.2	2.4 ± 0.5	5:1	26	0.44
10.0	2.120	9:1	2.0 ± 0.2	10.6 ± 0.2	2.1 ± 0.1	5:1	27	0.44

*Initially, the medium was supplemented with 3.0 g/L $(NH_4)_2 SO_4$ and 40 g/L biodiesel waste containing glycerol (28.3 g/L) and fatty acids (9.56 g/L). The content of carbon in glycerol is 11.06 g/L. The content of carbon in fatty acids (7.34 g/L) was calculated based on its content in the oleic and linoleic acids, which prevail in biodiesel waste. *n.d*, not determined

Table 3 Optimization of the elemental composition of nutrient medium

Chemical element	Concentration in Reader medium (mg/L)	Concentration in biomass grown in pH-auxostat (% of dry biomass)	Concentration in optimized medium (mg/L)
Mg	69.00	0.250	25.0
Ca	67.75	0.090	9.0
Na	230.00	2.280	228.0
Zn	0.01	0.061	6.1
Mn	0.01	0.003	0.3
Cu	0.01	0.014	1.4
Fe	0.05	0.030	3.0

formation q_{KGA} by these cells remained high (56 mg/g h) even at pH = 2. At the other extreme pH value (pH = 8), q_{KGA} was lower only by two times.

Taking into account all these results, further experiments were carried out controlling pH value at 5.0 during the growth phase and at 3.0–4.0 during the phase of KGA synthesis.

Effect of aeration

The effect of aeration on the growth of *Y. lipolytica* VKM Y-2412 and KGA synthesis was studied in two cultivation regimes differing in the rate of aeration (they are described in detail in the "Materials and methods" section). As seen from Fig. 1, yeast growth was similar within the first 2 days of cultivation in both regimes. Then, in the fermentor with high aeration, yeast growth ceased. However, in the fermentor with low aeration, the yeast continued to grow for 8 days. By this time, the biomass accumulated in this fermentor exceeded that in the fermentor with high aeration by 48%. In both cultivation regimes *Y. lipolytica* VKM Y-2412, the cells simultaneously consume glycerol and fatty acids at different rates, regardless of the growth phase and acid-forming activity of the producer. The effect of aeration on KGA synthesis was opposite; namely, the concentration of KGA in the fermentor with high aeration was 80.4 g/L in comparison with 56.8 g/L in the fermentor with low aeration. At high aeration, the KGA yield (Y_{KGA}) and the process productivity reached 1.01 g/g and 0.42 g/L h,

respectively, both parameters being lower by 29% in the fermentor with low aeration. In both cultivation regimes, the yeast strain also produced PA in small amounts, so that the selectivity of biodiesel waste conversion into KGA was as high as 96.8%.

Discussion

The biotechnology of KGA production from biodiesel waste with the aid of the yeast *Y. lipolytica* is based on its inability to synthesize the pyrimidine moiety of the thiamine molecule which is necessary for the normal synthesis of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase responsible for the oversynthesis of ketoacids (Finogenova et al. 2005; Stottmeister et al. 2005; Aurich et al. 2012; Otto et al. 2013; Guo et al. 2016; Fickers et al. 2020).

As can be seen from Table 1, a deep thiamine deficiency (0.15 μg/L) is a necessary condition of KGA oversynthesis from biodiesel waste by *Y. lipolytica* VKM Y-2412, is in agreement with the results of a study of other strains cultivated on various carbon sources. For example, the wild-type strain *Y. lipolytica* WSH-Z06 grown on glycerol produced KGA when the concentration of thiamine in the medium was between 0 and 0.2 μg/L and almost did not produce it in the presence of 1 μg/L thiamine (Zhou et al. 2010). Similarly, the oversynthesis of KGA from rapeseed oil by *Y. lipolytica* was observed within the narrow range of thiamine concentrations between 0.05 and 0.2 μg/L (Kamzolova and Morgunov 2013). In contrast, the same strain grown on ethanol required significantly higher thiamine concentrations for KGA oversynthesis (from 0.2 to 2 μg/L) (Kamzolova et al. 2012). Still higher requirements in thiamine (up to 20 μg/L) were observed for *Y. lipolytica* H355 and its genetically modified transformants with the superexpressed genes of fumarase and pyruvate carboxylase upon their cultivation in a bioreactor with the glycerol-containing biodiesel waste as the substrate (Otto et al. 2012).

