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Selection of Producer of α-Ketoglutaric Acid from Ethanol-Containing Wastes and Impact of Cultivation Conditions

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Abstract: Ester–aldehyde fraction (EAF) is a by-product of ethyl-alcohol-producing companies whose purification requires an expensive process. The results of this study illustrate the environmentally friendly and alternative possibility of using EAF to increase their value as substrate to produce α-ketoglutaric acid (KGA) using different yeasts. It was found that some species of the genera *Babjeviella*, *Diutina*, *Moesziomyces*, *Pichia*, *Saturnispora*, *Sugiyamaella*, *Yarrowia* and *Zygoascus* grown under thiamine deficiency accumulate KGA in the medium with an EAF as the sole carbon source. The strain *Y. lipolytica* VKM Y-2412 was selected as the producer. To reach the maximum production of KGA, the cultivation medium should contain 0.3 μg/L thiamine during cultivation in flasks and 2 μg/L in the fermentor; the concentration of $(NH_4)_2SO_4$ should range from 3 to 6 g/L; and the optimal concentrations of Zn^{2+}, Fe^{2+} and Cu^{2+} ions should be 1.2, 0.6 and 0.05 mg/L, respectively. EAF concentration should not exceed 1.5 g/L in the growth phase and 3 g/L in the KGA synthesis phase. At higher EAF concentrations, acetic acid was accumulated and inhibited yeast growth and KGA production. Under optimal conditions, the producer accumulated 53.8 g/L KGA with a yield (Yp/s) of 0.68 g/g substrate consumed.

Keywords: organic waste; by-product of ethyl alcohol industry; ester–aldehyde fraction (EAF); added-value products; α-ketoglutaric acid (KGA); pyruvic acid (PA); microbial synthesis; yeast *Yarrowia lipolytica*

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1. Introduction

The annual production of ethanol in the world is 6.2 billion gallons, the increase in demand being 3–5% [1]. Ethyl alcohol is obtained by distilling fermented raw materials (potatoes, cereal grains, fruits), and then raw spirit is rectified on rectification distillers to obtain a pure ethyl alcohol and reduce dangerous wastes such as the ester–aldehyde fraction (EAF). The fraction mainly contains ethanol (not less than 92 vol%), as well as several volatile substances (aldehydes, alcohols, esters and organic acids), which gives this fraction a specific smell and taste. EAF can be processed further, but this is economically unprofitable. For this reason, the EAF is mostly utilized by burning [2].

There is a need for development of alternative methods of organic waste transformation into commercially profitable products, such as lipids, biomass, organic acids, biomethane, biohydrogen and others [3,4]. Recently, it was indicated that the little-known waste EAF is an excellent substrate with which to produce isocitric acid using the yeast *Yarrowia lipolytica* [5]. Isocitric acid has found wide application in medicine; it is a more active antioxidant than ascorbic acid [6]. In experiments on rats, it favorably influenced their spatial memory [7].

Another compound that can be synthesized with the yeast *Y. lipolytica*, which is in demand in the medical field, is α-ketoglutaric acid (KGA) [8–16]. As noted in these reviews, KGA plays a key role in the cell: it is converted through glutamate dehydrogenase

into glutamate (prevailing in the brain tissue); through α-ketoglutarate dehydrogenase participates in the formation of succinate, the main energy-yielding substance of cells; through transaminases involves amino acids in various metabolic processes, especially energetic ones; and through hydroxylases plays an important role in the regulation of the hypoxia-induced factor (HIF) and in the synthesis of collagen and procollagen. KGA can be used for antitumor therapy and for the treatment of schizophrenia and alcoholism, as well as for the diagnosis of diseases (hepatitis, myocardial infections, muscle dystrophy, dermatitis and others).

Till now, KGA has been produced through chemical synthesis [17]. In recent years, there has been great interest in the data evidencing that KGA can be synthesized potentially in great quantities using selected wild, mutant and recombinant strains of the yeast *Y. lipolytica*. *Y. lipolytica* can produce KGA using a wide range of carbon sources, such as *n*-alcanes [8,10], glycerol [18–25], raw glycerol [26–30], plant oils [31–33], raw oil [32], fatty acids [32], a glycerol–oil mixture [33,34], cellulose [35] and pure ethanol [36,37]. The above works show that in all cases except ethanol, the cultivation medium contains not only KGA, but also the by-product pyruvic acid (PA). However, there are no data on the ability of yeast to produce KGA from EAFs.

The aim of this work was to carry out a comparative study on the ability of yeast from different taxonomic groups to produce KGA during cultivation on EAF and to determine conditions providing the maximum production of the target product.

2. Materials and Methods

2.1. Strains

In this study, the following strains from the All-Russian Collection of Microorganisms (VKM) were used: *Ambrosiozyma angophorae* VKM Y-2218, *Babjeviella inositovora* VKM Y-2494, *Debaryomyces tamarii* VKM Y-2626, *Diutina catenulata* VKM Y-5, *D. catenulata* VKM Y-65, *D. rugosa* VKM Y-67, *Moesziomyces antarcticus* VKM Y-2604, *M. aphidis* VKM Y-2090, *Pichia besseyi* VKM Y-2084, *P. fermentans* VKM Y-293, *P. haplophila* VKM Y-1286, *P. media* VKM Y-1381, *Saturnispora dispora* VKM Y-418, *Sugiyamaella paludigena* VKM Y-2443, *Yarrowia lipolytica* VKM Y-2373, *Y. lipolytica* VKM Y-2412 and *Zygoascus hellenicus* VKM Y-2154. The following strains from the laboratory's yeast collection of aerobic microbial metabolism of the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences were also used: *Candida tropicalis* (Castellani) 303, *Candida maltosa*, *Candida valida*, *Y. lipolytica* 69, *Y. lipolytica* 374/3, *Y. lipolytica* 387, *Y. lipolytica* 581, *Y. lipolytica* 607, *Y. lipolytica* 645 and *Y. lipolytica* 681. The strains were maintained on Reader agar with 1% paraffin at 4 °C.

