

Article

Factors Affecting the Yield in Formation of Fat-Derived Fragrance Compounds by *Yarrowia lipolytica* Yeast

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Abstract: *Yarrowia lipolytica* belongs to the group of microorganisms widely used in scientific research for environmentally friendly biotransformation reactions. This yeast produces a number of compounds important from the point of view of the food and cosmetics industries, including flavor and fragrance compounds. The results of selected studies on the possibility of biosynthesis of fat-derived fragrances, namely gamma-decalactone (GDL) and hexanal, are presented in this article. The wild-type *Yarrowia* yeast strain KKP379 and the LOX/HPL mutant were used in the studies. With the aim of improving the synthesis yield of both aroma compounds, parameters such as concentration of lipid substrate, the type of culture medium, and the addition of surfactants, and, not yet verified in the available literature in this context, the concentration of inoculum, the addition of heptane, and the emulsification of culture medium were analyzed. The research showed that the concentration of the lipid substrate and the degree of emulsification of the medium had a significant influence on the amount of GDL production. The higher the content of castor oil in the medium, the higher the concentration of the synthesized aroma compound, with a significant extension of the reaction time. By varying the concentration of castor oil in the medium in the range of 10–100 g/L, an increase in the lactone concentration was obtained from 1.86 ± 0.15 g/L to 3.06 ± 0.2 g/L, with a simultaneous extension of the reaction from 3 to 7 days. It is noteworthy that the additional application of the emulsification of the medium allowed the efficiency of GDL biosynthesis to be significantly increased by over 2 g/L to a lactone concentration of approximately 5.25 ± 0.10 g/L. This is one of the highest reported concentrations for the production of this biofragrance by the wild strain. In the case of hexanal synthesis, the increase of the concentration of linoleic acid in the culture medium from 25 g/L to 100 g/L resulted in a 5-fold higher concentration of aldehyde in the cell extract. At a higher concentration of the lipid substrate in the medium, the modified whole-cell catalyst showed a higher activity of lipoxygenase.



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Keywords: natural aroma; *Yarrowia lipolytica*; gamma-decalactone; green-note aroma compounds; hexanal; lipids

1. Introduction

Yarrowia lipolytica is an unconventional yeast which is very popular and widely used in the synthesis of natural products, thanks to the well-known functions of specific genes and favorable metabolic features [1]. This yeast is able to use a wide variety of carbon substrates, including industrial waste and hydrophobic substances [2]. The yeast produces many valuable enzymes extracellularly, including lipases, proteases, esterases, phosphatases, and RNases. *Yarrowia lipolytica* occurs naturally in dairy products and meat, affecting their sensory characteristics. From a biotechnological point of view, this yeast is a kind of microbial cell factory, and it plays an important role in the production of organic acids, polyols, surfactants, and flavor and fragrance compounds [3–6].

The aroma compounds that create the flavor and fragrance of food play a key role in the acceptance of food by the consumer, and represent an essential part of the food

additives market. It is estimated that the aroma compounds market will rise from 13.31 to 19.72 bn USD between 2018 and 2026, which puts the compound annual growth rate CAGR at 5% [7]. Factors like emerging health consciousness and personal well-being trends are expected to further drive the demand for natural fragrance ingredients and increase the natural aroma chemicals market. The natural fragrance segment accounts for 40% of the global natural and synthetic fragrance market, since natural aromas appear safer, healthier, and more sustainable [8,9].

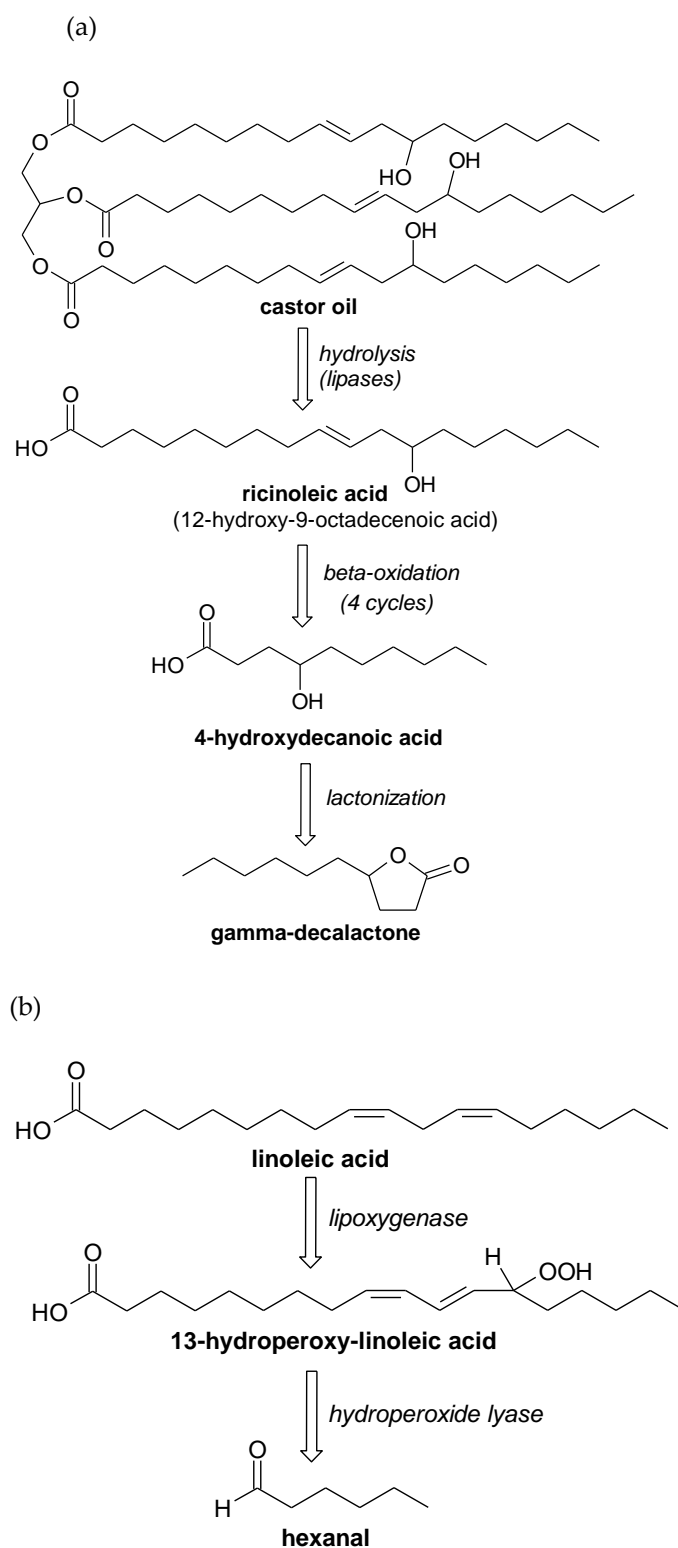
A route for natural fragrance synthesis that offers an alternative to extraction from plants is microbial biosynthesis or bioconversion [10–13]. Microorganisms can synthesize fragrances as secondary metabolites during fermentation. Fragrances can be generated in situ as integral parts of food and beverages (e.g., cheese, yogurt, and wine) that determine the organoleptic properties of the final product, or obtained in designed reactions catalyzed by microorganisms. A number of enzymes (e.g., glucosidases, lipases, proteases, oxidoreductases) catalyze the production of flavor and fragrance compounds from precursor molecules, including lipids.