Another necessary condition of KGA oversynthesis by *Y. lipolytica* VKM Y-2412 is a relatively high concentration of ammonium sulfate in the cultivation medium (no less than 2 g/L) with the C:N ratio no less than 43:1. Similar data were

Table 4 Effect of biotin on the growth of *Y. lipolytica* VKM Y-2412 and KGA production in the medium with thiamine deficiency and increased content of iron, copper, and manganese ions

Biotin (μg/L)	Biomass (g/L)	KGA (g/L)	PA (g/L)	KGA:PA	q_{KGA} (mg/g h)	Y_{KGA} (g/g)
0	2.7 ± 0.2	32.3 ± 2.5	1.2 ± 0.1	27:1	62	0.81
10	2.9 ± 0.4	33.1 ± 2.2	1.3 ± 0.1	25:1	59	0.83
20	3.0 ± 0.2	31.1 ± 1.5	1.1 ± 0.2	28:1	54	0.78
40	3.0 ± 0.3	29.8 ± 1.3	1.2 ± 0.1	25:1	52	0.75

Initially, the medium was supplemented with 3.0 g/L $(NH_4)_2SO_4$ and 40 g/L biodiesel waste containing glycerol (28.3 g/L) and fatty acids (9.56 g/L)

Table 5 Effect of pH on the growth of *Y. lipolytica* VKM Y-2412 and production of ketoacids

pH	Growing culture* (144 h)						Resting cells** incubated for 36 h	
	Biomass (g/L)	KGA (g/L)	PA (g/L)	KGA:PA	q_{KGA} (mg/g h)	Y_{KGA} (g/g)	q_{KGA} (mg/g h)	Y_{KGA} (g/g)
2.0	1.5 ± 0.2	6.1 ± 0.4	0.7 ± 0.1	9:1	21	0.15	56	0.73
3.0	2.5 ± 0.1	32.3 ± 0.5	1.1 ± 0.1	29:1	67	0.81	67	0.88
3.5	2.6 ± 0.3	33.1 ± 1.0	1.1 ± 0.2	30:1	66	0.83	67	0.88
4.0	2.7 ± 0.2	35.3 ± 2.5	1.2 ± 0.2	29:1	68	0.88	70	0.91
4.5	2.7 ± 0.2	32.3 ± 2.5	1.2 ± 0.2	27:1	62	0.81	63	0.82
5.0	3.0 ± 0.2	28.3 ± 2.5	1.2 ± 0.3	24:1	49	0.71	62	0.81
5.5	3.2 ± 0.4	29.3 ± 1.7	1.2 ± 0.2	24:1	48	0.73	62	0.81
6.0	3.5 ± 0.2	26.2 ± 1.7	1.0 ± 0.3	26:1	39	0.66	62	0.81
6.5	3.9 ± 0.3	24.2 ± 0.2	1.1 ± 0.2	22:1	32	0.61	57	0.73
7.0	3.9 ± 0.3	22.3 ± 0.4	1.0 ± 0.1	22:1	30	0.56	52	0.68
8.0	2.5 ± 0.2	6.3 ± 0.5	0.7 ± 0.1	9:1	13	0.16	36	0.47

*For growing culture experiments, the medium was supplemented with 3.0 g/L $(NH_4)_2SO_4$ and 40 g/L biodiesel waste containing glycerol (28.3 g/L) and fatty acids (9.56 g/L)

**For resting cell experiments, the medium was supplemented only with 20 g/L biodiesel waste containing glycerol (14.16 g/L) and fatty acids (4.78 g/L)

obtained earlier for the natural strain *Y. lipolytica* WSH-Z06 and its genetically modified transformants with the superexpressed genes of acetyl-CoA synthetase and pyruvate carboxylase. Namely, these strains grown on glycerol required the C:N ratio from 49:1 to 60:1 for the oversynthesis of ketoacids (Zhou et al. 2010, 2012; Yu et al. 2012; Yin et al. 2012).

