Alpha-Ketoglutaric Acid Production from a Mixture of Glycerol and Rapeseed Oil by Yarrowia lipolytica Using Different Substrate Feeding Strategies

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Abstract: The microbiological biosynthesis of α-ketoglutaric acid (KGA) has recently captured the attention of many scientists as an alternative to its common chemical synthesis. The present study aimed to evaluate the effect of the feeding strategy of substrates, i.e., glycerol (G = 20 g dm⁻³) and rapeseed oil (O = 20 g dm⁻³), on yeast growth and the parameters of KGA biosynthesis by a wild strain Yarrowia lipolytica A-8 in fed-batch and repeated-batch cultures. The effectiveness of KGA biosynthesis was demonstrated to depend on thiamine concentration and the substrate feeding method. In the fed-batch culture incubated with 3 µg dm⁻³ of thiamine and a substrate feeding variant 2G(OGO), KGA was produced in the amount of 62.1 g dm⁻³ at the volumetric production rate of 0.37 g dm⁻³ h⁻¹. These values of KGA production parameters were higher than those obtained in the control culture (with rapeseed oil only). During 10 cycles of the 1788-h repeated-batch culture carried out acc. to the feeding strategy 2G(OGO), in the last 5 cycles the yeast produced from 55.6 to 58.2 g dm⁻³ of KGA and maximally 2.9 g dm⁻³ of the pyruvic acid as a by-product.

Keywords: Yarrowia lipolytica; α-ketoglutaric acid; glycerol; rapeseed oil; substrate feeding strategies; fed-batch culture; repeated-batch culture

1. Introduction

α-Ketoglutaric acid (KGA), also referred to as 2-ketoglutaric acid or oxoglutaric acid, is a dicarboxylic acid, endogenous in a human body. It is a key molecule in the effective protein metabolism. As a precursor of glutamic acid and glutamine, it determines the removal of amine groups from the body in the form of ammonia [1]. Beneficial properties of ketoglutarate have been confirmed in studies with, e.g., pigs [2], turkeys [3], rats [4], sheep, but also in surveys with normal patients and patients hospitalized for various reasons [5–7].

KGA can contribute to an increase in the pool of amino acids essential for type I collagen synthesis, thereby having a positive impact on the length, mass, flexibility, and mechanical resistance of bones [6]. The osseous system has also been positively affected by its salts, including calcium and sodium ones [8]. Literature data indicate that under in vitro- or in vivo-induced oxidative stress, KGA exhibits strong antioxidative properties and, therefore, can be an effective weapon against reactive oxygen species [9]. In addition, KGA positively influences muscle formation in post-surgery patients. As a precursor of glutamic acid, it contributes to the maintenance of immune system homeostasis, which serves as a source of energy to lymphocytes and macrophages [3]. KGA also affects the endocrine system...
because it is converted to glutamate and then to arginine and ornithine, both of which stimulate the secretion of a growth hormone and an insulin-like growth factor 1 (IGF-1) [10]. It also improves the absorption of Fe\(^{2+}\) ions in anemic patients [11], while athletes use it to improve the blood flow to muscles, reduce catabolism, and enhance protein synthesis [12]. In addition, KGA has been shown to retard aging processes in \textit{Caenorhabditis elegans} cells [13].

KGA is delivered to a human body mainly via its synthesis by the enteral microbiome or its supplementation with an everyday diet. However, the latter provides mainly its precursors. Therefore, the use of pure KGA, its salts, or its complexes usually with amino acids as food additives has recently aroused a lot of interest from many scientific centers as a very interesting solution that increases its applicability.

Apart from food uses, KGA is also applied in the industry, e.g., as a substrate in the chemical synthesis of N-heterocyclic compounds exhibiting anticarcinogenic properties [14]. Its derivatives are used to synthesize pyridazines, i.e., compounds acknowledged as pesticides with antiviral and antibacterial properties [15]. In turn, a polymer produced based on KGA, i.e., poly(triol α-ketoglutarate), can be used as a synthetic biomaterial in tissue engineering and also as a drug carrier [16].

Today, KGA is produced with complicated chemical methods using such compounds as cyanides, toluene, chloroform, absolute ethanol, diethyl ether, sodium metal, or copper-containing catalysts, and its synthesis generates waste products toxic to the natural environment. Its microbiological biosynthesis can offer an alternative to its common chemical synthesis; therefore, it has recently captured the attention of many scientists. The capability to synthesize KGA has so far been investigated for many microorganisms, including, e.g., \textit{Pseudomonas fluorescens}, \textit{Corynebacterium glutamicum}, and \textit{Bacillus} spp. bacteria or \textit{Torulopsis glabrata} and \textit{Candida} spp. yeast [17,18]. Promising microorganisms in this respect seem to be the unconventional \textit{Yarrowia lipolytica} yeasts. They are non-pathogenic, and industrial biosynthesis with their use has been claimed to be Generally Recognized as Safe (GRAS) by the USA Food and Drug Agency [19]. The time of their cell generation is short, whereas their biomass production is relatively high and can be achieved at minimal environmental requirements [12].