The biosynthesis of fat-derived fragrance compounds is a multipath, complex process. It often begins with the biosynthesis of fatty acids, specifically with the biosynthesis of unsaturated fatty acids, where the activity of enzymes from the host's desaturase system is required [14].

The compounds synthesized from fatty acids and mediated by microorganisms include, among others, gamma-decalactone (GDL) and compounds from the so-called green fragrance notes. Gamma-decalactone ($C_{10}H_{18}O_2$) is a cyclic 4-hydroxydecanoic acid ester. Its pure form is an oily colorless or slightly yellow liquid, characterized by an intense peach-creamy aroma and perceptible at very low concentrations of 0.088 ppm in aqueous solutions [15]. The *R*-enantiomer of GDL naturally occurs in many fruits, especially in peaches, strawberries, and apricots. It has also been detected in butter, milk, cheese, beer, rum, and white and red wine [16]. GDL has GRAS (Generally Recognized As Safe) status and is considered a safe food additive approved by the FDA (Food and Drug Administration). It is widely used in the production of bakery products, confectionery, desserts, and beverages [17].

The biotechnological synthesis of GDL is based on the biotransformation of ricinoleic acid (*R*-12-hydroxy-9-*cis*-octadecenoic acid) or its derivatives (mainly methyl ester) in the presence of microorganisms. For economic purposes, castor oil is commonly used, in which ricinoleic acid constitutes nearly 90% of triglycerides. The pathway of degradation of lipids begins with extracellular lipases that hydrolyze triacylglycerols to free fatty acids (Scheme 1). In turn, ricinoleic acid participates in the β -oxidation pathway in which the chain shortens. As a result of four cycles of β -oxidation, 4-hydroxydecanoic acid is obtained, which, under appropriate conditions (after acidification to pH 2.0 and/or heating), undergoes intramolecular esterification—lactonization [18,19]. In the biotechnological production of GDL, both yeast and fungi [20] have been applied, including *Yarrowia lipolytica* [21], *Candida sorbophila* [22], *Sporobolomyces odoratus* [23], *Sporidiobolus salmonicolor* and *Sporidiobolus ruineni* [18,24], and *Rhodotorula aurantiaca* [25].

Green-note aroma compounds affect the green character of food products and the impression of freshness. They are popularly used in the food industry to reconstitute the fresh green odor of fruits and vegetables lost during processing. They are associated with the aromas of green apples, melons, cucumbers, and freshly cut grass. C_6 - C_9 carbon aldehydes and their corresponding alcohols such as hexanal, 3-*cis*-hexenal, 2-*trans*-hexenal, 3-*cis*-nonenal, 2-*trans*-nonenal, and (3-*cis*, 6-*cis*) and (2-*trans*, 6-*cis*) nonadienal are important components of green aroma notes [26]. One of the most popular green-note molecules is hexanal, characterized by a grease, grass, or leafy fresh odor.



Scheme 1. Scheme of the synthesis pathway of the fat-derived fragrances gamma-decalactone (a) and hexanal (b), mediated by microorganisms.

The biosynthesis of green-note aroma compounds takes place in the presence of lipoxygenase (LOX, EC. 1.13.11.12) from polyunsaturated fatty acids: linolenic and linoleic acid. In the lipoxygenase pathway, polyunsaturated fatty acids are transformed into aldehydes and alcohols by the enzymes lipoxygenase, hydroperoxide lyase (HPL, EC 4.1.2.92), and alcohol dehydrogenase (EC 1.1.1.1), respectively. Hexanal is synthesized

via the LOX and HPL degradation of linoleic acid (Scheme 1). When a plant oil is used as a reagent, then the hydrolysis of triacylglycerols by lipases into fatty acids needs to be conducted prior to the biosynthesis [15,26,27].

In most scientific reports concerning the biosynthesis of green aroma compounds, lipoxygenase from soy seeds has been used [28–30]. The biosynthesis of green aroma compounds is still being improved by means of genetic engineering, by incorporating genes responsible for the expression of lipoxygenase and hydroperoxide lyase into the cells of microorganisms. This is particularly important in the case of the creation of recombinants with genes for hydroperoxide lyase, which is an enzyme limiting biocatalysis [27].

The aim of this research was to analyze the influence of the selected factors on the catalytic activity of *Yarrowia lipolytica* in the synthesis of GDL and hexanal, a representative compound of the green aroma. In the biosynthesis of both compounds with the use of whole-cell catalysts, the influence of the concentration of lipid substrate (castor oil and linoleic acid, respectively) on the obtained levels of bioaroma compounds was analyzed. Moreover, in the synthesis of GDL, in order to improve the process, the effectiveness of adding heptane (organic phase) or surfactants to the biotransformation medium and the influences of the size of the inoculum used and the emulsification of the medium were determined. In the biotransformation aimed at hexanal synthesis, attention was paid to the activity of lipoxygenase (a key enzyme in the synthesis of green fragrance compounds) and the levels of aroma synthesis inside and outside yeast cells.

2. Materials and Methods

2.1. Materials

Gamma-decalactone > 98%, gamma-undecalactone 98%, hexanal 98%, isoamyl acetate \geq 99.5%, linoleic acid, heptane, and Tris-HCl were purchased from Sigma-Aldrich (Saint Louis, USA); diethyl ether, ethyl alcohol, acetone, and sodium hydroxide were from Avantor Performance Materials (Gliwice, Poland); Tween 80, Tween 20, and lecithin came from Acros Organics (Geel, Belgium); castor oil was from Carl Roth (Karlsruhe, Germany); and extra virgin olive oil (Ballester) was from Spain. The materials used to prepare the mediums—yeast extract, peptone, tryptone, glucose, and agar-agar—were obtained from BTL (Łódź, Poland). *p*-Nitrophenyl laurate was synthesized in our laboratory from lauryl chloride (99% Acros Organics) and *p*-nitrophenol (Sigma-Aldrich) in the presence of trimethylamine, as described by Vogel et al. [31]. The product was crystallized from methanol and tested for purity using gas chromatography (GC YL 6100 Young Lin Instrument).

2.2. Strains and Culture Mediums

Two strains of the yeast *Yarrowia lipolytica* were used in this study: KKP 379 and Po1d (double mutant LOX/HPL). The first was taken from the Collection of Industrial Microorganisms at the Institute of Agricultural and Food Biotechnology in Warsaw (Poland), and the second was from the collection of cultures at UMR PAM AgroSup-University de Bourgogne (France). The strains were stored at $-20\text{ }^{\circ}\text{C}$ in cryovials containing a cryoprotective agent and ceramic beads (Protect Select, Technical Service Consultants Ltd., Heywood, United Kingdom).

Yeast-peptone-glucose (YPG) medium contained of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. Yeast-peptone-glucose-agar (YPGA) medium was made by addition of 20 g/L of agar to YPG medium. Biotransformation medium for GDL production contained castor oil (from 10 g to 150 g/L), peptone (20 g/L), and Tween 80 (5 g/L). In the case of analysis of the influence of surfactant on the level of GDL synthesis, lecithin was added at the same concentration (5 g/L). Biotransformation medium for hexanal production consisted of 10 g/L tryptone, 5 g/L yeast extract, 5.3 g/L ammonium chloride, and linoleic acid from 25 g to 100 g/L.

2.3. Culture Conditions

Yarrowia lipolytica strains were multiplied for 48 h on Petri dishes with YPGA medium at 27 °C and used to inoculate 250 mL Erlenmeyer flasks containing 50 mL YPG medium. Flasks were shaken at 27 °C for 24 h at the speed of 140 rpm. This prepared medium was applied to inoculate the biotransformation mediums.

