Comparative analysis of L-carnitine production by Yarrowia lipolytica in different culture conditions in biofuel waste and fatty acid-poor medium for commercial purposes

Monika Elżbieta Jach (monijach@kul.pl)
John Paul II Catholic University in Lublin https://orcid.org/0000-0002-4932-3260
Konrad Kubiński
John Paul II Catholic University of Lublin: Katolicki Uniwersytet Lubelski Jana Pawla II
Marek Juda
Medical University of Lublin: Uniwersytet Medyczny w Lublinie
Ewa Sajnaga
John Paul II Catholic University of Lublin: Katolicki Uniwersytet Lubelski Jana Pawla II
Tomasz Baj
Medical University of Lublin: Uniwersytet Medyczny w Lublinie
Anna Malm
Medical University of Lublin: Uniwersytet Medyczny w Lublinie

Research

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Abstract

Background

Yarrowia lipolytica is an oleaginous yeast with the ability to grow in a variety of hydrophilic and hydrophobic substrates, including industrial wastes, in which it produces and accumulates various nutrients.

Methods

The aim of the present study was to examine the presence of free L-carnitine in the biomasses of two *Yarrowia lipolytica* strains (A-101 and ATCC 9793) growing in biofuel waste and YPD medium. The cultivations of *Y. lipolytica* were performed in aerobic conditions at different temperatures (20–30°C) and pH values (4.0–7.0) of the media with and without the addition of precursors for L-carnitine production (trimethyllysine, iron, and L-ascorbic acid) in a laboratory scale or other substances (chromium, selenite, or zinc) in a pilot plant scale.

Results

Both tested *Y. lipolytica* strains grown in fatty acid-poor YPD medium contained endogenous free L-carnitine in their biomass with a maximum of 22.85 mg/100 g of wet biomass. The addition of L-carnitine precursors to the YPD medium exerted a significant effect on L-carnitine concentration in the yeast biomass, increasing it up to 250%. In turn, the biomass of both tested *Y. lipolytica* strains cultivated in the biofuel waste, irrespective of the culture conditions, contained below 1 mg of L-carnitine/100 g of wet biomass. However, the supplementation of the culture media with the L-carnitine precursors significantly increased the yield of the yeast biomass by 20–30% in the non-fermentable biofuel waste cultures. Moreover, the addition of chromium (III) chloride into the biofuel waste caused an increase in the free L-carnitine concentration in the yeast biomass up to 2.24 mg/100 g of dry weight.

Conclusion

Biomass of *Y. lipolytica* grown in the fat-poor medium contained free L-carnitine, in contrast to the biomass grown in the fat-rich biofuel waste. The very low amounts of L-carnitine in the biomass of *Y. lipolytica* grown in the crude biofuel waste suggest that the yeast is able to utilize almost the entire pool of free L-carnitine for growth and nutritional biomass production. However, the addition of chromium to the biofuel waste contributed to an increase in L-carnitine concentration in *Y. lipolytica* biomass.

Background

L-carnitine or γ-trimethylamino-β-hydroxybutyric acid is a ubiquitous water-soluble quaternary amine compound [1, 2, 3]. It is synthesized by most eukaryotic organisms, including some yeast, from amino acids: lysine as a precursor and methionine or S-adenosyl methionine as a methyl donor [3, 4]. In humans, endogenous synthesis of L-carnitine occurs chiefly in the liver. However, it must be complemented through dietary uptake. Since it is regarded to be a quasi-nutrient or conditionally essential nutrient, L-carnitine deficiencies sometimes cause life-threatening disorders. As an important factor in cellular metabolism, L-carnitine binds fatty acids and transfers them to the mitochondria for β-oxidation required for generation of energy. Without L-carnitine, the mitochondrial inner membrane is impermeable to fatty acids [1, 5, 6]. L-carnitine transports long- to short-chain fatty acids out of the peroxisome, where β-oxidation is started, into the mitochondria, where the process is completed, by reversible esterification of the β-carbon hydroxyl group with a fatty acid to form *O*-acyl-carnitine. Cytosolic acyl-carnitine is then transported by carnitine acetyltransferases (CATs) into the mitochondrial matrix with a simultaneous 1:1 exchange with intramitochondrial free L-carnitine located within the mitochondrial inner membrane [1]. CATs transfer the coenzyme A (CoA) group of acetyl-CoA to carnitine; hence, acetyl-carnitine is biosynthesized, which can be transferred via the peroxisomal and mitochondrial membranes to the tricarboxylic acid (TCA) cycle [7]. Interestingly, the supplementation with L-carnitine results in more effective fatty acids transport to the mitochondria, where their decomposition occurs in the β-oxidation process [8].