2.2. Substrate

The ether–aldehyde fraction (EAF) (or the head fraction of ethyl alcohol) was used as the only carbon source for the growth of yeast and the synthesis of KGA. EAF was purchased from NTM Pharm (Nizhny Novgorod, Russia). The EAF was a by-product of rectification of raw spirits originating from agricultural distillers. EAF consisted of 90 vol% ethanol, 1 vol% methanol, 0.5 g/L aldehydes (in terms of acetaldehyde) and 0.4 g/L esters (in terms of ethyl acetate).

2.3. Medium and Cultivation Conditions

The inoculum was grown for 48 h at 30 °C in 750 mL Erlenmeyer flasks with 50 mL of Reader medium. This medium contained (g/L): $(NH_4)_2SO_4$, 3.0; $MgSO_4 \cdot 7H_2O$, 0.7; $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.4; NaCl, 0.5; KH_2PO_4, 1.0; and K_2HPO_4, 0.2. The medium was supplemented with the microelement solution containing (mg/L): KI, 0.1; B^{3+}, 0.01; Mn^{2+}, 0.01; Zn^{2+}, 0.3; Cu^{2+}, 0.01; Mo^{2+} and 0.01; Fe^{2+}, 0.05. The medium contained thiamine and biotin in concentrations of 2.0 or 20 µg/L, respectively. The flasks were shaken at 180–200 rpm. EAF was added twice a day at a concentration of 2.5 g/L. The pH of the medium was maintained during cultivation between 4.5 and 6.0 by the addition of a sterile 10% solution of KOH.

To select the producer or study the effect of thiamine, nitrogen and microelements, 750 mL Erlenmeyer flasks with 50 mL of Reader medium were inoculated with 5 mL of yeast inoculums and incubated at 30 °C on a shaker at 180–200 rpm. The concentrations of thiamine (B_1), biotin (H), $(NH_4)_2SO_4$, Zn^{2+}, Fe^{2+} and Cu^{2+} were varied as indicated in Section 3. EAF was added four times a day at a concentration of 1.25 g/L. The pH of the medium was maintained during cultivation between 4.5 and 6.0 by the addition of a sterile 10% solution of KOH. Cultivation lasted 8 days.

To study the effect of EAF concentration, *Y. lipolytica* VKM Y-2412 was cultivated in a 10 L ANKUM-2M fermentor (Institute of Biological Instrumentation of RAS, Pushchino, Moscow region, Russia) of 5 L. The medium contained (g/L): $(NH_4)_2SO_4$—6.0; $MgSO_4 \cdot 7\ H_2O$—1.4; $Ca(NO_3)_2 \cdot 4\ H_2O$—0.8; NaCl—0.5; KH_2PO_4—2.0; K_2HPO_4—0.2 and additionally was supplemented with microelements (mg/L): KJ, 0.1; B^{3+}, 0.01; Mn^{2+}, 0.01; Zn^{2+}, 1.2; Cu^{2+}, 0.05; Mo^{2+}, 0.01; and Fe^{2+}, 0.6. The pH of the medium during cultivation was maintained automatically at 4.5 by the addition of 20% KOH. The temperature was 30 °C. The concentration of dissolved oxygen was maintained at 50–55% saturation by the regulation of air supply to the fermentor. The agitation did not exceed 800 rpm. Cultivation lasted 8 days.

In the flask experiments, the sampling was carried out at the end of cultivation (192 h), but in the fermentor experiments, the daily sampling was carried out at each 3–6 h to determine the concentration of biomass, KGA, PA, acetate and ethanol and calculate production parameters.

2.4. Analytical Procedures

The biomass content was determined using the gravimetric method. The nitrogen content was determined potentiometrically as described earlier [5]. The concentration of ethanol (not EAF) in the medium was analyzed using gas–liquid chromatography on a Chrom-5 chromatograph (Laboratory Instruments, Praha, Czech Republic) equipped with a Chromaton N-AW column (0.160–0.200 mm) packed with 15% Reoplex 400; the column temperature was 65 °C. A sample for the determination of organic acids (KGA, PA, acetic acid and others) was prepared as described previously [5] and analyzed by high-performance liquid chromatography (HPLC) on a chromatograph (LKB, Stockholm, Sweden) equipped with an Inertsil ODS-3 reversed-phase column (250 × 4 mm, Elsiko, Moscow, Russia) at 210 nm; the column temperature was 35 °C. The mobile phase consisted of 20 mM phosphoric acid and had a flow rate of 1.0 mL/min.

2.5. Calculations

The production parameters, such as the KGA yield from the consumption of substrate (Yp/s) (g/g), the KGA yield relative to biomass formed (Yp/x) (g/g) and the volumetric productivity of KGA production (Qp) (g/L·h), were calculated using the equations: Yp/s = p/s, Yp/x = p/x and Qp = p/(v·t), where p is the total amount of KGA in the culture liquid at the end of cultivation (g), s is the total amount of ethanol (not EAF) consumed (g), x is the total amount of biomass in the culture liquid at the end of cultivation (g), v is the initial volume of culture liquid (L), and t is the time of incubation (h).

2.6. Statistical Analysis

All the data presented represent the mean ± standard deviation of three experiments and two measurements for each experiment. Data related to KGA biosynthesis were subjected to analysis of variance using Student's *t*-test ($p < 0.05$); the differences between the values were statistically significant if the confidence intervals did not overlap. The percentage of KGA and the values of $Y_{p/x}$, $Y_{p/s}$ and Q_p were calculated using the mean values of biomass, KGA and ethanol consumed.

3. Results and Discussion

3.1. Selection of Producer

Among the yeasts (28 strains) subject to selection, 25 strains were thiamine-auxotrophic (need thiamine for growth), of which 8 strains also required biotin, and only 3 strains were thiamine-independent (Table 1).

Table 1. KGA production using EAF with different yeast strains in shake flasks.