The experiments described in this paper show that the optimal proportions between some chemical elements in the cultivation medium favorably influence the preferential synthesis of KGA by *Y. lipolytica* VKM Y-2412. We are referring to those elements that serve as activators or inhibitors of enzymes involved in the assimilation of biodiesel waste and enzymes involved in the metabolism of ketoacids (pyruvate carboxylase, citrate synthase, aconitate hydratase, NAD-dependent isocitrate dehydrogenase, etc.). The requirements of *Y. lipolytica* VKM Y-2412 in elements such as Mg, Ca, Na, Zn, Mn, Cu, and Fe were studied in the cultivation regime of pH-auxostat when the nutrient medium is fed to the fermentor by the signal of deviation from the preset value of the culture liquid pH. In the steady state of pH-auxostat, all components of the nutrient medium are in excess and provide for all requirements of the culture in chemical elements. As a result, the planned amount of cell biomass can be obtained using the minimal values of nonlimiting concentrations of chemical elements. It was found that the optimum concentrations of Zn, Fe, Cu, and Mn ions determined in the cultivation regime of pH-auxostat enhanced KGA oversynthesis on average by 2.3 times in comparison with cultivation in Reader medium (Table 3). This result is in agreement with the positive effect of some elements on the rate of KGA synthesis. Also, it was reported the positive effect of Ca^{2+} ions (activator of pyruvate

carboxylase) on the rate of KGA synthesis and the KGA:PA ratio (Zhou et al. 2010, 2012; Cybulski et al. 2018). The increased concentrations of Cu^{2+} and Mn^{2+} ions in the medium stimulated the accumulation of citric acid and KGA by *Y. lipolytica* cultivated on glycerol for the purpose of erythritol production (Tomaszewska et al. 2014). The activity of citrate synthase strongly depends on the ionic strength of the solution: 0.01 M NaCl slightly activates the enzyme; 0.02 and 0.04 M NaCl do not influence it, and higher concentrations of NaCl inhibit it (Morgunov 2009). Presumably, the electrostatic interaction between the positively charged hydrated sodium ions and the negatively charged region close to the active center of citrate synthase may disturb the normal operation of this enzyme.

There is an opinion that the two-stage control of pH value (pH = 5 in the growth stage and pH = 3.5 in the phase of acid production) shifts the production of ketoacids by *Y. lipolytica* toward the formation of KGA (Zhou et al. 2010, 2012; Kamzolova et al. 2012; Kamzolova and Morgunov 2013; Otto et al. 2012; Yu et al. 2012; Yin et al. 2012; Guo et al. 2014; Zeng et al. 2017; Cybulski et al. 2018). However, experiments with the resting cells of *Y. lipolytica* VKM Y-2412 (Table 5) clearly demonstrate that the strong deficiency of thiamine rather than the pH of the cultivation medium is responsible for KGA excretion from the cells. Indeed, resting yeast cells produce KGA from biodiesel waste even at pH = 2 (Table 5). This is a very important observation because fermentation at low pH values reduces the amount of acid necessary for acidification of the fermentation broth upon the isolation and purification of KGA.

The low rate of culture aeration extends the period of its growth, thus stimulating the accumulation of higher biomass.

At the same time, the production of KGA at low aeration is less than at high aeration (Fig. 1). It can be assumed that an increase in biomass and a decrease in acid formation are not the result of a direct effect of low oxygen on the cell and its enzyme systems but rather is associated with the accumulation of excess CO_2 in the medium and an increase in the activity of carboxylating enzymes, primarily pyruvate carboxylase. As a result, part of the formed PA is converted into oxaloacetate, which is involved in the synthesis of various cellular components.

The optimized process of KGA production, presented in Fig. 1a, included the next conditions: during the first 2-day stage of cell growth, the aeration rate and pH value are maintained at 20% oxygen saturation and 5.0 units, respectively. Then, the aeration rate is elevated to 60% oxygen saturation. After 3 days of cultivation, pH is allowed to fall spontaneously to 3.0–4.0 and then is maintained at this level to the end of fermentation. Besides, after 3 days of cultivation, the medium

is additionally supplemented daily with ammonium sulfate in 1.2 g/L portions. If the concentration of dissolved oxygen pO_2 exceeds a value of 10% saturation, biodiesel waste is added to the medium in 40 g/L portions. This process ensured the production of 80.4 g/L KGA with a process selectivity of 96.8%.