In the GDL production, the cells in logarithmic growth phase (24 h) were transferred to the biotransformation medium at an initial concentration of $OD_{600} \approx 0.25$. The biotransformation was then conducted in 500 mL Erlenmeyer flasks at 27 °C and 140 rpm for 7–9 days.

In the hexanal production, the same procedure was followed, but the initial cell concentration in the biotransformation medium was at the level of $OD_{600} \approx 1$. The reaction was carried out in 500 mL Erlenmeyer flasks at 27 °C and 140 rpm for 4 days.

2.4. Gamma-Decalactone Extraction and Quantification

For the quantification of GDL, 1.5 mL medium samples were taken regularly from the biotransformation culture. After acidification to a pH of about 2 by the addition of 10 μ L of concentrated HCl in order to favor lactonization, 20 μ L of γ -undecalactone was added as an internal standard. GDL was extracted with 1.5 mL of diethyl ether (liquid–liquid extraction) by gently shaking 20 times, as described Gomes et al. [32]. After separation, the organic phase was analyzed using GC. All assays were performed in duplicate.

GC analyses were performed using a YL 6100 Young Lin Instrument equipped with a capillary column BPX (30 m \times 0.25 mm ID) and a flame ionization detector. The oven temperature was programmed to increase from 165 °C to 180 °C at a rate of 3 °C/min and then to 230 °C at a rate of 5 °C/min. The injector and detector temperature were set to 200 and 250 °C, respectively. The nitrogen as a carrier was adjusted to velocity of 1.1 mL/min and 0.6 bars. Samples of 1 μ L were manually injected. The retention times for GDL and γ -undecalactone were 11.5 min and 12.4 min, respectively.

2.5. Hexanal Extraction and Quantification

The hexanal concentration was analyzed in the supernatant and in the cell extract. For aldehyde extraction, 2 mL of supernatant was taken and 50 μ L of isoamyl acetate (as an internal standard) was added and extracted with 2 mL of diethyl ether. The organic phase was collected for GC analysis. For quantification of hexanal in cells, 2 mL of medium was taken and cells were separated by centrifugation (10,000 \times g, 5 min, 4 °C). After centrifugation, the cells were washed with distilled water and resuspended in 1 mL of Tris HCl buffer (100 mM, pH = 7.5). Next, 0.2 g of glass beads (50–100 μ m) were added and the suspension was homogenized into FastPrep (3 cycles, 60 s). The obtained cell extract was made up to 2 mL of Tris-HCl buffer, and the internal standard was added (50 μ L) and extracted with 2 mL of diethyl ether.

The extracted sample was automatically injected (1 μ L) into an HP6890 gas chromatograph with an HP-Innowax capillary column (0.25 μ m film thickness, 30 m \times 0.25 mm). The oven temperature was initially 35 °C, and increased after injection up to 40 °C at a rate of 0.5 °C/min and then at a rate of 10 °C/min to 100 °C. Finally, the temperature was kept at 100 °C for 1 min. Nitrogen was used as a carrier gas at 4 mL/min. The split injector and the flame ionization detector were adjusted to 250 and 300 °C, respectively.

2.6. Measurement of Lipase Activity

The measurement of enzymatic activity was conducted using a spectrophotometric method based on the hydrolysis of *p*-nitrophenyl laurate. First, 0.3 mmol of *p*-nitrophenyl laurate dissolved in 2 mL of *n*-heptane was added to 15 mL of supernatant. Reactions were carried out in Erlenmeyer flasks (100 mL) for 15 min at 37 °C with vigorous stirring. The lipolytic activity was measured using the Marcel UV-VIS spectrophotometer at a wavelength of 410 nm. Results were calculated using the standard curve and expressed as U/mL. Units of activity were defined as the enzyme quantity that liberated 1 μ mol of *p*-nitrophenol per minute under the assay conditions.

2.7. Measurement of Lipoxygenase Activity

To measure the lipoxygenase activity, 20 μL of the supernatant or cell extract was introduced into 100 μL of linoleic acid solution and 880 μL phosphate buffer (50 mM, pH = 5.5). After 5 min reaction, spectrophotometric measurement was performed at a wavelength of 234 nm. The linoleic acid solution was prepared by mixing 157.2 μL of linoleic acid with 157.2 μL of Tween 20 and filling to 10 mL of distilled water (molar concentration of solution was 0.050 mol/dm³). Subsequently, 1 mL of 1 N NaOH was added for clarification and made up to 200 mL with phosphate buffer pH = 5.5 (50 mM). The entire solution was homogenized (Ultra-Turrax, 10,000 rpm). A similar solution was prepared for each analysis.

2.8. Determination of Biomass

Yeast biomass was determined using the thermogravimetric method. Biomass yield was analyzed after the elimination of the lipid fraction of culture medium. A 20 mL measure of biotransformation medium was centrifuged at the speed of 8000 rpm for 10 min (Centrifuge MPW-223). The supernatant was eliminated and the biomass was washed with an acetone/ethanol mixture (10:10, *v/v*). Afterwards, the yeast cells were washed in distilled water and dried at 105 °C until they reached a constant weight. The results were given as biomass yield with respect to 1 L of medium (g d.w. of cells/L).

2.9. Statistical Analysis

Statistical analysis was carried out using Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) software. The obtained results were analyzed using analysis of variance (ANOVA). The significance of differences between means ($p < 0.05$) were tested using Tukey's test.

3. Results

To analyze the conditions for biotechnological synthesis of the fat-derived fragrance compounds GDL and hexanal, the following factors were taken into account: the influence of the concentrations of castor oil and linoleic acid, used as substrates in the respective biotransformation reactions; the influence of heptane addition to the medium; the influence of surfactant (Tween, lecithin) addition; the emulsification of the culture medium; and finally, the effect of the inoculum concentration introduced into the biotransformation reaction.

3.1. The Analysis of the Conditions for Gamma-Decalactone Biosynthesis

3.1.1. The Influence of Castor Oil Concentration on Gamma-Decalactone Production

The influence of the substrate concentration in the culture medium on GDL biosynthesis was analyzed. Due to the fact that castor oil is used by microorganisms as both a carbon source (necessary for growth) and a substrate from which ricinoleic acid is released as a result of lipase activity, relatively high concentrations of castor oil, from 10 g/L to 150 g/L, were analyzed (Figure 1).

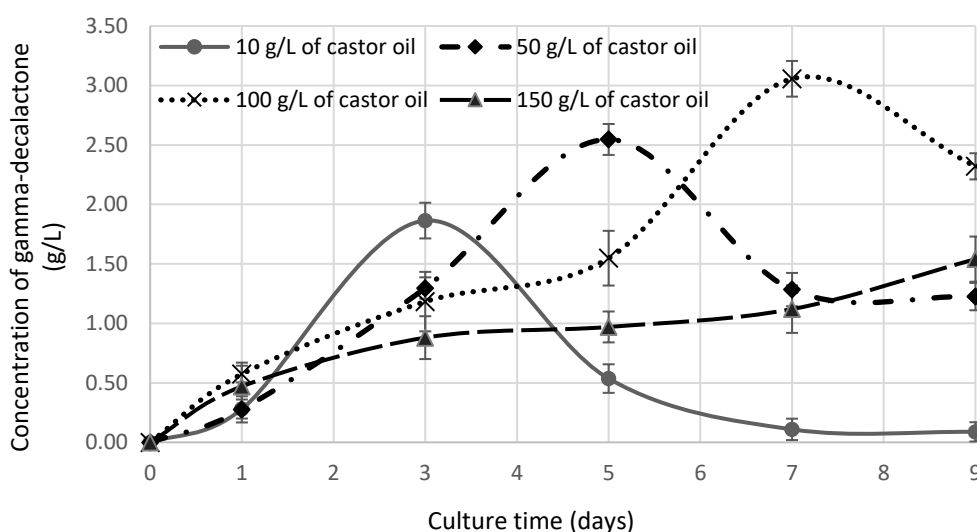


Figure 1. Effect of castor oil concentration in the medium on the amount of synthesized GDL.