Strains	Thiamine (μg/L)	Biotin (μg/L)	Biomass (g/L)	KGA (g/L)	PA (g/L)	KGA (% from Total Acids)	Yp/x (g/g)	Yp/s (g/g)
Ambrosiozyma angophorae VKM Y-2218	0.5	20	0.95 ± 0.07	0.49 ± 0.02	0.04 ± 0.0	92.59	0.51	0.02
Babjeviella inositovora VKM Y-2494	0.5	20	5.42 ± 0.4	5.31 ± 0.83	0.63 ± 0.1	89.45	0.98	0.22
Candida tropicalis (*Castellani*) 303	-	20	9.41 ± 0.7	0	0	nd	nd	nd
C. maltosa	-	20	9.50 ± 0.71	0	0	nd	nd	nd
C. valida	-	-	9.31 ± 0.69	0	0	nd	nd	nd
Debaryomyces tamarii VKM Y-2626	0.5	20	7.89 ± 0.59	0.10 ± 0.0	0.10 ± 0.0	50.00	0.01	nd
Diutina catenulata VKM Y-5	0.5	20	7.13 ± 0.53	2.31 ± 0.27	1.08 ± 0.17	68.17	0.32	0.10
D. catenulata VKM Y-65	0.5	20	7.79 ± 0.58	2.83 ± 0.10	0.15 ± 0.01	95.08	0.36	0.12
D.rugosa VKM Y-67	0.5	20	8.10 ± 1.27	2.45 ± 0.49	0.20 ± 0.01	92.63	0.30	0.10
Moesziomyces antarcticus VKM Y-2604	0.5	-	8.55 ± 0.64	4.00 ± 0.14	0.20 ± 0.01	95.35	0.47	0.17
Moesziomyces aphidis VKM Y-2090	0.5	-	1.81 ± 0.13	1.17 ± 0.04	0.59 ± 0.02	66.67	0.65	0.05
Pichia besseyi VKM Y-2084	0.5	-	8.10 ± 1.13	5.07 ± 0.18	0.20 ± 0.01	96.30	0.63	0.21
Pichia fermentans VKM Y-293	0.5	-	3.64 ± 0.51	0.88 ± 0.03	0.20 ± 0.01	81.82	0.24	0.04
Pichia haplophila VKM Y-1286	0.5	20	2.37 ± 0.33	0.06 ± 0.00	0.07 ± 0.00	46.15	0.02	nd
Pichia media VKM Y-1381	0.5	20	7.41 ± 0.55	4.97 ± 0.18	0.24 ± 0.01	95.33	0.67	0.21
Saturnispora dispora VKM Y-418	0.5	-	7.64 ± 1.07	5.50 ± 0.71	0.20 ± 0.01	96.58	0.72	0.23
Sugiyamaella paludigena VKM Y-2443	0.5	-	5.99 ± 0.45	4.00 ± 0.14	1.56 ± 0.06	71.93	0.67	0.17
Yarrowia lipolytica VKM Y-2373	0.5	-	4.00 ± 0.56	4.97 ± 0.18	0.59± 0.02	89.47	1.24	0.21
Y. lipolytica VKM Y-2412	0.5	-	3.42 ± 0.25	8.00 ± 0.29	0.78 ± 0.03	91.11	2.34	0.34
Y. lipolytica 47	0.5	-	3.02 ± 0.42	5.45 ± 0.20	0.20 ± 0.01	96.55	1.80	0.23
Y. lipolytica 69	0.5	-	2.73 ± 0.38	3.32 ± 0.12	0.07± 0.00	97.98	1.21	0.14
Y. lipolytica 374	0.5	-	2.74 ± 0.20	3.67 ± 0.13	0.20 ± 0.01	94.95	1.34	0.15
Y. lipolytica 387	0.5	-	2.85 ± 0.40	3.59 ± 0.13	0.29 ± 0.01	92.46	1.26	0.15
Y. lipolytica 581	0.5	-	3.33 ± 0.25	4.76 ± 0.17	0	100.00	1.43	0.20
Y. lipolytica 607	0.5	-	3.56 ± 0.27	3.39 ± 0.12	0.20 ± 0.01	94.57	0.95	0.14
Y. lipolytica 645	0.5	-	3.98 ± 0.30	2.95 ± 0.11	0.07 ± 0.00	97.74	0.74	0.12
Y. lipolytica 681	0.5	-	3.41 ± 0.48	2.67 ± 0.10	0.59 ± 0.02	82.04	0.78	0.11
Zygoascus hellenicus VKM Y-2154	0.5	-	1.09 ± 0.15	2.24 ± 0.08	0.29 ± 0.01	88.46	2.05	0.09

"-"—does not need vitamin; "nd"—not determined; KGA—α-ketoglutaric acid; PA—pyruvic acid; Yp/x—KGA yield relative to biomass formed; Yp/s—KGA yield from the consumption of substrate. Culture conditions: growth on flasks for 8 days; the sampling was carried out at the end of cultivation.

As seen from the data in Table 1, most strains (25) out of the 28 investigated were able to produce KGA in amounts from 0.1 to 8 g/L during growth on the ester–aldehyde fraction (EAF) under thiamine deficiency (0.5 μg/L). These strains belonged to the species *Ambrosiozyma angophorae*, *Babjeviella inositovora*, *Debaryomyces tamarii*, *Diutina catenulata*, *D. rugosa*, *Moesziomyces antarcticus*, *M. aphidis*, *Pichia besseyi*, *P. fermentans*, *P. haplophila*, *P. media*, *Saturnispora dispora*, *Sugiyamaella paludigena*, *Yarrowia lipolytica* and *Zygoascus hellenicus*. The distribution of strains with respect to KGA production was as follows: 4 strains (up to 1 g/L), 7 strains (1–3 g/L) and 14 strains (over 3 g/L). Besides KGA, the strains also accumulated PA (0.04–1.56 g/L) as a by-product. The most efficient 14 strains

accumulated predominantly KGA (71–100% of the acid sum). The biomass accumulated by the strains grown on the EAF varied from 0.95 to 8.55 g/L. The KGA yield relative to biomass formed (Yp/x) and the KGA yield from the consumption of the substrate (Yp/s) among 14 active strains varied from 0.47 to 2.34 g/g and from 0.17 to 0.34 g/g, respectively.

The most efficient strain turned out to be *Y. lipolytica* VKM Y-2412, which had the following parameters: 8 g/L KGA, a Yp/x value of 2.34 g/g, a Yp/s value of 0.34 g/g and a KGA proportion of 91.11%. This strain was chosen for further studies.

It should be noted that the selected strain, *Y. lipolytica* VKM Y-2412, also actively synthesized KGA on purified ethanol [37], rapeseed oil [31] and glycerol-containing biodiesel waste [30].