The goal of the present study was to evaluate the effect of the feeding mode of substrates, i.e., glycerol and rapeseed oil, on yeast growth and parameters of KGA biosynthesis by a wild strain \textit{Yarrowia lipolytica} A-8 under conditions of its growth inhibition by thiamine in fed-batch and repeated-batch cultures. The study demonstrated that the substrate feeding strategy had a great impact on the effective production of KGA from a mixture of these substrates containing even up to 80% of glycerol.

2. Materials and Methods

2.1. Microorganism

The study was conducted with a natural strain of \textit{Yarrowia lipolytica} A-8 yeasts from the culture collection of the Department of Biotechnology and Food Microbiology at the University of Environmental and Life Sciences in Wroclaw, Poland, which was stored on YM agar slants at a temperature of 4 °C.

2.2. Media and Culture Conditions

Cosmetic glycerol (Wratislavia-Bio; Wroclaw, Poland) and edible rapeseed oil purchased in a popular network of supermarkets were used as sources of carbon and energy in the culture media. The inoculation medium contained (g \(\cdot\) dm\(^{-3}\)): rapeseed oil (O)—20.0, (NH\(_4\))\(_2\)SO\(_4\)—4.5; KH\(_2\)PO\(_4\)—1.0; K\(_2\)HPO\(_4\)—0.1; MgSO\(_4\) \(\cdot\) 7 H\(_2\)O—0.7; Ca(NO\(_3\))\(_2\)—0.4; NaCl—0.5; thiamine—0.2 \(\mu\)g \(\cdot\) dm\(^{-3}\); and distilled water–up to 1 dm\(^{3}\). The production medium was prepared with (g \(\cdot\) dm\(^{-3}\)): glycerol (G)—20.0 or 40.0, or rapeseed oil—20.0; (NH\(_4\))\(_2\) \(\cdot\) SO\(_4\)—12.0; KH\(_2\)PO\(_4\)—2.0; K\(_2\)HPO\(_4\)—0.2; MgSO\(_4\) \(\cdot\) 7 H\(_2\)O—1.4; Ca(NO\(_3\))\(_2\)—0.8; NaCl—0.5; thiamine—0.6, 1, 2, or 3 \(\mu\)g \(\cdot\) dm\(^{-3}\); and tap water–up to 1 dm\(^{3}\). Throughout the cultivation time, a total of 60 or 80 g dm\(^{-3}\) of the substrate were added in batches (G, O = 20 g \(\cdot\) dm\(^{-3}\)) that were fed in 24-h or
48-h intervals, according to culture conditions provided in legends and footnotes to tables and figures. Culture media prepared and substrate batches were sterilized at 121 °C for 20 min.

The inoculation cultures were incubated in 300-cm³ Erlenmeyer flasks containing 50 cm³ of the inoculation medium, on a CERTOMAT IS rotary shaker (Sartorius, Germany) at 140 rpm and 29.5 °C for 3 days. The production culture was initiated by inoculating the production medium with 100 cm³ of the inoculation culture, in a 5 dm³ BIOSTAT B Plus bioreactor (Sartorius, Germany), and continued at an agitator speed of 800 rpm, an air flow rate of 0.6vvm, a temperature of 29.5 °C, and pH kept at 4.5 within the first 24 h and then at 3.5 using a 20% KOH solution. Considering all batches of the substrate fed to the bioreactor and inoculation culture, its final volume was 2 dm³.

2.3. Analytical Methods

During production culture, samples of the culture fluid were collected from the bioreactor with the frequency denoted by points in figures presented in the Results section. The collected samples were centrifuged (5000 rpm, 5 min), extracted with petroleum ether, and determined for biomass concentration—with the gravimetric method, and for concentrations of glycerol (G), α-ketoglutaric acid (KGA), and pyruvic acid (PA)—with the high-performance liquid chromatography method (HPLC) according to the methodology provided by Cybulski et al. [20].

3. Results

3.1. Fed-Batch Culture at a Thiamine Concentration of 0.6 µg·dm⁻³ and Individual Substrates as Carbon Sources in Batches

The first stage of our experiment was aimed at comparing the growth, glycerol consumption kinetics, and production of KGA and PA in fed-batch cultures incubated with a thiamine, i.e., vitamin B1, concentration of 0.6 µg·dm⁻³, and with single substrates used as carbon sources in batches (Figure 1). The initial source of carbon was provided by glycerol in the concentration of 20 g·dm⁻³, followed by glycerol or rapeseed oil (in total—80 g·dm⁻³) fed in batches of 20 g·dm⁻³ each in the 20, 44, 66, and 92 h of the cultivation in three variants: glycerol, glycerol, oil, and oil (GGOO); oil, glycerol, glycerol, and oil (OGGO); and oil, oil, glycerol, and glycerol (OOGG). Two control cultures were made for comparison: one in the culture medium containing only glycerol (G(GGGG)) and the with rapeseed oil as the sole carbon source (O(OOOO)). All cultures were incubated for 189 h.