The results presented in Figure 1 showed that the concentration of castor oil in the medium influenced the amount of lactone produced by the yeast. When castor oil was used in the medium at a concentration of 10 g/L, the microorganisms produced a maximum of approximately 1.86 ± 0.15 g/L of GDL. The highest biotransformation efficiency in this cultivation variant was observed on the 3rd day. After that period, the GDL concentration decreased to values of 0.54 ± 0.12 g/L (on the 5th day of cultivation) and $0.088 \text{ g/L} \pm 0.02 \text{ g/L}$ (on the 9th day of cultivation), respectively. It is assumed that the almost complete disappearance of lactone in the medium may have resulted from the yeast's ability to use it as a carbon source.

The highest lactone concentration among the analyzed mediums was recorded for the oil content of 100 g/L. *Yarrowia lipolytica* yeast produced approximately 3.06 ± 0.20 g/L of the aroma compound and the maximum of the reaction occurred on the 7th day of cultivation. For castor oil concentrations used in the range of 10–100 g/L, it was observed that higher oil concentrations delayed reaching the reaction maximum. At a concentration of 10 g/L, the reaction maximum was reached after 3 days of cultivation, at 50 g/L after 5 days of the reaction, and at 100 g of oil per liter on 7th day of the reaction.

In the above research, the highest concentration of castor oil, used at the level of 150 g/L, allowed for the production of only about 1.54 ± 0.11 g of lactone per liter (during the analyzed 9 day reaction period). Such a large amount of oil in the medium resulted in much weaker cell growth (unpublished data), and thus a lower lactone concentration.

In order to better visualize the influence of substrate concentration on the level of GDL biosynthesis, the curves of changes in GDL concentration over time were described using a five-parameter Pearson IV equation using TableCurve 2D v.5.01 software. High coefficients of determination from 0.9393 to 0.9939 were obtained. On this basis, the rates of change in GDL concentration depending on the substrate concentration in the biotransformation mediums were determined and described with the following equation:

$$y^{-1} = 1.1389798 + 0.060850278x + 0.0064025173x^{1.5} \quad (1)$$

where x is the concentration of castor oil (substrate) in g/L and y is the concentration of gamma-decalactone in g/L.

3.1.2. Effect of Heptane Presence on Gamma-Decalactone Production

The decrease in the concentration of GDL after a certain cultivation time (Figure 1) may have been related, as mentioned, to the use of lactone as a carbon source by yeasts, or it may have resulted from lactone hydrolysis. In either case, the contact of the reactants with the aqueous phase seemed to be decisive for the course of the process. Hence, an attempt was made to conduct a cultivation with the addition of an organic solvent: heptane

(1 mL/100 mL). The aim of this experiment was to examine whether the presence of an organic phase in which GDL could dissolve and be less accessible to microorganisms, and at the same time less susceptible to hydrolysis, would limit the decrease in its concentration in the medium. Heptane was chosen as a solvent due to its relatively low toxicity and neutrality to the growing yeasts *Yarrowia lipolytica* [33].

The results of the experiment (Figure 2) for two castor oil concentrations in the medium, 50 and 100 g/L, did not show a clear correlation between the addition of heptane and the maximum amount of GDL synthesized by yeast. In the cultures with the addition of heptane, as in the control sample, maxima of the reaction were observed, after which the lactone concentration decreased.

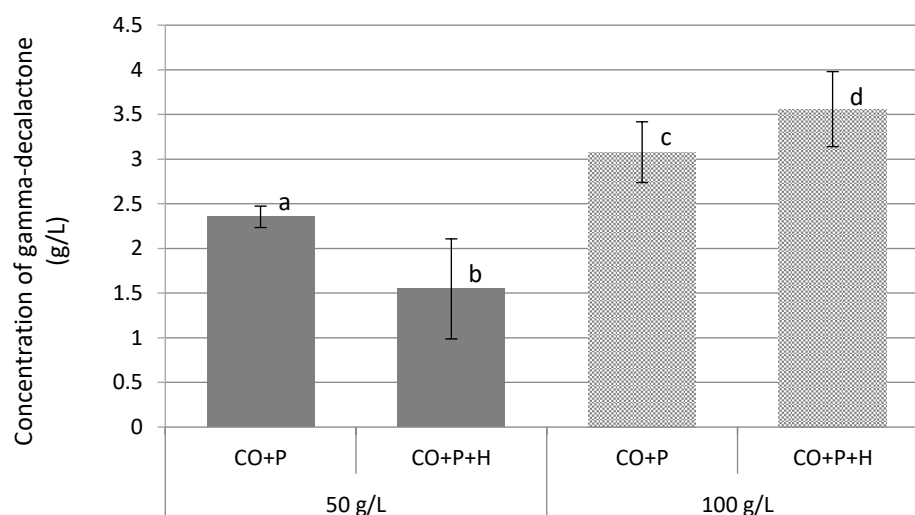


Figure 2. Effect of heptane presence on the maximum level of GDL concentration in biotransformation medium. CO+P: medium with castor oil and peptone; CO+P+H: medium with castor oil, peptone and heptane. Different letters denote significant differences at $p < 0.05$.

3.1.3. Effect of Surfactant Presence on Gamma-Decalactone Production

Since the medium used in the biotechnological synthesis of GDL is an oil-in-water emulsion, the influence of surfactant addition on the GDL biosynthesis efficiency was also analyzed. Surfactants are chemical compounds with the ability to change the surface properties of the liquid in which they are dissolved. The decrease of the surface tension by these molecules at the water/oil interface contributes to the formation of stable emulsions [34]. In our research, the addition of two nonionic and one amphoteric surfactants on the biosynthesis of GDL was analyzed. The selected surfactants are included in the list of food additives and are commonly used in the food industry: nonionic: Tween 20-E432 and Tween 80-E433 and amphoteric: lecithin-E322.

Figure 3 presents data on the concentration of GDL in media with added surfactants on particular days of a 7 day yeast cultivation. A control culture was carried out simultaneously without the addition of a surfactant. The collected data did not show a significant statistical effect of surfactant supplementation on the level of GDL biosynthesis. In case of the addition of Tween 20 (polyoxyethylenesorbitan monolaurate), the maximum lactone concentration on the 6th day of culture 2.44 ± 0.07 g/L was almost identical to that of the control medium 2.45 ± 0.25 g/L (maximum on the 7th day of the reaction). In the case of the addition of soy lecithin, the maximum level of the flavor compound was 2.05 ± 0.11 g/L. The highest content of lactone was achieved in the medium with added Tween 80 (polyoxyethylenesorbitan monooleate) 2.99 ± 0.24 g/L.