According to the literature data, various natural *Y. lipolytica* strains grown on glycerol-containing waste can produce as much as 39.2–72 g/L KGA (Zhou et al. 2010, 2012; Otto et al. 2012; Yu et al. 2012; Yin et al. 2012; Zeng et al. 2017; Cybulski et al. 2018). At the same time, the product yield (Y_{KGA}) was found to be relatively low (0.39–0.71 g/g) because of the significant formation of PA. The predominant synthesis of KGA was achieved using the genetically modified *Y. lipolytica* strain with the superexpressed genes of fumarase and pyruvate carboxylase, which accumulated 138 g/L KGA with a yield of 0.94 g/g during 94-h fermentation (Otto et al. 2012). Another genetically modified *Y. lipolytica* strain with the superexpressed genes of NADP-

Fig. 1 Time courses of the biomass accumulation, ketoacids production, pO_2 profile, glycerol, and fatty acids consumption during cultivation of *Y. lipolytica* on biodiesel waste under high aeration (**a**) and low aeration (**b**)
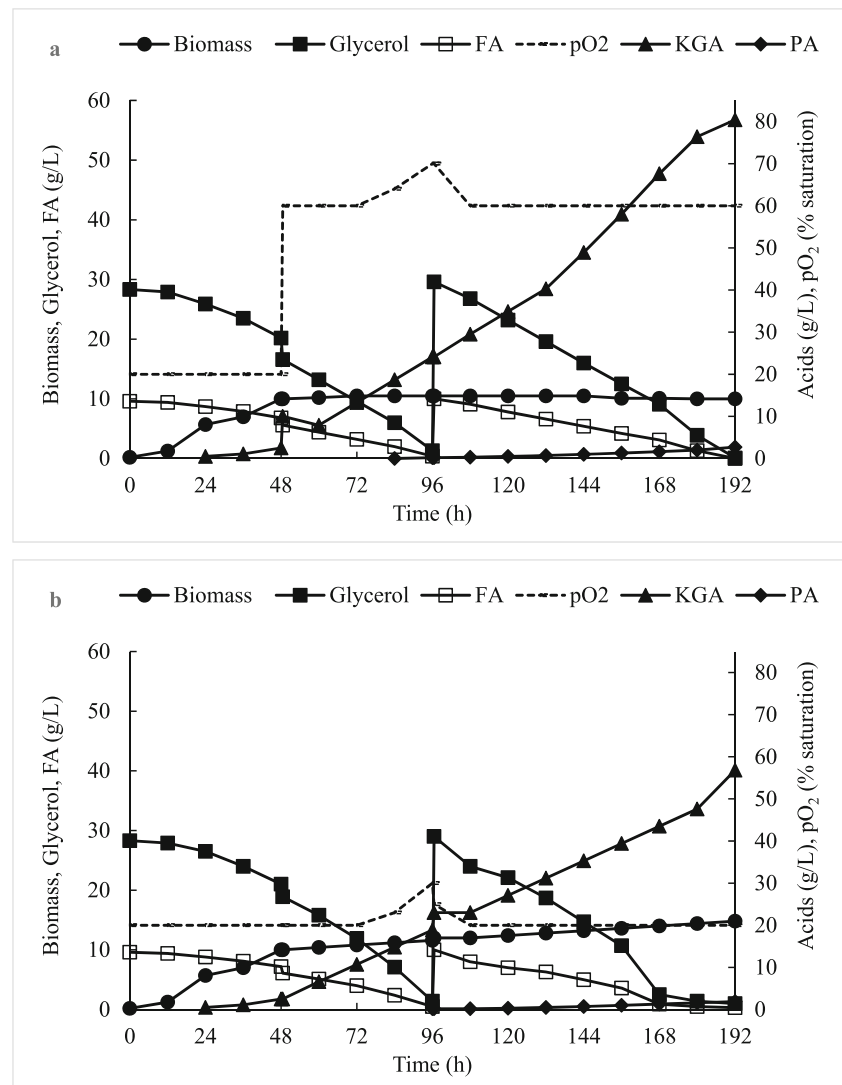
dependent isocitrate dehydrogenase (*IDP1*) and pyruvate carboxylase (*PYC1*) produced 186 g/L KGA with a selectivity of 95% (Yovkova et al. 2014). The superexpression of the carboxylate transporter YALI0B19470g allowed researchers to reach the accumulation of 46.7 g/L KGA and 12.3 g/L PA (Guo et al. 2015).