The best-known yeast *Saccharomyces cerevisiae* is unable to synthesize L-carnitine. However, this yeast possesses enzymatic activities, especially CATs, which allow the uptake of L-carnitine from the environment [4, 7, 10]. Studies reported elsewhere [3, 12–14] revealed that some oleaginous yeast, *e.g. Candida albicans* and *Yarrowia lipolytica*, synthesize *de novo* endogenous L-carnitine. Similar to *S. cerevisiae*, *C. albicans* and *Y. lipolytica* have the activity of three forms of CATs; however, the oleaginous yeast is able to grow in a medium containing fatty acids without supplementary carnitine [3, 15]. Additionally, *Y. lipolytica* possesses proteins for transport and activation of fatty acids (*i.e.* Faa1p, Pxa1p, Pxa2p, and Ant1p) similar to those of *S. cerevisiae.* However, the activation and mechanism of the peroxisomal transport of fatty acids into *Y. lipolytica* mitochondria differ considerably from that in *S. cerevisiae* [15]. In contrast to *S. cerevisiae*, *Y. lipolytica* has six additional acetyl-CoA oxidases (encoded by *POX1-6* genes) with different chain-length specificities, which allow direct utilization of fatty acid-rich feedstock through complete oxidation using intermediates of β-oxidation [9, 16–18]. When yeasts grow on a fatty acid-poor or fat-fee carbon source, peroximal structures cannot be identified. In turn, when they grow on fatty acid-containing media, the proliferation of peroxisomes is clearly detected. Thus, peroxisomes seem to be strictly required for yeasts to catabolize fatty acids. They play the sole role in β-oxidation in the fatty acid degradation process causing the formation of acetyl-CoA in the peroxisome. Therefore, L-carnitine is important for the acetyl shuttle mechanism, transferring long-chain fatty acids between peroxisomes and mitochondria for subsequent β-oxidation during yeast growth on non-fermentable carbon sources such as fatty acids, acetate, and ethanol [4, 7]. During yeast growth on these carbon sources, acetyl units are only produced in the cytosol and need to be transferred to the mitochondrial TCA cycle and to the peroxisomal/cytosolic glyoxylate cycle [9].

The availability of fatty acids from industrial by-products promotes the use of fatty acid-enriched feedstock as a cheap carbon source by oleaginous yeast [12]. It has been shown that *Y. lipolytica* grown on a medium with different fatty wastes is a natural source of such nutritional components as single cell oils (especially mono-unsaturated fatty acids and saturated cocoa-butter equivalents), protein (*i.e.* single cell protein, SCP), amino acids, and B-group vitamins, including vitamin B12 [19–27]. In this respect, this yeast occupies an important place in pharmaceutical, feed, and food industry [23]. Moreover, the use of this yeast biomass as an additional nutritional supplement can support a solution to the problem of food scarcity in the ever-growing human population, especially in developing countries such as India and Burkina Faso [23, 28]. In 2019, the European Food and Safety Authority (EFSA) authorized the use of dried and heat-killed *Y. lipolytica* biomass as a novel food in dietary supplements intended for the general population above 3 years of age [29]. Furthermore, fatty waste biodegradation by this yeast is regarded as particularly relevant for environmental protection [27, 30–33]. Moreover, growing in nitrogen starvation conditions during lipogenesis, *Y. lipolytica* is also used for production of diesel-like fuels and oleochemicals from carbohydrate resources [34].

Previously, we reported that *Y. lipolytica* is able to produce protein, amino acids and generally an appropriate amount of vitamin B-enriched biomass when grown in biofuel waste [21, 22, 26, 35]. The mentioned studies were focused on *Y. lipolytica* A-101, which is a valuable industrial strain used to produce biomass rich in various nutrients. Dried biomass of the A-101 strain cultivated in biofuel waste is sold on the European market as a commercial nutritional feed additive. However, there is hardly any information about the concentrations of free L-carnitine in *Y. lipolytica* biomass in literature. Therefore, in this study, two *Y. lipolytica* strains, namely the industrial A-101 strain and the standard reference strain from the American Type Culture Collection (ATCC), were examined for L-carnitine content in their biomasses depending on the medium, fat-rich biofuel waste, and fatty acid-poor YPD medium.