According to the literature data, different wild producers isolated include *Y. lipolytica* WSH-ZO6 cultivated on glycerol [18,20] and crude glycerol [25]; *Y. lipolytica* H355 on *n*-alkane [10], crude glycerol [26] and plant oils [10]; *Y. lipolytica* A-101 on plant oils [32]; and *Y. lipolytica* 695 on acetate and glucose [38]. Based on the strain *Y. lipolytica* WSH-Z06, the recombinant strains *Y. lipolytica*-ScPYC-1 and *Y. lipolytica*-RoPYC2 with the superexpression of the gene *PYC1* encoding pyruvate carboxylase were created [13]. This approach increased the carboxylation of PA and triggered yeast metabolism toward KGA excretion via the reductive pathway of the TCA cycle. The recombinant strains *Y. lipolytica*-ACS1 and *Y. lipolytica*-ACL1 derived from the wild-strain *Y. lipolytica* WSH-Z06 showed the superexpression of the gene *ACS1* encoding acetyl-CoA synthetase and the gene *ACL1* encoding ATP-citrate lyase; as a result, the high level of acetyl-CoA promoted KGA formation [20]. Based on the strain *Y. lipolytica* H355, the recombinant strains with overexpression of pyruvate carboxylase and NADP+ isocitrate dehydrogenase were created [26,27]. Transformants of *Y. lipolytica* were constructed to overexpress genes encoding glycerol kinase, methylcitrate synthase and a mitochondrial organic acid transporter to improve KGA production in glycerol- and oil-based media [34]. The random mutagenesis of *Y. lipolytica* WSH-Z06 allowed for several mutants with enhanced KGA production to be derived. Their comparative genomic analysis showed that manipulation with genes regulating mitochondrial biogenesis and energetic metabolism can enhance KGA production, while genes involved in the regulation of transformation between ketoacids and amino acids inhibit KGA production [28].

3.2. Effect of Thiamine

The species *Y. lipolytica* is not able to synthesize the pyrimidine part of thiamine molecules. As a result, thiamine shortage drastically decreases the activity of α-ketoglutarate dehydrogenase with thiamine pyrophosphate as a cofactor. The excess of KGA in cells is excreted into the cultivation medium [8–12,14,36–39].

The effect of thiamine on the growth of *Y. lipolytica* VKM Y-2412 and KGA production was studied using concentrations from 0 to 10 μg/L (Table 2). Cell growth was observed in the thiamine-free medium apparently due to the traces of thiamine available from the inoculum. Linear dependence between the biomass of *Y. lipolytica* VKM Y-2412 and thiamine concentration was observed to range from 0 to 2.5 μg/L, indicating that cell growth was limited only by thiamine. The increase in thiamine concentration from 2.5 to 10 μg/L did not improve cell growth notably. The active excretion of KGA (8.07–8.95 g/L) was observed in a very narrow range of thiamine concentrations from 0.3 to 0.5 μg/L. Below this range, KGA production decreased by 2.1 times. The increase in thiamine concentration from 0.5 to 2.5 μg/L decreased KGA formation by 16 times. Thiamine in concentrations above 5 μg/L totally inhibited KGA production. The proportion of KGA was high (90.81–100% of the acid sum) at all thiamine concentrations studied. The maximal values of Yp/x (3.73 g/g) and Yp/s (0.38 g/g) were observed at 0.3 μg/L thiamine.

Table 2. The effect of thiamine for KGA production.

Thiamine (μg/L)	Biomass (g/L)	KGA (g/L)	PA (g/L)	KGA (% from Total Acids)	Yp/x (g/g)	Yp/s (g/g)
0	1.70 ± 0.08	4.21 ± 0.20	0.00 ± 0.01	100.00	2.48	0.18
0.1	2.10 ± 0.08	5.84 ± 0.15	0.13 ± 0.01	99.90	2.78	0.25
0.3	2.40 ± 0.17	8.95 ± 0.10	0.76 ± 0.04	92.18	3.73	0.38
0.5	3.55 ± 0.27	8.07 ± 0.19	0.81 ± 0.05	90.85	2.27	0.34
1.0	4.21 ± 0.17	4.38 ± 0.10	0.43 ± 0.04	91.05	1.04	0.18
1.5	4.87 ± 0.29	3.04 ± 0.31	0.31 ± 0.02	90.81	0.62	0.13
2.0	6.18 ± 0.19	1.70 ± 0.17	0.15 ± 0.02	91.77	0.27	0.07
2.5	6.47 ± 0.37	0.51 ± 0.03	0.03 ± 0.03	95.31	0.08	0.02
5.0	6.94 ± 0.27	0	0	nd	nd	nd
10.0	8.37 ± 0.30	0	0	nd	nd	nd

"nd"—not determined; KGA—α-ketoglutaric acid; PA—pyruvic acid; Yp/x—KGA yield relative to biomass formed; Yp/s—KGA yield from the consumption of substrate. Culture conditions: growth on flasks for 8 days; the sampling was carried out at the end of cultivation.

Earlier studies showed that the mutant *Y. lipolytica* N 1 grown on purified ethanol produces KGA most actively (3.0–8.0 g/L) also in the narrow range of thiamine concentrations (0.2–0.4 μg/L) [36]. The same result was obtained for other yeasts grown on *n*-alkanes, while optimal concentrations of thiamine for yeast grown on glucose were 3–4 times higher [40]. This fact can be explained by the difference in the metabolism of glucose and *n*-alkanes, as well as a varying degree of the involvement of thiamine-dependent enzymes. The optimal concentration of thiamine for *Y. lipolytica* H222-27-11 producing KGA from rapeseed oil was found to be 0.1 μg/L [9,11]. For the strain *Y. lipolytica* WSH-Z06 grown on glycerol, the optimal thiamine concentration was 0.2 μg/L, and higher thiamine concentrations promoted growth and inhibited acid formation [18].

Further experiments were carried out with 0.3 μg/L thiamine in the cultivation medium.