Although the factor limiting yeast growth was the same in all cultures (i.e., a low thiamine concentration), the growth of yeasts greatly varied among cultures (Figure 1A). After 24-h incubation, biomass growth (ca. 4 g·dm⁻³) was observed only in the culture with oil used as the sole carbon source, whereas it was insignificant in the other cultures. After 48-h culture, yeasts achieved the stationary phase of growth also in this culture, whereas a stable but slow biomass growth till the end of incubation was still observed in the other cultures. The course of Y. lipolytica A-8 strain growth curve was similar in G(OOGO) and G(OOGG) variants and in G(GGGG) and G(GGOO) variants. At the end of the incubation, the highest concentration of biomass, reaching 17.4 g·dm⁻³, was obtained in the culture with oil used as the sole carbon source (O(OOOO)) and the lowest one, reaching ca. 3 g·dm⁻³, in the G(GGGG) and G(GGOO) variants.

Glycerol consumption by Y. lipolytica A-8 strain is presented in Figure 1B. Depending on substrate feeding variant, the consumption of the carbon source was strongly correlated with yeast biomass concentration (Figure 1A). In the control culture with glycerol, substrate consumption was minimal, and its concentration increased with each batch fed to the bioreactor until 93 h, when it exceeded 90 g·dm⁻³. At the end of the incubation process, the lowest concentration of this substrate (8 g·dm⁻³) was determined in the culture variant G(OOGO).

In each culture, KGA production started within the first 24 h of incubation and increased successively till the end of the process (Figure 1C). In the 189 h, definitely the highest concentration of
this metabolite was obtained in the culture with oil (59.2 g·dm⁻³) and the lowest one in the culture with glycerol used as the sole carbon sources (G(GGGG)).

Figure 1. The growth (A), consumption of glycerol (B), and production of \( \alpha \)-ketoglutaric (KGA) and pyruvic (PA) acid (C) by \textit{Y. lipolytica} A-8 depending on carbon source at the beginning of the culture and on the order of glycerol (G = 20 g·dm⁻³) and rapeseed oil (O = 20 g·dm⁻³) feeding. Culture conditions: thiamine concentration 0.6 µg·dm⁻³, substrate feeding time 20 h, 44 h, 66 h, and 93 h. Error bars indicate the standard deviations of mean data values.
PA was produced as a waste product of KGA biosynthesis in various amounts depending on the initial carbon source and the order of oil and glycerol feeding (Figure 1C). Its minimal amounts, below 2.9 g·dm⁻³, were determined in the culture with oil. In contrast, the highest PA concentration was determined in the G(OGGO) culture after 4 days of incubation. In the consecutive days of incubation, the substrate was successively consumed by cells. The highest PA concentration at the end of incubation, reaching ca. 28 g·dm⁻³, was determined in the cultures run in G(GGOO) and G(OOGG) variants.

Fed-Batch Culture at a Thiamine Concentration 0.6 µg·dm⁻³ and with a Mixture of Substrates as a Carbon Source in Batches

At the second stage of the experiment, two cultures were conducted with mixtures of glycerol and oil in ratios of 1:1 and 3:1, and fed in batches of 10G + 10O(4 × (10G + 10O)) and 15G + 5O(4 × (15G + 5O)) (Figure 2). In the first variant (1:1), the total amounts of glycerol and oil fed to the bioreactor reached 50 g·dm⁻³ and 50 g·dm⁻³, respectively. In the second variant (3:1), the respective values were at 75 g·dm⁻³ and 25 g·dm⁻³.

![Figure 2](image-url). The growth, consumption of glycerol, and production of α-ketoglutaric (KGA) and pyruvic (PA) acid by Y. lipolytica A-8 in cultures with glycerol and rapeseed oil mixtures in ratios of 1:1 and 3:1 at the beginning of incubation and in subsequently fed batches. Culture conditions: thiamine concentration 0.6 µg·dm⁻³, substrate addition time 21 h, 46 h, 66 h and 92 h. Variant 1:1 → 10G + 10O(4 × (10G + 10O)); variant 3:1 → 15G + 5O(4 × (15G + 5O)). Culture conditions: thiamine concentration 3 µg·dm⁻³, the substrate addition time 20 h, 44 h, 66 h and 93 h. Error bars indicate the standard deviations of mean data values.

In both variants of cultures, the yeasts reached the stationary growth phase after the last batch fed to the bioreactor and produced a similar biomass concentration. In the culture with a higher glycerol concentration, they produced significantly more PA, whose concentration increased until 144 h when it reached 42.5 g·dm⁻³, and then decreased to 32.6 g·dm⁻³ within the two subsequent days of incubation. At the end of this culture incubation, KGA concentration reached 25 g·dm⁻³. In turn, in the culture
run in the 1:1 variant, the final biomass concentration was at 8.7 g·dm⁻³, KGA concentration was at 42.5 g·dm⁻³, and PA concentration was 5.7 g·dm⁻³.

3.2. Fed-Batch Culture at a Thiamine Concentration of 3.0 µg·dm⁻³

At the next stage of the study, four other cultures were conducted with a thiamine concentration increased to 3 µg·dm⁻³. In two cultures, the total amounts of substrates fed to the bioreactor were: 60 g·dm⁻³ of glycerol and 40 g·dm⁻³ of oil. In these culture variants, oil was initially fed to the reactor at a concentration of 20 g·dm⁻³, and then carbon sources were applied in different substrate batches, i.e., (GGGO) and 4 × (15G + 5O). In another two cultures, glycerol concentration was increased to 80 g·dm⁻³ at the expense of oil. In these culture variants, glycerol was added alone at the beginning of incubation, and then in batches as above. The yeast growth curve and glycerol consumption in these four cultures are presented in Figure 3, whereas production of KGA and PA is in Figure 4.