Results

Influence of culture conditions on the L-carnitine concentration in Y. lipolytica biomass

The L-carnitine concentration in the yeast biomass was tested using both *Y. lipolytica* A-101 and *Y. lipolytica* ATCC 9793 strains growing in two culture media: biofuel waste (SK medium) and standard laboratory YPD. The first step consisted in the determination of the L-carnitine concentration in the biomass of the industrial A-101 strain cultivated in biofuel waste. The level of L-carnitine in the A-101 biomass grown in biofuel waste was below 1 mg/100 g of wet biomass in all tested culture conditions (data not shown). In the light of the obtained results, we decided to use the standard YPD medium as another culture medium. As shown in Fig. 1A, B, free L-carnitine was detected in the biomass of both *Y. lipolytica* strains cultured in the YPD medium in the different culture conditions. The obtained biomasses of *Y. lipolytica* strains enriched in L-carnitine exhibit different sensitivities to temperature and pH. This trial evaluated the effect of different values of pH (from 4.0 to 7.0) and temperature (form 20 to 30°C). In

the case of *Y. lipolytica* ATCC 9793, the maximum concentration of L-carnitine (22.85 mg/100 g of wet biomass) was reached at the temperature of 20°C and pH 6.0 (Fig. 1B). This result was comparable to that obtained at the temperature of 30°C and pH 5.0 (Fig. 1A). The differences were not statistically significant ($P > 0.05$). On the contrary, in the industrial standard conditions (30°C, pH 6.0) in the YPD medium, the L-carnitine level produced by *Y. lipolytica* ATCC 9793 was 2 times lower (9.93 mg/100 g of wet biomass) than that obtained at the temperature of 30°C and pH 5.0. These differences were statistically significant ($P < 0.01$). The concentration of L-carnitine in the biomass of *Y. lipolytica* A-101 was statistically lower than in the *Y. lipolytica* ATCC 9793 biomass ($P < 0.01$) obtained in the same conditions (Fig. 1). However, *Y. lipolytica* A-101 growing in the YPD medium at the temperature of 30°C and pH 5.0 produced a 2.5 times higher level of L-carnitine (7.09 mg/100 g of wet biomass) than in the industrial standard culture conditions (30°C, pH 6.0) (Fig. 1A). These differences were statistically significant ($P < 0.01$).

A Bland-Altman plot (Fig. 2) comparing the concentration of L-carnitine and protein [21] in the biomasses of *Y. lipolytica* A-101 and ATCC 9793 was also performed to determine the difference in the L-carnitine and protein production ability between the two strains. Irrespective of the culture parameters, both of the strains produced comparable amount of proteins but it existed significant difference in production of L-carnitine between these strains. *Y. lipolytica* ATCC 9793 produced significantly more L-carnitine in comparison with A-101 strain.

Influence of precursors on the L-carnitine concentration in Y. lipolytica biomass yields

The addition of a low concentration of trimethyllysine hydrochloride (0.01 g/L), L-ascorbic acid (0.002 g/L), and iron(II) sulfate (0.001 g/L), as precursors of L-carnitine synthesis to the YPD medium [3, 4, 9] only slightly influenced the L-carnitine concentration in the *Y. lipolytica* ATCC 9793 biomass (Table 1). The differences in the L-carnitine concentration were not statistically significant in comparison to the L-carnitine level in the biomass of the ATCC 9793 strain grown in the medium without these supplementations. However, a 25% increase in the L-carnitine content ($P < 0.05$) was reported in the biomass of the reference *Y. lipolytica* ATCC 9793 strain grown in the YPD medium supplemented with the higher concentration of iron(II) sulfate (0.01 g/L) and trimethyllysine hydrochloride (0.1 g/L) and the same concentration of L-ascorbic acid (0.002 g/L). In the case of the *Y. lipolytica* A-101 strain, the addition of the low concentration of iron (II) sulfate (0.001 g/L), trimethyllysine hydrochloride (0.01 g/L), and L-ascorbic acid (0.002 g/L) resulted in a 250% increase in the L-carnitine concentration in the yeast biomass in comparison to the A-101 strain cultivated in the medium without these additives (Table 1). These differences were statistically significant ($P < 0.01$). The further increase in the concentrations of iron (II) sulfate (0.01 g/L) and trimethyllysine hydrochloride (0.1 g/L) in the YPD medium with L-ascorbic acid (0.002 g/L) caused no statistically significant change in the L-carnitine level in the A-101 strain biomass in comparison with results obtained in the biomass of *Y. lipolytica* grown with lower amount of these compounds.