3.3. Effect of Nitrogen

The effect of nitrogen on KGA production using *Y. lipolytica* VKM Y-2412 was studied in the range of $(NH_4)_2SO_4$ concentrations from 0.3 to 10.0 g/L (nitrogen content from 0.06 to 2.12 g/L) at a constant concentration of the EAF (24.12 g/L). As seen from the data in Table 3, KGA synthesis depended on the nitrogen content in the cultivation medium. The amount of synthesized KGA increased from 0.49 to 8.53 g/L when the nitrogen concentration in the medium increased from 0.06 g/L (C/N ratio 197) to 0.21 g/L (C/N ratio 59). A further increase in the nitrogen content to 0.64 g/L did not enhance KGA production and even suppressed it at concentrations greater than 0.85 g/L.

Based on the data on nitrogen consumed and biomass synthesized, we calculated the nitrogen content (mg of nitrogen per g of biomass). Data in Table 3 show that the maximal values of Yp/x (3.63–3.73 g/g) were observed at a nitrogen content of 90–110 mg/g. In this case, the KGA yield from the consumption of the substrate (Yp/s) was 0.35–0.37 g/g. The increase in the nitrogen content to 970 mg/g reduced these parameters by 3 and 3.7 times, respectively.

Similar results have been obtained for *Y. lipolytica* grown on other substrates. The strain *Y. lipolytica* VKM Y-2412 grown on glycerol-containing waste from biodiesel production could actively synthesize KGA only if the content of ammonium sulfate in the medium was not less than 2 g/L (C/N ratio not less than 43:1) [30]. In the case of media with *n*-alkanes, the concentration of ketoacids synthesized by unit biomass increased from 0.23 g/g (C/N = 300) to 1.53 g/g (C/N = 30) [38].

Table 3. The effect of nitrogen for KGA production.

$(NH_4)_2SO_4$ (g/L)	Nitrogen (g/L)	C/N Ratio	Biomass (g/L)	KGA (g/L)	PA (g/L)	N content (mg/g)	KGA (% from Total Acids)	Yp/x (g/g)	Yp/s (g/g)
0.3	0.06	197	2.16 ± 0.14	3.49 ± 0.03	0.04 ± 0.01	30	92.02	0.23	0.02
0.5	0.11	119	2.29 ± 0.17	3.37 ± 0.26	0.38 ± 0.05	50	89.98	1.47	0.14
1.0	0.21	59	2.35 ± 0.13	8.53 ± 0.28	0.68 ± 0.06	90	92.66	3.63	0.35
2.0	0.42	30	2.36 ± 0.11	8.81 ± 0.14	0.70 ± 0.04	100	92.62	3.73	0.37
3.0	0.64	20	2.40 ± 0.17	8.95 ± 0.10	0.76 ± 0.04	110	92.18	3.73	0.37
4.0	0.85	15	2.88 ± 0.13	6.89 ± 0.35	0.52 ± 0.09	220	93.01	2.40	0.29
6.0	1.27	10	3.15 ± 0.13	3.31 ± 0.21	0.27 ± 0.07	340	92.51	1.05	0.14
8.0	1.70	7	2.25 ± 0.14	2.68 ± 0.10	0.27 ± 0.05	680	90.75	1.19	0.11
10.0	2.12	6	2.03 ± 0.13	2.53 ± 0.12	0.23 ± 0.07	970	91.73	1.25	0.10

KGA—α-ketoglutaric acid; PA—pyruvic acid; Yp/x—KGA yield relative to biomass formed; Yp/s—KGA yield from the consumption of substrate. Culture conditions: growth on flasks for 8 days; the sampling was carried out at the end of cultivation.

In the case of the wild-strain *Y. lipolytica* WSH-Z06 and its transformants with the superexpression of genes encoding acetyl-CoA synthetase or pyruvate carboxylase, ketoacids are actively synthesized at a C/N ratio ranging from 49:1 to 60:1 [18,20–22]. The use of polypeptone as a nitrogen source favored yeast growth but suppressed KGA production [18]. This result can be explained by the fact that polypeptone contains various nutrients, including thiamine.

The importance of nitrogen content in the cultivation medium for the excretion of organic acids (KGA and citric acid) has been shown for the mutant *Y. lipolytica* N 1 grown on purified ethanol. A nitrogen content equal to 0.64 g/L becomes a growth-limiting factor and triggers cells to synthesize citric acid instead of KGA. KGA production was most active at a concentration of ammonium sulfate equal to 10 g/L [36].

Further experiments in flasks were carried out with media containing 3 g/L $(NH_4)_2SO_4$.

3.4. Effect of Microelements

During the last few decades, more attention has been given to the effect of microelements (mostly Zn^{2+}, Fe^{2+} and Cu^{2+}) on the physiology of *Y. lipolytica* [30,41–44]. It became clear that different strains of *Y. lipolytica* have different needs in these ions, which are either activators or inhibitors of many enzymes. Taking this into account, we determined the optimal concentrations of these microelements for *Y. lipolytica* VKM Y-2412 cultivated in media with the EAF.

3.4.1. Zinc

Figure 1 shows the dependence of biomass and acid formation on the concentration of zinc in the medium (0.3–5 mg/L) at a constant concentration of Fe^{2+} (0.05 mg/L) and Cu^{2+} (0.01 mg/L). The increase in zinc concentration from 0.3 to 1.2 mg/L stimulated KGA production by 29%. Higher zinc concentrations (up to 5 mg/L) suppressed KGA production. In all experiments, the content of PA did not exceed 10% of the acid sum. The maximal values of Yp/x (3.89 g/g) and Yp/s (0.49 g/g) occurred when the zinc concentration was 1.2 mg/L and decreased to 1.25 g/g and 0.15 g/g, respectively, at 5 mg/L Zn.

The strain *Y. lipolytica* VKM Y-2412, when cultivated on pure ethanol, also needs Zn for KGA overproduction [30], while in experiments with glycerol-containing substrates, zinc was not added to the medium [18,20,22,29,30]. The elevated need for Zn of *Y. lipolytica* cultivated on ethanol and EAF can be explained by activation of zinc-containing alcohol dehydrogenase (EC 1.1.1.1), which initiates ethanol assimilation.

Further experiments were carried out with media containing 1.2 mg/L Zn.