Figure 3. The growth and consumption of glycerol by Y. lipolytica A-8 depending on carbon source at the beginning of the culture (glycerol, G or rapeseed oil, O) and the method of its addition as a single carbon source, GGGO (A) or in a mixture 4 × (15G + 5O) (B).
Figure 3B shows yeast growth and glycerol consumption rate in the cultures in which glycerol and oil substrates were fed in mixtures at 3:1, i.e., \( 4 \times (15G + 5O) \). Biomass concentration reached 9.9 and 15.2 g·dm\(^{-3}\) in the cultures incubated in the media fed initially with glycerol G(\( 4 \times (15G + 5O) \)) and oil O(\( 4 \times (15G + 5O) \)), respectively, and was higher than in the analogous cultures run with 80 g·dm\(^{-3}\) of glycerol presented in Figure 3A.

Figure 4. Production of α-ketoglutaric (KGA) and pyruvic (PA) acid by \( Y. \ lipolytica \) A-8 depending on the method of carbon source addition as a single, GGGO or in a mixture \( 4 \times (15G + 5O) \) in the culture with glycerol, G (A) and rapeseed oil, O (B) at the beginning of the process. Culture conditions: thiamine concentration 3 µg·dm\(^{-3}\), the substrate addition time 20 h, 44 h, 66 h, and 93 h.
and 60 g·dm\(^{-3}\) of glycerol presented in Figure 3A. In both variants with a mixed source of carbon in batches, glycerol was completely consumed before the end of incubation.

Figure 4 presents a comparison of KGA and PA production in the cultures initiated in the medium with the same carbon source but differing in the strategy of feeding the remaining part of the substrate.

This presentation of results allows also for a direct comparison of acid production with a total of 80 g·dm\(^{-3}\) of glycerol (Figure 4A) and 60 g·dm\(^{-3}\) of glycerol (Figure 4B). Results obtained show that the Y. lipolytica A-8 strain was able to effectively produce KGA even in the presence of 80 g·dm\(^{-3}\) glycerol; however, when used in this concentration, glycerol needs to be fed to the medium together with oil (Figure 4A). The culture variant G(4 × (15G + 5O)) enabled producing 42.2 g·dm\(^{-3}\) of KGA and 11.4 g·dm\(^{-3}\) of PA. In the culture in which glycerol was fed in the first four batches, PA production was higher than that of KGA and reached 38.1 g·dm\(^{-3}\) at the end of the process (Figure 4A). The composition of batches was not that important in the cultures initiated by medium feeding with oil. In their both variants, KGA and PA production was similar (Figure 4B).

### 3.3. Repeated-Batch Cultivation

In the next stage of the study, in-depth analyses were performed to determine the course of semi-continuous biosynthesis of KGA by Y. lipolytica A-8 strain, and their results are presented in Figure 5. Because at the previous stage, good production parameters were achieved in the culture started with glycerol feeding to the medium (Table 1), the process of KGA biosynthesis in the semi-continuous culture was initiated as a fed-batch culture, but with the initial glycerol concentration increased to 40 g·dm\(^{-3}\) (2G). Another glycerol batch in the amount of 20 g·dm\(^{-3}\) was fed in the 72 h, whereas in the 46 and 97 h oil was added in batches of 20 g·dm\(^{-3}\) each. After 166.5 h of incubation, the culture medium was exchanged by withdrawing 75% of a cell suspension from the bioreactor and completing this volume with a fresh medium under sterile conditions. The fresh medium composition was adjusted so as to ensure the initial concentration of glycerol again at 40 g·dm\(^{-3}\). Then, substrates were fed in the subsequent batches as follows: 20 g·dm\(^{-3}\) of oil after 2 days, 20 g·dm\(^{-3}\) of glycerol after 3 days, and 20 g·dm\(^{-3}\) of oil after 4 days of culture incubation. In total, 9 similar exchanges of the culture medium were performed, and final results of KGA production in the cultures with these media are presented in Figure 6.