Table 1
L-carnitine concentration in the wet biomass of *Yarrowia lipolytica* strains cultured in biofuel waste and YPD medium.

medium	L-carnitine concentration (mg/100g wet biomass) Mean ± Standard deviation (SD)			
	Y. lipolytica A-101		*Y. lipolytica* ATCC 9793	
	Biofuel waste	YPD medium	Biofuel waste	YPD medium
unsupplemented	< 1.00	2.80 ± 0.05	< 1.00	9.93 ± 0.50
supplemented trimethyllysine (0.01 g/L), iron(II) (0.001 g/L), L-ascorbic acid (0.002 g/L)	< 1.00	9.80 ± 0.49 **	< 1.00	10.99 ± 0.55
supplemented trimethyllysine (0.1 g/L), iron(II) (0.01 g/L), L-ascorbic acid (0.002 g/L)	< 1.00	10.74 ± 0.52 **	< 1.00	12.41 ± 0.62 **
**P < 0.01 indicates significant difference compared with the reference unsupplemented cultivation.				
The yeast were cultivated 12 h, at 30°C, pH 6.0, 12 h.				

We also tested the effect of the addition of the mixture of the factors, *i.e.* trimethyllysine hydrochloride (0.1 g/L), iron(II) sulfate (0.01 mg/L), and L-ascorbic acid (0.002 g/L), to the SK medium (biofuel waste) on the yeast biomass yield (growth density, OD_{600}), in comparison to the medium without these supplements (Fig. 3A, B). After 12-hour cultivation, the biomass quantity of both yeast strains cultured in the SK medium without the additives was quite satisfactory (OD_{600} 1.5). However, the addition of these L-carnitine precursors caused a significant increase in the yield of both *Y. lipolytica* strains ($P < 0.05$). After 10-hour yeast cultivation, the addition of the mixture led to a 20% and 30% increase in the biomass yield of the reference *Y. lipolytica* ATCC 9793 strain and the A-101 strain, respectively.

Concentration of free L-carnitine in the Yarrowia powder

The content of free L-carnitine in the dried Y. lipolytica A-101 biomass (so-called *Yarrowia* powder) was determined in probes obtained through standard production of seven independent batches in a pilot plant scale in biofermentors. Additionally, chromium-, selenium-, or zinc-enriched yeast biomass was derived from cultures of the A-101 strain grown in the presence of a source of an appropriate micro or macro element. Therefore, six batches (per two batches) were cultivated using supplemented biofuel waste (the SK medium): chromium(III) chloride (15 mg/L), sodium hydrogen selenite (15 mg/L), and zinc sulfate

(1200 mg/L). After drying, we obtained Yarrowia powder, which was amorphous hygroscopic beige-coloured powder with a slight yeast odor. L-carnitine was only detected in the dry biomass of Y. lipolytica cultured in the chromium-supplemented SK medium. In this case, the free L-carnitine concentration was comparable in both batches (mean 2.31 mg ± 0.13 /100 g of dried biomass) (Table 2). However, the dry biomass of *Y. lipolytica*, grown in all studied chromium-unsupplemented biofuel waste contained below 1 mg of L-carnitine/100 g of dry weight.

Table 2
Concentration of protein and free L-carnitine in the dried *Yarrowia lipolytica* A-101 biomass (*Yarrowia* powder) obtained after culturing in the SK medium (biofuel waste) in a pilot plant scale.

Batch number[1]	**Protein content (% of dry weight)**[2]	**Concentration of L-carnitine (mg/100g of dry weight) Mean ± Standard deviation (SD)**
1 (unsupplemented)	49.3	< 1.00
2 (supplemented $CrCl_3$)	41.9	2.37 ± 0.13
3 (supplemented $NaHSeO_3$)	42.6	< 1.00
4 (supplemented $ZnSO_4$)	43.7	< 1.00
5 (supplemented $ZnSO_4$)	44.8	< 1.00
6 (supplemented $NaHSeO_3$)	42.0	< 1.00
7 (supplemented $CrCl_3$)	41.9	2.24 ± 0.11
Mean	45.6	2.31

[1]Each batch was obtained from a different and independent biofermentor culture; [2]Jach et al., 2017; Conditions of cultivation: 100 L, 30°C, pH 5.0, 40% oxidation, 12 h.