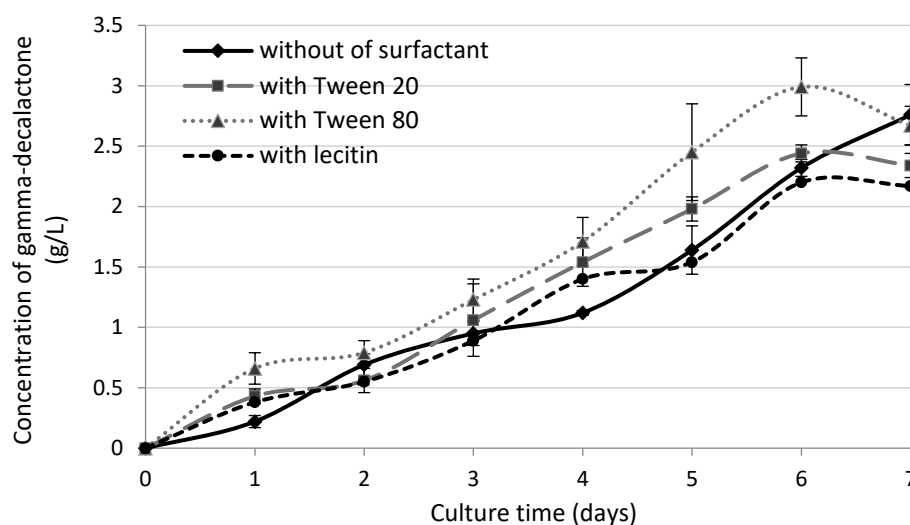


Figure 3. Effect of surfactant addition (5 g/L) on biotransformation of ricinoleic acid into GDL by yeast *Yarrowia lipolytica*.

3.1.4. Effect of the Emulsification of the Medium on Gamma-Decalactone Production

Another element of the present research was to analyze the influence of medium emulsification on the GDL production. It is believed that the contact surface of fat droplets with cells of multiplied organisms and the contact surface between two liquid phases may be a factor affecting the degradation of the hydrophobic substrate, and thus cell growth and aroma production. Without emulsification, the lipid and water phases clearly separate, which causes problems when taking a reliable sample [35]. Attention was paid to the level of multiplication of yeast cells and the lipolytic activity they exhibited. Moreover, the conversion yield of GDL per biomass formed was compared and the specific rate of GDL production was calculated (Table 1).

Table 1. Effect of the emulsification of the medium on growth and catalytic activity of the yeast *Yarrowia lipolytica*.

Culture Time	Medium	Concentration of GDL L (g/L)	Lipolytic Activity (U/mL)	Biomass Yield X (g _{d.w.} /L)	Conversion Yield of GDL Per Biomass Formed $Y_{L/X}$ (g/g _{d.w.})	Specific Rate of GDL Production q_L (g/g _{d.w.} /h)
3rd day	non-emulsified	2.02 ± 0.06 ^a	0.13 ± 0.02 ^a	21.85 ± 2.99 ^c	0.092 ^a	0.0013 ^b
	emulsified	1.66 ± 0.08 ^a	1.46 ± 0.18 ^c	8.48 ± 0.87 ^a	0.196 ^c	0.0027 ^d
7th day	non-emulsified	3.21 ± 0.14 ^b	0.23 ± 0.04 ^b	22.03 ± 3.08 ^c	0.146 ^b	0.0009 ^a
	emulsified	5.25 ± 0.10 ^c	0.26 ± 0.05 ^b	18.58 ± 1.55 ^b	0.283 ^d	0.0017 ^c

Letters compare values in the same column, different letters denote significant differences at $p < 0.05$.

The data presented in Table 1 show that the emulsification of the medium allowed higher biotransformation yields to be obtained. On both the 3rd and 7th days of the reaction, the amount of GDL produced in relation to the dry weight of the cells in the emulsified media was twice as high as that in the nonemulsified medium. The $Y_{L/X}$ parameter was the highest on the 7th day of the reaction (0.283 g/g_{d.w.}), and on that day the highest concentration of lactone was recorded, reaching the level of 5.25 ± 0.10 g/L. This value was about 2 g higher than in the nonemulsified medium. It may have been related to the high extracellular lipolytic activity of *Yarrowia* yeast in this medium observed on the 3rd day of cultivation (1.46 ± 0.18 U/mL). The activity of lipases is important for the hydrolysis of castor oil present in the medium in order to release ricinoleic acid, the precursor of GDL.

The yield of biomass shows that the emulsification of the medium contributes to slower and less intensive multiplication of cells. It is assumed that in nonemulsified media,

yeast cells reached the stationary phase of growth much earlier (on the 3rd day of the reaction), because the difference in the biomass yield between the 3rd and 7th days was statistically insignificant. The rate of multiplication of microorganisms was much lower in the emulsified media. On the third day of the reaction, the cells were still in log phase of growth. The yield of yeast biomass between the 3rd and the 7th days for this culture increased approximately twice (from the value of 8.48 ± 0.87 to 18.58 ± 1.55 g d.w. cells/L).

3.1.5. Effect of Inoculum Size on Gamma-Decalactone Concentration

In the present research on GDL biosynthesis, the influence of inoculum concentration introduced into the medium was also analyzed. The concentration of microorganism cells seemed to be quite an important factor influencing the content of lipolytic enzymes in the medium and the achieved level of castor oil hydrolysis, and thus the biotransformation efficiency. For this purpose, a 7 day cultivation was carried out, differentiating the addition of the inoculum in the range of 0.25–5 mL per 100 mL of medium (Table 2). The optical density (OD_{600}) of yeast cells in the inoculum preculture ranged from 2 to 2.2 (average $OD_{600} 2.09 \pm 0.11$).

Table 2. Effect of inoculum size on the concentration of gamma-decalactone in the medium during the 7-day culture.

Inoculum Volume (mL/100 mL of Medium)	Concentration of Gamma-Decalactone (g/L)				
	Culture Time (days)				
	1	2	3	5	7
0.25 mL	0.15 ± 0.02	0.48 ± 0.02	0.67 ± 0.01	1.00 ± 0.11	1.92 ± 0.14
0.5 mL	0.14 ± 0.01	0.31 ± 0.03	0.45 ± 0.04	1.32 ± 0.14	2.10 ± 0.04
1 mL	0.13 ± 0.04	0.45 ± 0.03	0.57 ± 0.02	1.71 ± 0.07	3.01 ± 0.15
2 mL	0.11 ± 0.03	0.25 ± 0.09	0.64 ± 0.07	0.97 ± 0.01	1.29 ± 0.06
5 mL	0.08 ± 0.01	0.21 ± 0.07	0.58 ± 0.10	0.95 ± 0.06	1.37 ± 0.08

The collected data indicate that the concentration of inoculum in the medium had a significant impact on the achieved GDL concentration. The highest content of aroma compound, at the level of 3.01 ± 0.15 g/L, was obtained with the addition of 1 mL of inoculum per 100 mL of medium. In the first 2 days of the reaction, the concentration of GDL was inversely proportional to the concentration of the inoculum used. Regardless of the initial amount of yeast cells in the biotransformation medium, the concentration of GDL increased in all variants during the 7 day cultivation.

3.2. The Analysis of the Selected Conditions for Hexanal Biosynthesis

A *Yarrowia lipolytica* strain (Po1d/LOX/HPL) expressing lipooxygenase and hydroperoxide lyase from the green bell pepper fruit was used in the present research on the biosynthesis of hexanal, a representative of the green-note aroma.