The culture incubation was initiated at a thiamine concentration of 3 μg·dm\(^{-3}\). In cycles II and III, B1 vitamin concentration was at 2 μg·dm\(^{-3}\), in cycles IV and V at 1μg·dm\(^{-3}\), and since cycle VI at 3 μg·dm\(^{-3}\). At the end of cycle I, KGA and PA concentrations reached 62.1 g·dm\(^{-3}\) and 3.1 g·dm\(^{-3}\), respectively. As shown in Figure 6 and Table 1, the best KGA production parameters were achieved at the end of this cycle, including production effectiveness of 0.62 g·g\(^{-1}\), volumetric production rate of 0.37 g·dm\(^{-3}\)·h\(^{-1}\), and specific production rate of 0.25 g·g\(^{-1}\)·h\(^{-1}\). In cycles II, III, and IV, the volumetric KGA production rate was significantly lower than in cycle I (Figure 6). Besides, in cycle III, KGA concentration began to decrease along with glycerol depletion in the culture medium. Therefore, in another cycle, analyses were conducted for a thiamine concentration of 1 μg dm\(^{-3}\). Under these conditions, the yeasts produced firstly PA, the concentration of which in cycle V in the 738 h was as high as 37.6 g·dm\(^{-3}\). Despite a satisfying KGA concentration obtained in this cycle (55.6 g·dm\(^{-3}\)), thiamine was used in the concentration of 3 μg·dm\(^{-3}\) in the next cycles because of the long duration of this cycle (267 h). This modification resulted in the successive shortening of the consecutive cycles, i.e., from 137.5 h (VIII) to 263.5 h (VI), at the similar final concentration of KGA which ranged from 54.6 g·dm\(^{-3}\) (X) to 58.2 g·dm\(^{-3}\) (VII) (Figure 5). Since cycle V, KGA production effectiveness also became stable and fitted within the range from 0.49 to 0.53 g·g\(^{-1}\). It is worth noticing that in the cycles VI to X, the final concentration of PA did not exceed 2.9 g·dm\(^{-3}\).
Figure 5. Concentration of biomass, α-ketoglutaric acid (KGA), pyruvic acid (PA), and glycerol during repeated batch culture with *Y. lipolytica* A-8 in the medium with glycerol and rapeseed oil according to 2G(OGO) substrate feeding strategy. Error bars indicate the standard deviations of mean data values.
Table 1. Comparison of parameters of KGA biosynthesis by *Y. lipolytica* A-8 yeast strain depending on thiamine concentration in the culture medium, substrate feeding strategy, and cultivation regime.

| Cultivation Regime | Thiamine (µg dm⁻³) | Substrate Feeding Strategy | Y (g g⁻¹) | Q (g dm⁻³ h⁻¹) | q (g g⁻¹ h⁻¹) | Selectivity (%) |
|--------------------|--------------------|---------------------------|-----------|----------------|---------------|-----------------|
| Fed-batch          | 0.6                | G(GGGG)                    | 0.05      | 0.029          | 0.0097        | 21              |
| Fed-batch          | 0.6                | G(GGGO)                    | 0.1       | 0.054          | 0.0174        | 27              |
| Fed-batch          | 0.6                | G(GGGO)                    | 0.3       | 0.16           | 0.017         | 68              |
| Fed-batch          | 0.6                | G(GGGO)                    | 0.13      | 0.069          | 0.0071        | 32              |
| Fed-batch          | 0.6                | G(GGGO)                    | 0.59      | 0.31           | 0.018         | 99              |
| Fed-batch          | 0.6                | 10G + 10O(4 × (10G + 10O)) | 0.42      | 0.22           | 0.025         | 88              |
| Fed-batch          | 0.6                | 15G + 5O(4 × (15G + 5O))   | 0.25      | 0.13           | 0.016         | 43              |
| Fed-batch          | 3.0                | O(GGGG)                    | 0.38      | 0.2            | 0.019         | 84              |
| Fed-batch          | 3.0                | G(GGGO)                    | 0.19      | 0.1            | 0.01          | 33              |
| Fed-batch          | 3.0                | O(4 × (15G + 5O))          | 0.4       | 0.21           | 0.014         | 74              |
| Fed-batch          | 3.0                | G(4 × (15G + 5O))          | 0.42      | 0.22           | 0.022         | 79              |
| Repeated fed-batch (no. I) | 3.0 | 2G(OGO)                    | 0.62      | 0.37           | 0.025         | 95              |
| Repeated fed-batch (no. II–III) | 2.0 | 2G(OGO)                    | 0.2–0.3   | 0.17–0.22      | 0.01–0.013    | 58–82           |
| Repeated fed-batch (no. IV–V) | 1.0 | 2G(OGO)                    | 0.27–0.53 | 0.16–0.2       | 0.014         | 57–85           |
| Repeated fed-batch (no. VI–X) | 3.0 | 2G(OGO)                    | 0.49–0.53 | 0.19–0.35      | 0.013–0.019   | 94–99           |
by Kamzolova et al. [14], while glycerol has been used by Förster et al. [15]. The effectiveness of KGA production from glycerol achieved by both research groups mentioned above did not exceed 0.4 g·g\(^{-1}\) [15,24]. In contrast, the effectiveness of KGA production with rapeseed oil used as a substrate was shown to reach even 130% [15]. Usually, these are the economic reasons that prompt scientists to search for novel carbon sources. This may be associated with the price of raw material used so far or with the appearance of large quantities of a new, cheap substrate on the market, as it happened in the case of glycerol over a decade ago [25]. Results presented in this work indicate, for the first time ever, the feasibility of using two substrates in one culture, i.e., rapeseed oil and glycerol, for KGA biosynthesis. The strategy of using two carbon sources has already been employed for the biosynthesis of citric acid or erythritol by \(Y.\ lipolytica\) yeast [26,27]. Citric acid was produced with glycerol and glucose, and results obtained were similar to those achieved with a single carbon source used as a substrate [27]. According to a study by Rakicka et al. [26], molasses combined with glycerol promoted economical erythritol biosynthesis.