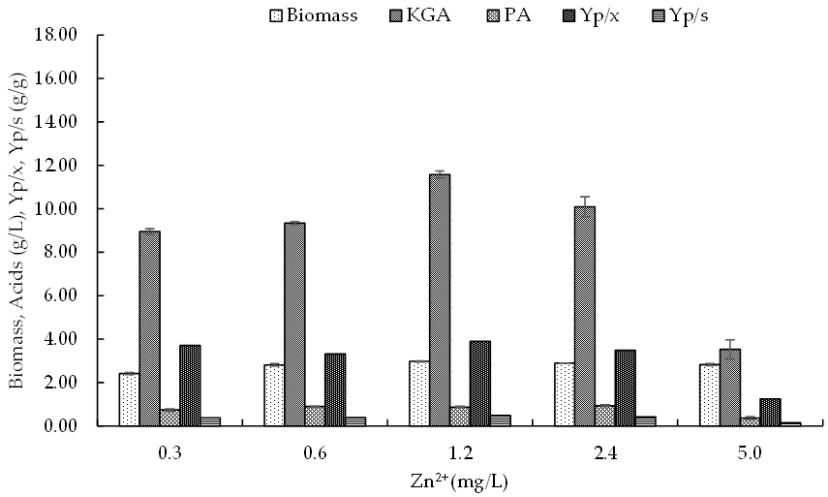

Figure 1. Effect of zinc ions on the growth of *Y. lipolytica* VKM Y-2412 and KGA synthesis in media with EAF. KGA—α-ketoglutaric acid; PA—pyruvic acid; Yp/x—KGA yield relative to biomass formed; Yp/s—KGA yield from the consumption of substrate. Culture conditions: growth on flasks for 8 days; the sampling was carried out at the end of cultivation.

3.4.2. Iron

It is known that iron ions influence the metabolism of microorganisms, being components of many enzymes and proteins, such as aconitate hydratase (EC 4.2.1.3), which is involved in the synthesis of isocitric acid (a precursor of KGA), as well as cytochrome *c*, catalases, etc. The alcohol dehydrogenases of *Zymomonas mobilis* [45] and *Acinetobacter baumannii* [46] contain iron instead of zinc in the catalytic center. In media with an iron shortage, microbial growth is limited by available energy resources in the cell [47]. Under iron deficiency, the conversion of organic substance falls; most acetyl-CoA does not enter the TCA cycle and is condensed with the formation of ethyl acetate [48]. As shown in the work [49], the increase in iron concentration in cultivation media activates the TCA cycle, respiratory chain and oxidative phosphorylation in *Y. lipolytica*.

Results from the present work also demonstrate the essential effect of iron on the metabolism of *Y. lipolytica* VKM Y-2412 grown in media with the EAF (Figure 2). As seen from Figure 2, at 0.1 mg/L Fe^{2+} in the medium (the ratio Zn^{2+}/Fe^{2+} = 12:1), the biomass and KGA were lower than when the concentration of Fe^{2+} ions was 0.6 mg/L (the ratio Zn^{2+}/Fe^{2+} = 2:1). The increase in iron concentration from 0.6 to 5 mg/L stimulated growth by 26%, but suppressed KGA synthesis by 13 times. The concentration of PA increased from 7 to 24% when the concentration of Fe^{2+} was raised from 0.1 to 5 mg/L. The maximal values of Yp/x (3.84 g/g) and Yp/s (0.62 g/g) occurred when the Fe^{2+} concentration was 0.6 mg/L and sharply decreased by 18 and 13 times, respectively, at 5 mg/L Fe^{2+}. These data may indicate both the inhibiting action of Fe^{2+} ions and nonoptimal proportion of iron and zinc in the medium.

According to literature data, the yeasts *Torulopsis* and *Yarrowia* cultivated on ethanol excrete the acetate at high Zn^{2+}/Fe^{2+} ratios. The increase in iron proportion hinders the accumulation of acetate due to activation of iron-dependent aldehyde dehydrogenase and stimulates yeast growth [49]. As the authors of this review believed, aldehyde dehydrogenase is activated not only through de novo synthesis, but also through the partial or complete change of enzymes involved in the primary step of ethanol oxidation. As a result, the intracellular pool of NAD rises and activates aldehyde dehydrogenase.

Further experiments were carried out at the ratio Zn^{2+}/Fe^{2+} = 2:1.

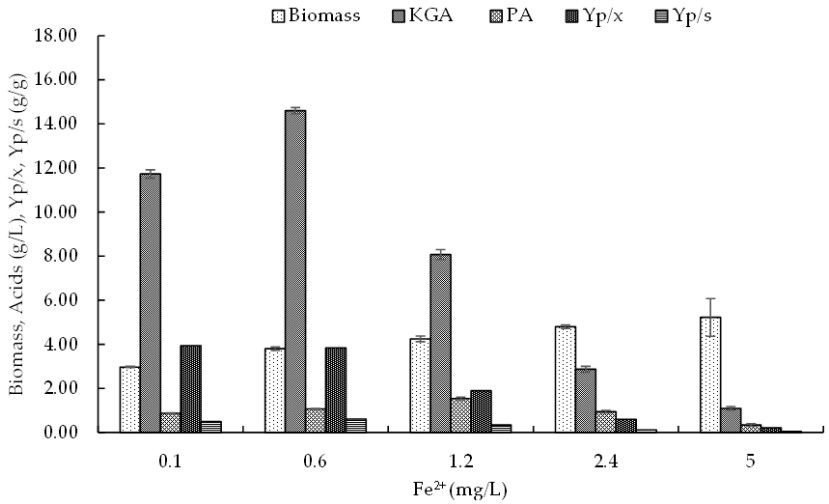

Figure 2. Effect of iron ions on the growth of *Y. lipolytica* VKM Y-2412 and KGA synthesis in media with EAF. KGA—α-ketoglutaric acid; PA—pyruvic acid; Yp/x—KGA yield relative to biomass formed; Yp/s—KGA yield from the consumption of substrate. Culture conditions: growth on flasks for 8 days; the sampling was carried out at the end of cultivation.

3.4.3. Copper

As seen from Figure 3, the increase in Cu^{2+} concentration from 0.01 to 0.15 mg/L promoted growth by 1.4 times and provided a high level of KGA production (13.03–15.83 g/L). Above 0.15 mg/L Cu^{2+}, the production of KGA fell by 5.3 times from the maximum value at 0.05 mg/L Cu^{2+}. The maximal values of Yp/x (3.84 g/g) and Yp/s (0.67 g/g) occurred when the Cu^{2+} concentration was 0.05 mg/L and sharply decreased by 8.6 and 4.5 times, respectively, at 0.2 mg/L Cu^{2+}.