3.2.1. Lipooxygenase Activity and Hexanal Production in the Cellular Extract and Supernatant

One of the key parameters in the synthesis of hexanal by microorganisms is the activity of lipooxygenase, an enzyme that catalyzes the conversion of linoleic acid into 13-hydroperoxy-9,11-octadecadienoic acid. Figure 4 shows the data on the growth of yeast cells expressed in dry cell weight and the data on lipooxygenase activity in the supernatant and cellular extracts. LOX activity was measured at 12 h intervals for a period of 96 h during the growth of *Yarrowia lipolytica* yeast on media supplemented with linoleic acid or olive oil (5 g/100 mL). Olive oil was used for a comparative purpose to test how active LOX was in the presence of a mixture of fatty acids in the medium. According to Fabiszewska and Białeczka [36], olive oil contains approximately 6.2% linoleic acid, the precursor of hexanal.

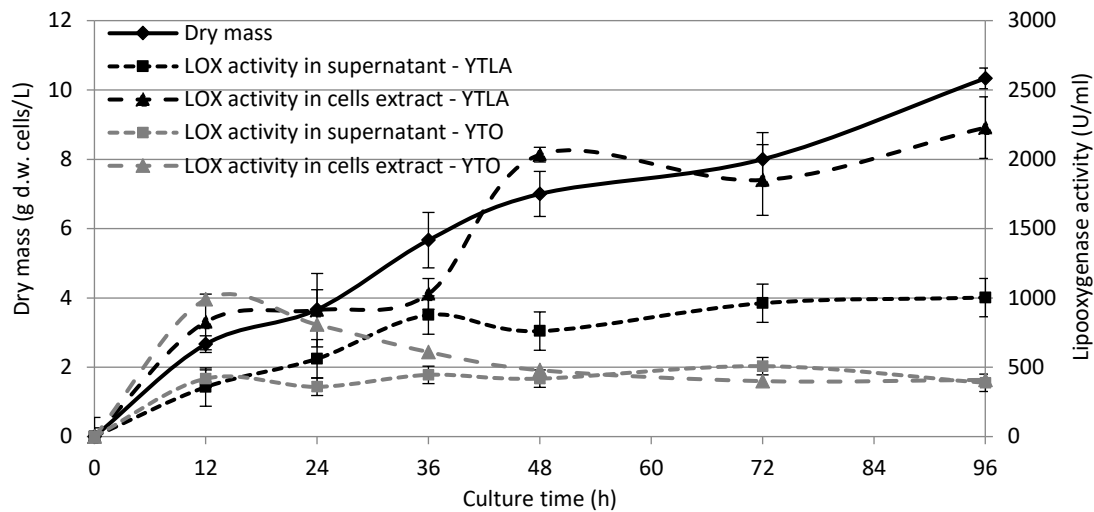


Figure 4. The dry mass of cells and lipoxygenase activity in cell extracts and supernatants during the growth of *Yarrowia lipolytica* in media with linoleic acid and olive oil.

The data shown in Figure 4 indicate that the yeast showed lipoxygenase activity both on a medium enriched with pure linoleic acid and on a medium with added olive oil. On the first day of the reaction, the LOX activity in the cell extract or the supernatant was comparable in both media. After 24 h, this activity varied significantly. Much higher LOX activity (2.5–5 fold) was observed in the linoleic acid medium. This is probably due to the fact that in the medium with olive oil, linoleic acid as a LOX activator was not directly available, but had to be released from triacylglycerols through lipase-mediated hydrolysis. In addition, the efficiency of the olive oil hydrolysis process should also be considered in order to optimize its concentration in the culture medium. In case of the medium with the addition of linoleic acid, the activity of lipoxygenase in the cell extract after 2 days of reaction was about 2.2–2.5 times higher than the activity in the supernatant. In the analyzed period, up to 96 h of culture, the cells were in the logarithmic growth phase—the dry cell mass yield increased during this period from 2.67 ± 0.24 g/L to 10.33 ± 0.30 g/L. The highest LOX activity (2228.37 ± 222.03 U/mL in the cell extract and 1002.26 ± 153.71 U/mL in the supernatant) was obtained for this medium after 96 h of culture.

The mutant yeast strain was active in converting linoleic acid to green aldehyde (Table 3). The higher activities of lipoxygenase in the cell extracts corresponded to the higher contents of hexanal in this fraction (the maximum concentration of hexanal was approximately 16 times higher relative to the supernatant fraction: 352.09 ± 22.69 mg/L and 21.71 ± 2.33 mg/L, respectively). In the initial period of cultivation, the concentration of hexanal in the cell extract increased, reaching its maximum concentration after about 48 h. In the supernatant, the highest concentration of hexanal was obtained in the first day of the reaction. After this period, its content decreased.

Table 3. The concentration of hexanal synthesized by *Yarrowia lipolytica* on the medium with linoleic acid.

Culture Time (h)	Concentration of Hexanal (mg/L)	
	Cellular Extract	Supernatant
12	120.14 ± 12.22	10.86 ± 2.01
24	137.09 ± 22.12	21.71 ± 2.33
36	281.07 ± 17.54	10.31 ± 1.87
48	352.09 ± 22.69	7.89 ± 0.87
72	290.46 ± 8.98	3.19 ± 0.65
96	241.32 ± 23.78	3.54 ± 1.03

3.2.2. Effect of Linoleic Acid Concentration on Lipoxygenase Activity and Hexanal Production

In the present studies on the production of hexanal, the impact of the substrate concentration (linoleic acid) on the level of its consumption by yeasts and the lipoxygenase activity demonstrated was analyzed (Figure 5). In the research, 25, 50, and 100 g/L additions of linoleic acid were used.

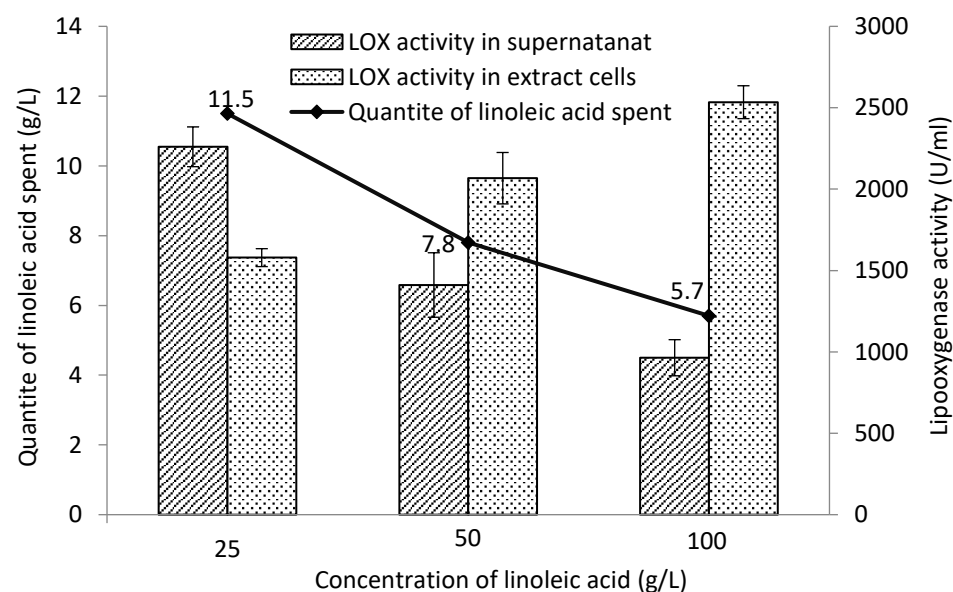


Figure 5. The influence of the concentration of linoleic acid on the quantity consumed by the yeast *Yarrowia lipolytica* and its lipoxygenase activity.