### 4. Discussion

The capability of \(Y.\ lipolytica\) for KGA overproduction has been known since the 1960s; however, \(n\)-paraffins have been the source of carbon in related studies until the 1990s [9,19,21]. Further investigations have also shown ethanol to be a good substrate in KGA biosynthesis [22]. In turn, Aurich and Stottmeister [23] achieved very good results with refined plant oils, including olive oil, canola oil, and sunflower oil. Rapeseed oil has been used for the first time for KGA biosynthesis by \(Y.\ lipolytica\) by Kamzolova et al. [14], while glycerol has been used by Förster et al. [15]. The effects of various factors on the parameters of KGA production from glycerol were also investigated by Zhou et al. [24]. However, the effectiveness of KGA production from glycerol achieved by both research groups mentioned above did not exceed 0.4 g·g\(^{-1}\) [15,24]. In contrast, the effectiveness of its production with rapeseed oil used as a substrate was shown to reach even 130% [15]. Usually, these are the economic reasons that prompt scientists to search for novel carbon sources. This may be associated with the price of raw material used so far or with the appearance of large quantities of a new, cheap substrate on the market, as it happened in the case of glycerol over a decade ago [25].

![Figure 6](image.png)

**Figure 6.** Results of the \(\alpha\)-ketoglutaric acid (KGA) production from glycerol and rapeseed oil in repeated-batch culture by \(Y.\ lipolytica\) A-8 according to 2G(OGO) substrate feeding strategy. Error bars indicate the standard deviations of mean data values.

Both rapeseed oil and glycerol are very good sources of carbon to the yeasts they differ significantly in their consumption rate and in yeast growth effectiveness. At the first stage of fermentations, paraflins have been the source of carbon in related studies until the 1990s [9,19,21]. Further investigations have also shown ethanol to be a good substrate in KGA biosynthesis [22]. The capability of \(Y.\ lipolytica\) for KGA overproduction has been known since the 1960s; however, \(n\)-paraffins have been the source of carbon in related studies until the 1990s [9,19,21]. Further investigations have also shown ethanol to be a good substrate in KGA biosynthesis [22]. In turn, Aurich and Stottmeister [23] achieved very good results with refined plant oils, including olive oil, canola oil, and sunflower oil. Rapeseed oil has been used for the first time for KGA biosynthesis by \(Y.\ lipolytica\) by Kamzolova et al. [14], while glycerol has been used by Förster et al. [15]. The effects of various factors on the parameters of KGA production from glycerol were also investigated by Zhou et al. [24]. However, the effectiveness of KGA production from glycerol achieved by both research groups mentioned above did not exceed 0.4 g·g\(^{-1}\) [15,24]. In contrast, the effectiveness of its production with rapeseed oil used as a substrate was shown to reach even 130% [15]. Usually, these are the economic reasons that prompt scientists to search for novel carbon sources. This may be associated with the price of raw material used so far or with the appearance of large quantities of a new, cheap substrate on the market, as it happened in the case of glycerol over a decade ago [25].

Results presented in this work indicate, for the first time ever, the feasibility of using two substrates in one culture, i.e., rapeseed oil and glycerol, for KGA biosynthesis. The strategy of using two carbon sources has already been employed for the biosynthesis of citric acid or erythritol by \(Y.\ lipolytica\) yeast [26,27]. Citric acid was produced with glycerol and glucose, and results obtained were similar to those achieved with a single carbon source used as a substrate [27]. According to a study by Rakicka et al. [26], molasses combined with glycerol promoted economical erythritol biosynthesis.

![Diagram](image.png)
Both rapeseed oil and glycerol are very good sources of carbon to *Y. lipolytica* yeast; however, they differ significantly in their consumption rate and in yeast growth effectiveness. At the first stage of the study presented in this work, the process of KGA biosynthesis was conducted under conditions of yeast growth inhibition by thiamine, used at a concentration of 0.6 µg·dm⁻³. The concentration of this vitamin ranging from 0.6 to 1.0 µg·dm⁻³ was indicated in a previous study as beneficial for KGA biosynthesis by *Y. lipolytica* yeast from both rapeseed oil and fatty acids [20]. In the present work, in the culture variant with oil used as the sole carbon source (O(0)), the yeast growth was promoted, and biomass concentration was as high as 17.4 g·dm⁻³. In contrast, in the culture variant with glycerol as the only source of carbon (G(GGGG)), it reached barely 3 g·dm⁻³. In the strategy, in which the bioreactor was fed with 40 g·dm⁻³ batches of glycerol and oil in total, i.e., in cultures G(GGOO), G(OGGO), and G(OOGG), glycerol was not completely consumed in any of the culture variants. Biomass growth was observed already after oil addition to the culture medium, and glycerol consumption was strongly correlated with biomass concentration. Interestingly, the yeasts consumed more readily fatty acids from the oil than glycerol. Both substrates differ in the metabolic pathway; glycerol is converted in the course of glycolysis, whereas oil (fatty acids) in the process of β-oxidation, which has a strong influence principally on KGA production selectivity. Nevertheless, both metabolic transformations-glycolysis and β-oxidation result in the formation of acetyl coenzyme A, which is included into the Kreb’s cycle. Its total pool determines the growth rate and the formation of certain metabolites. PA is the final metabolite of the glycolysis pathway. Its oxidative decarboxylation is catalyzed by pyruvate dehydrogenase and leads to the synthesis of acetyl coenzyme A, which is the basic substrate in the Kreb’s cycle. Even at this stage, a low concentration of thiamine decreases the growth rate because this vitamin is a co-factor of pyruvate dehydrogenase. Therefore, thiamine deficiency, low pH, and substrate degraded via glycolysis, e.g., glycerol, impair the Kreb’s cycle and contribute to the secretion of not only KGA (due to the suppressed activity of α-ketoglutarate dehydrogenase) but also PA [14,28]. These acids are synthesized in the ratio of 20%–25% KGA to 75%–80% PA [29]. In turn, in the β-oxidation pathway, the acetyl coenzyme A is formed directly as a product of the shortening of individual molecules of fatty acids, whereas PA is not synthesized at any of the β-oxidation stages. Therefore, PA is not produced when oil is used as a source of carbon in KGA biosynthesis. This was confirmed in our study, where PA concentration in the course of and at the end of the culture was at trace levels and KGA concentration reached 59.2 g·dm⁻³ in the culture with rapeseed oil. In contrast, PA was produced always when glycerol was available in the culture medium; however, yeast cells began to metabolize it when biomass concentration reached a certain level. This trend was also observed for a wild strain *Y. lipolytica* H355 and its transformants [30,31], and also for *Y. lipolytica* H222 and its multicopy transformant H222-MH1 [32]. In turn, in the case of *Y. lipolytica* WSH-Z06 strain and its recombinants, PA concentration remained stable over the stationary phase [33]. In the present study, after feeding the last oil batch in the 93 h in the culture variant G(OOGO), biomass concentration was at ca. 6 g·dm⁻³, and PA concentration was as high as 27 g·dm⁻³; however, it decreased significantly afterward. Until the end of the culture, the yeasts consumed 13 g·dm⁻³ of PA and 11 g·dm⁻³ of glycerol. In the same time span, biomass concentration increased by 4 g.