The putative scheme of assimilation of biodiesel waste by *Y. lipolytica* and oversynthesis of ketoacids is depicted in Fig. 2. The strain *Y. lipolytica* VKM Y-2412 realizes concurrent uptake of glycerol and fatty acid fractions during the conversion of biodiesel waste, although glycerol was utilized at a higher rate than fatty acids. These data are comparable with those obtained for *Y. lipolytica* cultivated on mixtures of saturated free fatty acids (an industrial derivative of animal fat called stearin) and technical glycerol (the main byproduct of biodiesel production facilities) (Papanikolaou et al. 2003). Of interest is the literature data that the mixture of glycerol and fatty acids acts synergetically on the expression of the β-oxidation gene *POX2* (Shabbir Hussain et al. 2017). Fatty acid molecules, free or attached to the cell surface, are transported into the cell interior either by diffusion or by special carriers.

Inside the cell, fatty acids are converted into acyl-CoA and then undergo a series of β-oxidation reactions giving rise to acetyl-CoA (Fickers et al. 2005; Strijbis and Distel 2010; Sekova et al. 2015). The yeast *Y. lipolytica* can also assimilate fatty acids by ω-oxidation with the formation of ω-acids, which are further oxidized into aldoacids and then dicarbonic acids. The latter acids undergo β-oxidation to form the respective number of acetyl-CoA molecules (Gatter et al. 2014).

As seen from the scheme in Fig. 2, glycerol is assimilated through its phosphorylation by glycerol kinase; the generated glycerol-3-phosphate is oxidized by NAD-dependent glycerol-3-phosphate dehydrogenase into dihydroxyacetone phosphate (Ermakova and Morgunov 1987; Morgunov et al. 2004; Makri et al. 2010; Workman et al. 2013; Morgunov and Kamzolova 2015). There is evidence that the assimilation of glycerol can be enhanced by superexpression of the *GUT1* gene coding for glycerol kinase and the *GUT2* gene coding for NAD-dependent glycerol-3-phosphate dehydrogenase. The respective transformant assimilated glycerol and produced acids by 14 times more active than the parent strain (Mirończuk et al. 2016). Dihydroxyacetone phosphate is
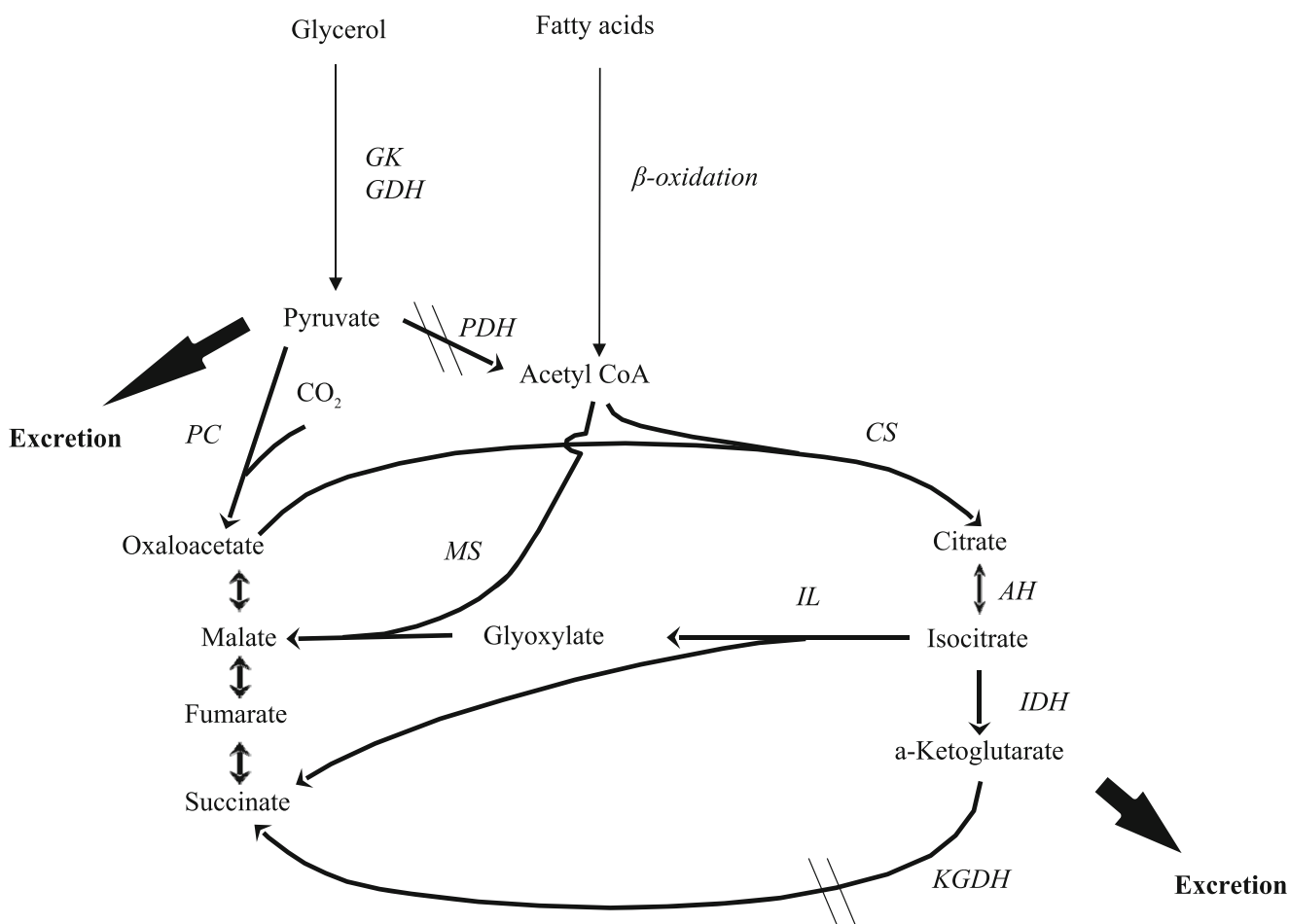