Studies have shown that the higher the concentration of linoleic acid in the substrate, the lower the efficiency of its biotransformation. With the increasing concentration of the substrate in the medium, the activity of lipoxygenase increases in the cell extract and decreases in the supernatant. With the addition of 25 g/L linoleic acid, the LOX activity in the supernatant was 2260 ± 122 U/mL and that in the cellular extract 1579 ± 55 U/mL. When the addition of acid was 100 g/L, the LOX activity in the supernatant was about 2.5 times lower and amounted to 964 ± 111 U/mL, while in the cell extract it increased to 2534 ± 101 U/mL. Along with the increase in the concentration of linoleic acid in the medium, not only did the LOX activity in the cell extract increase, but so did the concentration of the synthesized hexanal (Table 4). When using 100 g/L of linoleic acid, the concentration of hexanal in the cell extract was 189.44 ± 29.23 mg/L, nearly 5 times higher than in the culture where 2.5 g/L of linoleic acid was used. The results showed no correlation between the hexanal concentration in the supernatant and the amount of linoleic acid in the yeast culture medium.

Table 4. Influence of linoleic acid concentration on the level of hexanal biosynthesis by *Yarrowia lipolytica* yeasts.

Concentration of Linoleic Acid (g/L)	Concentration of Hexanal (mg/L)	
	Cellular Extract	Supernatant
25	38.68 ± 4.87	8.32 ± 2.22
50	163.82 ± 19.98	47.10 ± 8.81
100	189.44 ± 29.23	15.40 ± 4.46

4. Discussion

Although research on the biosynthesis of GDL and green-note aroma compounds has been carried out for over a dozen years, the search continues for strains and the optimal

reaction conditions to increase the efficiency of the process and make it more competitive on the market. The yield of synthesis of the fat-derived fragrance compounds is significantly dependent on the amount of lipid substrate in the medium.

The present research on the biosynthesis of GDL showed that the higher the concentration of castor oil used in the medium, the longer the time needed to obtain the maximum concentration of lactone—from three days at an oil concentration of 10 g/L to 7 days at an oil concentration of 100 g/L. The optimal concentration of the lipid substrate in the biosynthesis of GDL is the result of the level of castor oil consumption as a source of carbon necessary for the growth of microorganisms, the intensity of multiplication of the microorganisms, the secretion of extracellular lipases, the level of hydrolysis of castor oil to ricinoleic acid, and finally the inclusion of the acid in the β -oxidation pathway.

Similar conclusions were presented by Gomes et al. [37], who also dealt with the optimization of castor oil concentrations in the context of obtaining the highest concentrations of GDL in the reaction mixture. They added 10, 30, and 50 g/L of oil to the culture media and monitored the time needed to reach the highest concentrations of GDL. The results of their experiments, as well as ours, showed that the higher the concentration of castor oil in the medium, the longer the time needed to obtain the maximum concentration of lactone. However, the team showed that the concentration of GDL in the medium was not proportional to the amount of lipid substrate used. The addition of 30 g/L castor oil resulted in about 1.84 g/L of lactone production, whereas the addition of oil at a level of 10 or 50 g/L caused the GDL synthesis to be less efficient, resulting in lower concentration of about 1.35 g/L.

The decrease in lactone concentration after reaching the maximum biotransformation, observed in each medium regardless of the concentration of castor oil, was confirmed by the studies by Groguenin et al. [38] and Gomes et al. [39]. In the first case, using methyl ricinoleate in a medium at a concentration of 5 g/L, the team obtained 0.15 g of lactone per liter after 24 h of cultivation. After 40 h, the concentration of GDL decreased more than three times (to 0.05–0.06 g/L), and in the final stage of the reaction it was only 0.02 g/L (a decrease of 7.5 times). Using 10 times the amount of substrate (50 g/L of methyl ricinoleate), Gomes et al. [39] achieved a maximum of about 1 g/L, with a subsequent decrease of 10 times in the next days of cultivation. Additionally, during solid-state fermentation castor seed used as the source of substrate, a decrease in the concentration of GDL in the medium was observed from the value of about 0.19 g/L obtained in the 28th hour of culture to 0.06 g/L at 90 h of fermentation [40].

There are several theories explaining the reasons for this decrease in lactone concentration in the medium. Research on *Yarrowia* yeast has shown that this microorganism has genes coding for enzymes specializing in the degradation of hydrophobic substrates [33]. *Yarrowia lipolytica* cells contain six acyl-CoA oxidases (Aox1 to Aox6, encoded by the POX1 to POX6 genes). POX gene products show different substrate carbon chain degradation abilities. Aox2 oxidase is specific for long-chain substrates, while Aox3 shows activity towards short-chain compounds, leading the oxidation process of the 10-carbon chain and thus contributing to the degradation of the GDL produced [21]. This was confirmed in the studies by Małajowicz et al. [35] comparing the production of GDL by the wild W29 strain and the MLYP40-2p mutant strain of the yeast *Yarrowia lipolytica*.

According to Braga and Belo [41] the most probable route for GDL degradation is the opening of the lactone ring through gamma-lactonase activity, followed by the activation of the CoA esters and β -oxidation. The lactone oxidation pathway is also possible, producing a dicarboxylic acid after delactonization. According to Waché et al. [42], an intense decrease in lactone concentration may also occur after the depletion of ricinoleic acid in the medium.

Regarding the efficient hydrolysis of castor oil and biotransformation of ricinoleic acid, the contact of the lipid droplet surfaces with the cells of microorganisms is also important. Although the present research did not show a statistically significant difference between the efficiency of GDL synthesis on substrates with added surfactants and the control ones, the literature from this field includes some reports confirming that surfactants play a key

role in the degradation of hydrophobic substrates, and thus indirectly influence the results of GDL synthesis [13]. It is believed, among other factors, that they can accelerate the rate of transport of substrates into the interior of cells and contribute to a greater degree of development of the interface surface. Aguedo et al. [13] drew these conclusions on the basis of research carried out on the synthesis of GDL, in which both ionic and nonionic surfactants were added to the medium. When using saponin, Triton X-100, Tween 80, CTAB (hexadecyltrimethylammonium bromide), and SDS (sodium dodecyl sulfate) in the biotransformation of methyl ricinoleate to GDL, it was found that the highest concentration of aroma compound (0.234 g/L) was possible in the presence of Tween 80. This is consistent with our test results, in which the highest concentration of GDL in the tested media was also recorded in the presence of this surfactant. In the authors' research, the efficiency was approximately 2.5–3 times lower when using Triton X-100, the presence of which was associated with the shorter viability of yeast cells. The authors also suggested that the ionic surfactants used (SDS or CTAB) had a toxic effect on microorganisms (especially due to interactions with cell membranes), which may explain the complete lack of GDL in the samples extracted from these media.

The presented research showed that mechanical emulsification of the substrate was extremely important from the point of view of the level of GDL synthesis, which is not mentioned in the extensive literature on the biosynthesis of GDL by microorganism cells. Creating finely dispersed droplets contributes heavily to a medium's stability. The dispersed lipid phase ensures better contact of the substrate with the enzyme, higher lipolytic activity of cells, higher level of castor oil hydrolysis, and thus higher biotransformation efficiency. Thanks to emulsification, the conversion yield of GDL per biomass formed and specific rate of GDL production were more than twice as high as in nonemulsified substrates.