In the consecutive experiments, conducted also at a thiamine concentration of 0.6 µg·dm⁻³, glycerol and oil were used in mixtures prepared at ratios of 1:1 and 3:1. The final biomass concentration was very similar in both cultures, but the use of a mixture of substrates resulted in the complete consumption of glycerol after five (1:1) and six (3:1) days of the process. In addition, results obtained indicate the 1:1 variant to be very beneficial for KGA biosynthesis.

Summing up the preliminary stage of the study conducted at a thiamine concentration of 0.6 µg·dm⁻³, it can be concluded that oil replacement with glycerol requires adjusting thiamine concentration, as the same concentration of this vitamin cannot be used for both these carbon sources. In addition, the effective and selective biosynthesis of KGA in cultures with media containing oil and glycerol is also determined by the appropriate feeding of these substrates. At a low thiamine
concentration, a medium feeding with oil is recommended at the early and at the final stage of incubation because this substrate promotes yeast growth, which in turn facilitates the selective production of KGA.

In the next experiments, the thiamine concentration was increased to 3 µg·dm\(^{-3}\) and two variants of substrate feeding: (GGGO) and 4 × (15G + 5O), were compared in the cultures initiated in the presence of either glycerol (G) or oil (O). The culture media from variants began with glycerol, i.e., G(GGGO) and G(4 × (15G + 5O)), contained a total of 80 g·dm\(^{-3}\) of glycerol, whereas these from variants initiated with oil, i.e., O(GGGO) and O(4 × (15G + 5O)), contained by 20 g·dm\(^{-3}\) less of this carbon source. Glycerol used at the onset of the culture turned out to be less beneficial for yeast growth in both variants of its feeding, as it ensured a biomass weight lower by ca. 5 g than the analogous cultures with oil. In contrast, like in the cultures incubated at the lower thiamine concentration, mixing the substrate in batches had a positive effect on glycerol consumption rate. Glycerol was not completely consumed in the culture variant G(GGGO), where its concentration determined in the 189 h in the post-culture fluid reached 9.5 g·dm\(^{-3}\). In turn, its trace amounts were found as early as after 7 days of incubation in the culture variant G(4 × (15G + 5O)). This variant turned out to be the most beneficial considering KGA production (42.2 g·dm\(^{-3}\)). It needs to be emphasized, however, that at this stage of the study, KGA production was unsatisfactory only in the variant G(GGGO), whereas PA prevailed in the sum of metabolites.

To recapitulate this part of our study, at the higher thiamine concentration tested (3 µg·dm\(^{-3}\)) the analyzed \textit{Y. lipolytica} A-8 strain can lead effective biosynthesis of KGA in the culture medium containing 60% and even 80% of glycerol in the total pool of substrates. At the higher glycerol contribution, like at the low thiamine concentration, it is necessary to begin culture incubation in the presence of oil or/and to feed the medium with a mixture of substrates. The proposed conditions, despite the significant share of glycerol in the substrates pool, allow for an efficient production of KGA with a low proportion of PA.