Fig. 2 Putative scheme of oxidation of biodiesel waste and the formation of ketoacids in *Y. lipolytica*. Enzymes: GK, glycerol kinase; GDH, NAD-dependent glycerol-3-phosphate dehydrogenase; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; CS, citrate synthase; AH, aconitate hydratase; IDH, isocitrate dehydrogenase; KGDH, α-ketoglutarate dehydrogenase; IL, isocitrate lyase; MS, malate synthase

transformed into pyruvate by glyceraldehyde phosphate dehydrogenase and pyruvate kinase. The pyruvate molecule is converted into acetyl-CoA by two ways: either through decarboxylation by the pyruvate dehydrogenase complex to form acetyl-CoA or through carboxylation by pyruvate carboxylase to form oxaloacetate (Ermakova and Morgunov 1987; Morgunov et al. 2004; Makri et al. 2010; Otto et al. 2012; Yin et al. 2012; Yovkova et al. 2014). Thiamine deficiency makes the complete oxidation of pyruvate impossible because of the impairment of pyruvate dehydrogenase. As a result, pyruvate accumulates in the cell and then is excreted into the environment.

Acetyl-CoA generated during the oxidation of fatty acids is metabolized in the TCA cycle to form KGA (Fig. 2). Under thiamine deficiency, the generated KGA cannot be oxidized further in the TCA cycle because of the limitation of the thiamine-dependent α-ketoglutarate dehydrogenase. As a result, the accumulated KGA is excreted into the culture liquid. Theoretically, the removal of KGA from the cell should disrupt the TCA cycle and stop the generation of KGA. Actually, this does not happen because the removal of intermediates from the TCA cycle is replenished by isocitrate lyase and malate synthase involved in the glyoxylate cycle. The resultant oxaloacetate enters the TCA cycle, thus ensuring its continual operation (Ermakova et al. 1986; Fickers et al. 2005; Otto et al. 2013; Morgunov and Kamzolova 2015; Sabra et al. 2017). As a result, the production of KGA continues until exhaustion of the biodiesel waste. For additional details on α-KG synthesis in *Y. lipolytica*, refer to the reviews of Otto et al. (2011, 2013), Guo et al. (2015, 2016), and Fickers et al. (2020).

The fact that glycerol and fatty acids were consumed simultaneously is of special importance for the development of the efficient regime of biodiesel waste feeding in the KGA production process by *Y. lipolytica*. According to the auxiliary concept of Babel (2009), the mixed utilization of physiologically analogous substrates increases the yield of the process (in our case, up to 1.01 g/g) which is of a very important economic consideration.

To conclude, the result of this study and those of other researchers demonstrate that the cheap and renewable biodiesel waste is a great carbon source for the development of microbial technology of KGA production with a minimum content of the main byproduct PA. The promising microorganism for such technology is the yeast *Y. lipolytica*, which are generally recognized as safe (GRAS) (Groenewald et al. 2014; Zinjarde 2014).

Authors' contributions S.K. and I.M. conceived and designed research. S.K. conducted experiments. S.K. and I.M. analyzed data. S.K. wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This review does not include any researches with human participants or animals performed by any of the authors.

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