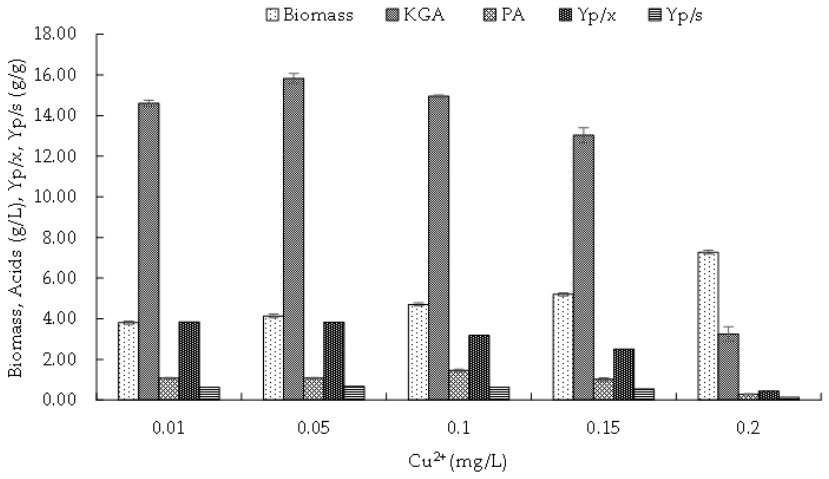

Figure 3. Effect of copper ions on the growth of *Y. lipolytica* VKM Y-2412 and KGA synthesis in media with EAF. KGA—α-ketoglutaric acid; PA—pyruvic acid; Yp/x—KGA yield relative to biomass formed; Yp/s—KGA yield from the consumption of substrate. Culture conditions: growth on flasks for 8 days; the sampling was carried out at the end of cultivation.

The favorable effect of copper ions on the KGA synthesis by *Y. lipolytica* is known. For instance, there is evidence that 2.5–5 mg/L Cu^{2+} in the cultivation medium of *Y. lipolytica* cultivated on glycerol stimulates the accumulation of erythritol, citric acid and KGA [43]. Similarly, the addition of 1.4 mg/L copper stimulated KGA formation in media with glycerol-containing waste from biodiesel production [30].

The foregoing shows that both the concentration of microelements Zn^{2+}, Fe^{2+} and Cu^{2+} and their proportions are important for the cultivation of *Y. lipolytica* VKM Y-2412, producing KGA.

3.5. Effect of EAFs

The strain *Y. lipolytica* VKM Y-2412 was cultivated in the fermentor in two variants of EAF supply, the small-dose feeding (1.5 g/L EAF during growth and 3 g/L in the phase of synthesis) and the large-dose feeding (3 g/L EAF during growth and 6 g/L in the phase of synthesis) (Figure 4). To increase cell density, the thiamine and $(NH_4)_2SO_4$ concentrations in the medium were increased to 2 μg/L and 6 g/L, respectively.

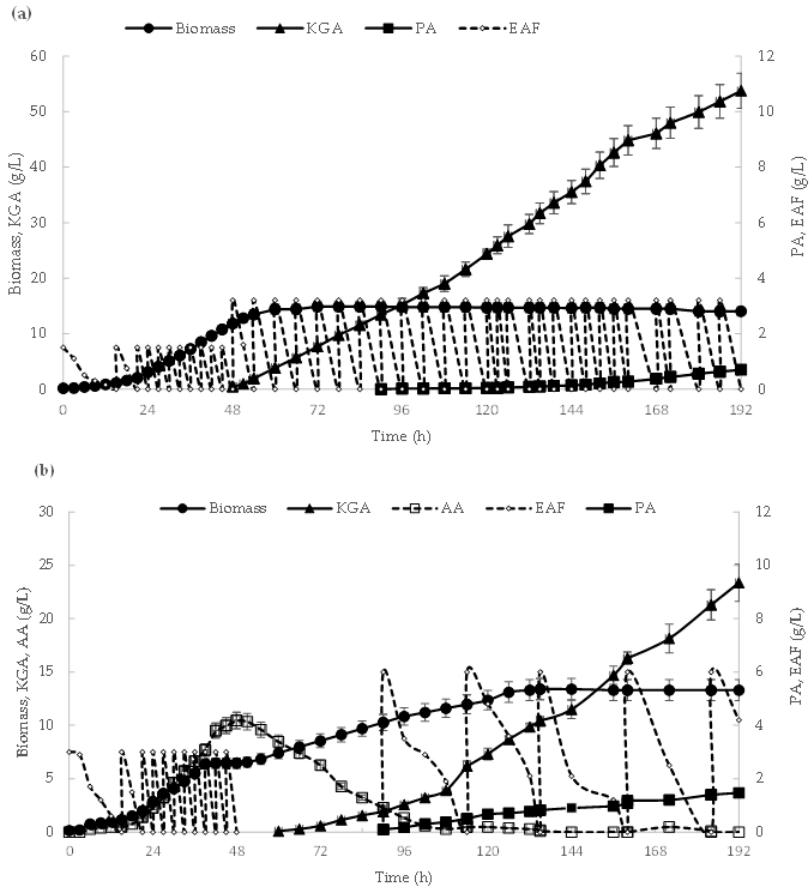

Figure 4. Effect of EAF concentration on the growth of *Y. lipolytica* VKM Y-2412 and synthesis of organic acids: (**a**) the small-dose feeding; (**b**) the large-dose feeding. KGA—α-ketoglutaric acid; PA—pyruvic acid; AA—acetic acid; EAF—ester–aldehyde fraction. Culture conditions: growth on fermentor for 8 days. Error bars represent the standard deviation from the mean.

At the small-dose feeding (Figure 4a), the yeast well grew for 48 h and accumulated 11.8 g/L biomass. Then, growth was retarded, and *Y. lipolytica* began to synthesize KGA. Synthesis continued in the stationary phase until the EAF was exhausted. By the end of cultivation (192 h), the yeast had accumulated 53.8 g/L KGA and 3.5 g/L PA.