Nevertheless, this study confirmed the feasibility of effective KGA biosynthesis in the cultures began in the medium with glycerol. Therefore, another culture was incubated with initial glycerol concentration increased to 40 g·dm\(^{-3}\) (2G), and with consecutive 20 g·dm\(^{-3}\) substrate batches fed to the bioreactor after 48, 72, and 96 h of incubation (O,G,O). Outcomes of this process were very good, as KGA production reached 62.1 g·dm\(^{-3}\) after 166 h. Production effectiveness and volumetric production rate reaching 0.62 g·g\(^{-1}\) and 0.37 g·dm\(^{-3}\)·h\(^{-1}\), respectively, were higher than these obtained in the culture with oil (0.59 g·g\(^{-1}\) and 0.31 g·dm\(^{-3}\)·h\(^{-1}\), respectively). This culture process was used to initiate a semi-continuous culture. Even though the consecutive cycles/medium exchanges failed to improve production parameters compared to the fed-batch culture initiating the entire process, the cycles from V to X allowed for the achievement of higher values of the production parameters than in the earlier fed-batch cultures (Supplementary Table S1). The effectiveness of KGA biosynthesis in the consecutive cycles of the semi-continuous culture ranged from 0.49 to 0.53 g·g\(^{-1}\), which means that it fitted within the highest values obtained so far from glycerol [12,24,30,31]. The long-lasting cultivation processes, including semi-continuous culture, can pose various problems, because sterility maintenance can be challenging, and downstream processing complicated [34].

Despite that, such cultures have recently gained popularity among researchers. Many research works have appeared concerning their use in algae cultures [35]. Different feeding strategies used for glucose as a substrate in lipid accumulation by \textit{Rhodosporidium toruloides} Y4 were tested by [36]. Definitely, the best results were obtained by these authors in the repeated fed-batch process, which allowed them to indicate this process as applicable on the industrial scale. The repeated batch culture was also used for citric acid production by \textit{Y. lipolytica} from inulin [37] and glycerol [38], for erythritol biosynthesis from glycerol [39], or for the production of lipid-rich biomass of \textit{Y. lipolytica} yeast [40]. In the present study, we used a wild yeast strain \textit{Y. lipolytica} A-8, isolated in 1974 from the soil around a car repair shop in Wroclaw, Poland. It needs to be emphasized that this strain remained stable throughout the entire cultivation time of 1788 h. The available literature lacks work on KGA production in other than batch and fed-batch cultivation regimes. According to literature data, the maximal time of KGA biosynthesis from glycerol with the use of \textit{Y. lipolytica} reached 240 h [31], and from ethanol—324 h [14].
An important element strongly affecting the costs of KGA secretion from the post-culture fluid is the contribution of waste metabolites in the total pool of metabolites produced. As mentioned earlier in the work, a significant amount of PA poses some problems during KGA biosynthesis, particularly from glycerol, but the post-culture fluid can contain also small amounts of other acids from the Kreb’s cycle, like malic, fumaric, succinic, or citric acid [30]. Many recombinant strains of \textit{Y. lipolytica} yeast were constructed to eliminate waste products and improve parameters of KGA production. These modifications concerned, most of all, the overexpression of genes encoding Kreb’s cycle enzymes, including NADP$^+$ dependent isocitrate dehydrogenase (\textit{IDP1}), pyruvate carboxylase (\textit{PYC1}) [31], fumarase (\textit{FUM1}) [30], alpha-ketoglutarate dehydrogenase (KGDH) [32], or genes involved in the metabolism of acetyl-CoA [33,41]. Apart from PA, the wild strain investigated in our study did not produce any other waste metabolites in concentrations exceeding 1 g·dm$^{-3}$. It is worth emphasizing that during the last five cycles of the semi-continuous culture, PA concentration was below 2.9 g·dm$^{-3}$, hence KGA production selectivity reached 94%–99%.

5. Conclusions

Although the production parameters of KGA by \textit{Y. lipolytica} yeast are higher with the use of oil than with glycerol as a carbon source, it is very difficult to conduct long-term cultivation processes using hydrophobic substrates. To respond to the industry’s needs, this work proposes a strategy for using two sources of carbon (oil and glycerol) in one production culture. Therefore, the amount of oil needed for the process was reduced and exchanged by glycerol, which seems to be of great practical importance. Finally, replacing the hydrophobic substrate with water-soluble glycerol can significantly facilitate the industrial-scale production of KGA, especially if wild strains are used in the process. It is worth noticing that the use of two substrates for KGA biosynthesis has not been investigated so far. The novel solutions proposed in this work regarding substrate feeding strategy foster a chance for developing an effective process of KGA production by wild strains of \textit{Y. lipolytica} in a wider scale. Considering such variables as: thiamine concentration in the culture medium, substrate type at the onset of cultivation as well as amounts and ratios of oil and glycerol in batches, satisfactory parameters of KGA production can be achieved in the culture medium containing in total 60% and even 80% of glycerol. The maximum concentration and the volumetric production rate of KGA reaching 62.1 g·dm$^{-3}$ and 0.37 g·dm$^{-3}$·h$^{-1}$, respectively, were obtained in the culture variant 2G(_OGO). For comparison, rapeseed oil used as the sole source of carbon ensured KGA concentration of 59.2 g·dm$^{-3}$ achieved at the volumetric production rate of 0.31 g·dm$^{-3}$·h$^{-1}$. In addition, good production parameters obtained in the repeated batch culture lasting in total 1788 h point to the feasibility of making the production process of this acid continuous.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/12/15/6109/s1, Table S1: Results of KGA biosynthesis depending on thiamine concentration in the culture medium, substrate feeding strategy, and cultivation regime.

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