Another factor that determines the level of GDL biosynthesis is the size of the inoculum introduced into the reaction. The present research showed that with an optical density of the substrate in the range of 2 to 2.2 (average $OD_{600} 2.09 \pm 0.11$), the best results in the aroma production were obtained for an inoculum size of 1 mL. Larger inoculum volumes (2 mL or 5 mL) resulted in a lower concentration of aroma compound. Literature data indicate that a smaller inoculate can result in higher viable counts. Davis et al. [43] analyzed the effect of inoculum size on the cultivation of soil bacteria. The authors showed that decreasing the inoculum size resulted in significant increases in the viable cell count. This may explain the much higher GDL concentrations achieved within 48 h for the lower inoculum size— 0.48 ± 0.02 g/L on the 2nd day of reaction for an inoculum size of 0.25 mL/100 mL and two-fold less lactone for the inoculum of 5 mL/100 mL of medium. However, the kinetics of colony development did not differ for the different inoculum sizes.

Liu et al. [44] investigated the influence of inoculum volume on biomass, lipid content, and lipid production by *Lipomyces starkeyi*. Their analyses showed that the inoculum size had an important influence on the biomass and lipid accumulation. Experiments were conducted with different inoculum volumes ranging from 5 to 25%. The authors believe that the low inoculation concentration and the rich nutrition component in the fermentation medium were used for cell growth. A high cell concentration in the medium with the same nutrient content resulted in a lack of nutrients for metabolite synthesis. Perhaps, in our experiments, the low concentration of nutrients and ricinoleic acid was a factor contributing to the lower concentrations of GDL in the media with 2 mL and 5 mL of inoculum.

Studies have shown that *Yarrowia lipolytica* yeast, Po1d strain (a genetically modified derivative of W29 wild-type isolate, obtained at INRA France), with the selection of the appropriate lipid substrate and its concentration, also synthesizes a hexanal or "green aldehyde". Cultures containing linoleic acid or olive oil confirmed the substrate specificity of lipoxygenase, one of the key enzymes in the synthesis of green-note aroma compounds. Both the supernatant and the cell extract showed enzymatic activity with respect to both linoleic acid and olive oil as a lipid substrate, but the activity was much higher with linoleic

acid in the medium. These results are consistent with the studies by Bisakowski et al. [45]. This team investigated the substrate specificity of the partially purified LOX (FIIa) fraction of *Morchella esculenta* using free fatty acids and mono-, di-, and triacylglycerols. The highest relative enzymic activity was obtained with the use of linoleic acid (100%), followed by arachidonic acid (83.3%). The results also showed that the lowest relative LOX activity of 7.7–17.8% was exhibited towards the mono-, di-, and triacylglycerols. Bisakowski et al. [46] reached a similar conclusion for the catalytic activity of partially purified extracts of the yeast *Saccharomyces cerevisiae*.

The level of hexanal production is also determined by the activity of hydroperoxidase, which converts the hydroperoxides of fatty acids into short-chain C₆–C₉ forms. This enzyme is believed to be the major limiting factor in bioformation efficiency, due to the suicide inactivation phenomena and low storage and process stability. The activity of this enzyme was not determined in the conducted biotransformations.

The different concentrations of linoleic acid used in the assessment of hexanal productivity by the *Yarrowia lipolytica* Po1d strain showed that when using a higher concentration of linoleic acid, a lower level of its conversion was obtained. The determined activity of LOX was correlated with the level of hexanal production. The higher LOX activity in the cell extract observed in the culture with the higher concentration of lipid substrate corresponded to a higher aldehyde content. However, the level of biotransformation efficiency, compared to the literature data, was about 2–2.5 times lower—for comparison, Márczy et al. [47] achieved an efficiency of about 54% in their research.

Lower levels of synthesized hexanal may result from the loss of proper HPL activity in yeast, or may be the result of partial conversion of hexanal into the corresponding alcohol, the presence of which in the medium was not determined. According to Bourel et al. [48], lower concentrations of hexanal in the medium may be the result of fatty aldehyde dehydrogenase (FALDH) enzymes. A survey of *Yarrowia lipolytica* sequences by this team showed four putative FALDH genes in this yeast which might be induced by the production of hexanal, resulting in its degradation into the alcohol.

According to Schade et al. [49] and Buchhaupt et al. [30], in biotransformation reactions with the participation of whole-cell catalysts or isolated enzymes, a decrease in the concentration of green-note aroma compounds is observed after a specific period of biotransformation, which may result from strong inhibition of the reaction products against enzymes. Hence, it is extremely important to terminate the reaction when the correct level of hexanal is obtained. The time taken to reach the maximum hexanal synthesis, after which a decrease in aldehyde concentration was observed, varied in our studies—it was 24 h for the supernatant and 48 h for the cell extract (Table 3). This time was also taken into account when determining the aldehyde contents in media with different concentrations of the lipid substrate.

5. Conclusions

Yarrowia lipolytica yeast is able to synthesize potentially valuable flavor compounds, and the examples presented in this article demonstrate the potential catalytic power of these microorganisms in the biosynthesis of GDL and hexanal, both fat-derived fragrance compounds. The efficiency of biotechnological synthesis of these compounds can be significantly modified by selecting the appropriate process parameters, which is done in many research centers. This study showed that the concentration of the lipid substrate and the degree of emulsification of the medium had the greatest impact on the amounts of aroma compound produced. When using the *Yarrowia lipolytica* strain KKP379 in a mechanically emulsified medium (homogenization 5 min, 10,000 rpm) not previously described in the literature, with a castor oil content of 100 g/L, it was possible to obtain over 5 g of lactone per liter of medium (5.25 ± 0.10 g/L). This amount of the fragrance compound is several times higher (from 2.8 to 5) than the examples presented in the cited publications, where concentrations ranging from 1 to 1.84 g/L were achieved. The use of higher concentrations of the lipid substrate in the medium was connected with the extension of reaching the maximum reaction time.

By increasing the concentration of castor oil in the medium from 10 to 100 g/L, the time taken to reach the maximum concentration of the product increased from 3 to 7 days. The addition of an organic phase (heptane) to the biotransformation medium, in terms of better solubility of hydrophobic GDL, did not increase the concentration of the aroma compound and did not protect against its degradation after the specified reaction time. The whole-cell catalyst used in the present study, with the activity of LOX and HPL enzymes (Po1d, double mutant LOX/HPL), may be a promising approach towards an efficient synthesis of green-note fragrance compounds by microorganisms. In the range of analyzed concentrations of linoleic acid (25–100 g/L), the higher the concentration of lipid substrate in the medium, the higher the activity of Po1d lipoxygenase and the higher concentration of hexanal in the cell extract. Comparing the extreme values of linoleic acid added to the substrate, lipoxygenase activity increased by approximately 1.6 times (from 1579 ± 55 U/mL to 2534 ± 101 U/mL) and the hexanal concentration increased by approximately 4.9 times (from 38.68 ± 4.87 mg/L to 189.44 ± 29.23 mg/L).

The presented results are only a part of the work that should be put into the development of the bioprocess in order to make it competitive on the market. Further research is necessary to overcome the limitations identified so far, namely the activity of the mutant HPL and potential toxicity of GDL to yeast at higher concentrations in the medium.

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Abbreviations

GDL	gamma-decalactone;
LOX	lipoxygenase;
HPL	hydroperoxide lyase;
CAGR	compound annual growth rate;
GRAS	generally recognized as safe;
FDA	Food and Drug Administration;
OD	optical density

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