At the large-dose feeding (Figure 4b), the yeast grew for 48 h and accumulated 10.5 g/L acetic acid (AA), which was then consumed. As a result, the biomass was 1.8 times lower than in the small-dose feeding. Then, the supply of the EAF to the fermentor was stopped to exhaust AA. This resulted in a biomass enhancement from 6.46 to 10.26 g/L, KGA synthesis being low (2 g/L). When the EAF supply was restored (after 90 h of cultivation), KGA synthesis continued without notable formation of AA. By the end of cultivation, the yeast had accumulated 23.4 g/L KGA and 1.47 g/L PA.

These data show that the high content of the EAF during the growth phase of *Y. lipolytica* VKM Y-2412 influenced the excretion of AA, which inhibited yeast growth. At the same time, a high EAF concentration did not inhibit active KGA synthesis at high cell density. The same concentration of the EAF could inhibit the growth of a sparse cell population and did not affect the growth of the dense population.

There are few data concerning the effect of AA on the physiology of *Y. lipolytica*. It has been shown that AA decreases the specific growth rate of *Y. lipolytica* cultivated on glucose [50]. The plasmatic membrane protein Gpr1p found in *Y. lipolytica* helps microbial cells to adapt to AA; the synthesis of this protein is induced by AA and slightly suppressed by glucose [51]. It has been shown that elevated concentrations of glucose- or glycerol-containing waste from biodiesel production led to the secretion of AA in significant amounts in *Y. lipolytica* [52,53].

The most evident factor that inhibits the growth of *Y. lipolytica* VKM Y-2412 at high EAF concentrations is acetaldehyde, which is oxidized enzymatically or spontaneously to acetic acid due to its high reactivity. On the other hand, acetate may accumulate in the medium due to a sharp decrease in the activity of citrate synthase responsible for the biosynthesis of citric acid [54] and is involved in biosynthesis of KGA in the TCA cycle.

Table 4 presents data on the production parameters of *Y. lipolytica* VKM Y-2412 for two feeding modes.

Table 4. The effect of EAF for production parameters.

Type of Feeding	Yp/x (g/g)	Yp/s (g/g)	Qp (g/L·h)
Small-dose feeding	3.84	0.68	0.38
Large-dose feeding	1.76	0.52	0.15

Small-dose feeding—1.5 g/L EAF during growth and 3 g/L in the phase of synthesis; large-dose feeding—3 g/L EAF during growth and 6 g/L in the phase of synthesis; Yp/x—KGA yield relative to biomass formed; Yp/s—KGA yield from the consumption of substrate; Qp—volumetric productivity of KGA production.

Data in Table 4 show that the highest values of the volumetric productivity of KGA production (Qp) (0.38 g/L h), the KGA yield relative to biomass formed (Yp/x) (3.84 g/g) and the KGA yield from the consumption of the substrate (Yp/s) (0.68 g/g) were observed for the small-dose feeding. An increase in the dose of the EAF during the period of growth and acid formation led to the decrease in the values of Qp, Yp/x and Yp/s by 2.5, 2.2 and 1.31 times, respectively.

It should be noted that the maximal yield (Yp/s) of 0.68 g/g obtained in this study was a 53.5% theoretical maximal yield on this substrate. Earlier, we indicated that the same strain produced KGA with the same yield (Yp/s) (0.70 g/g) from pure ethanol [40]. Thus, the low yield (Yp/s) in media with the EAF was not associated with harmful impurities (aldehydes, esters and methanol) originating from this substance. The subsequent optimization of cultivation conditions to increase production parameters may be the subject of further research.

The literature indicates that *Y. lipolytica* can synthesize KGA on other organic wastes. The selected wild-strain *Y. lipolytica* WSH-Z06 grown on raw glycerol produced 78.1 g/L KGA with a yield (Yp/s) of 0.52 g/g and a productivity (Qp) of 0.38 g/L·h [29]. Another wild-strain *Y. lipolytica* A-101 cultivated on waste products originating from rapeseed processing produced 48 g/L KGA with a yield (Yp/s) of 0.48 g/g and a productivity of 0.26 g/L·h in the raw oil medium [32]. The wild-strain *Y. lipolytica* H355 and its transformants with the superexpression of genes *IDP1*, *PYC1* and *FUM1* encoding NADP-dependent isocitrate dehydrogenase, pyruvate carboxylase and fumarase produced 156.9–186.0 g/L KGA with a productivity (Qp) of 1.47–1.75 g/L·h; however, the yield from raw glycerol (Yp/s) was low (0.30–0.52 g/g) [26,27]. When a mixed glycerol–oil medium was used, the recombinant strain *Y. lipolytica* 1.31.GUT1/6.CIT1/3.E34672 with the superexpression of genes *GUT1*, *CIT1* and *YALI0E34672g* encoding glycerol kinase, methylcitrate synthase and mitochondrial organic acid transporter produced 53 g/L KGA with a product yield (Yp/s) of 0.53 g/g and a productivity (Qp) of 0.35 g/L·h [34]. The maximum product

yields (Yp/s) were achieved using *n*-alkanes (0.90 g/g) [10], glycerol (0.90 g/g) [19] and rapeseed oil (0.95 g/g) [31].

4. Conclusions

The search for new types of raw materials with which to produce organic acids is an important task in the field of microbial synthesis. Of great interest are cheap substrates, such as wastes of ethanol production (technical alcohol, ester–aldehyde fraction (EAF)), which contain a number of valuable organic substances. This work has shown the possibility of α-ketoglutaric acid (KGA) production from the EAF with the aid of the yeast strain *Y. lipolytica* VKM Y-2412. The developed cultivation conditions, including the concentrations of thiamine, nitrogen, microelements and EAF, provided for the accumulation of 53.8 g/L KGA in the cultivation medium with a low content of by-products. It should be emphasized that the yeast *Y. lipolytica*, as well as products based on its synthesis, are generally recognized as safe (GRAS) and can be used for food and medical purposes [55].

Author Contributions: Conceptualization, S.V.K. and I.G.M.; investigation, S.V.K.; methodology, S.V.K.; resources, I.G.M.; supervision, I.G.M.; writing—original draft, S.V.K. All authors have read and agreed to the published version of the manuscript.

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