Discussion

In the present study, we examined the effect of the culture conditions, two different media, and supplementation of the media on the level of free L-carnitine in the biomass of *Y. lipolytica* strains. The results confirm other findings [7, 22, 35–38] revealing that the fermentation process parameters can have a significant impact on improvement of the nutrient content in the biomass of the studied yeast strains. However, we showed that the concentration of free L-carnitine in the yeast biomass depended primarily on the medium used, and, to some extent, on the strains and culture conditions.

The first medium used was biofuel waste (the SK medium) with high contents of fatty acids. It is known that *Y. lipolytica* growing in fatty substrates is able to accumulate and store lipids [18, 24, 37, 39–40]. Since extracellular carbon sources were depleted, the yeast utilized its own storage lipids (body lipids) as

a carbon and energy source, increasing the production of proteins [19, 20, 41, 42]. Interestingly, a knockout of the sextuple *POX* genes in *Y. lipolytica* causes inability of this yeast to degrade storage lipids, leading to over-accumulation of fats in yeast cells [15, 18]. Therefore, the biosynthesis of cellular proteins or polysaccharides and the fat-free biomass production are competitive to lipid accumulation [42, 43]. We found previously that both *Y. lipolytica* strains (ATCC 9793 and A-101) utilized biofuel waste (the SK medium) to produce biomass with a high concentration of protein and amino acids, especially the industrial A-101 strain [21, 35]. In the present study of both *Y. lipolytica* strains grown in the biofuel waste at a temperature range from 20°C to 30°C and different pH values (from 4.0 to 7.0), we did not notice a significant influence in the L-carnitine concentrations. The level of L-carnitine was below 1 mg/100 g of wet biomass in all fermentation samples (data not shown). We can hypothesize that, irrespective of the culture conditions, both *Y. lipolytica* strains used the entire pool of endogenous free L-carnitine to utilize fatty acids from biofuel waste to grow and produce protein-enriched biomass. It is worth emphasizing that carnitine can also be used as a sole nitrogen source, most commonly through the glycine betaine pathway, where glycine conversion to serine is followed by deamination to form pyruvate and ammonia [1]. It was proved that *Y. lipolytica* grown in biofuel waste was able to produce all amino acids [21, 35]. However, it should be added that the reference *Y. lipolytica* ATCC 9793 strain did not grow at low pH (4.0 or 5.0) in the biofuel waste, in contrast to the growth in the standard YPD medium. In turn, the *Y. lipolytica* A-101 strain was able to grow at low pH (4.0 or 5.0) in the biofuel waste. Our results coincide with those reported by Swigers et al. [4], who found that L-carnitine was strictly required for yeast growth on non-fermentable carbon sources such as acetate, ethanol, fatty acids, and glycerol, which do not contain carnitine. Mutants with deletion of all three CAT genes (Δ*cit2* strain) were unable to grow in media containing these carbon sources. However, supplementation of yeast extract, which contains a sufficient amount of carnitine, or free L-carnitine into these media, caused growth of the Δ*cit2* strain due to absorption of carnitine from the environment [4].

Another study [3] showed that another oleaginous yeast *C. albicans* strain, with deletion of all four genes determining the L-carnitine synthesis pathway, was unable to grow on fatty acids and to utilize either acetate or ethanol as carbon sources. In turn, a transfer of the gene encoding acetyl-CoA oxidase from *Y. lipolytica* to *S. cerevisiae* enabled *S. cerevisiae* to grow on fatty acid-rich feedstock [12]. The possibility of biofuel waste utilization as a substrate by *Y. lipolytica* mainly depended on the strains and culture conditions. Our previous studies revealed that the temperature of 30°C and pH 5.0 were more suitable for production of SPC, amino acids, and B-group vitamins by *Y. lipolytica* strains cultivated in both YPD and SK media (biofuel waste) than at standard industrial conditions (30°C, pH 6.0) [21, 22, 26, 35]. Noteworthy, the culture parameters (i.e. temperature and pH) also strongly affect lipase activities. The maximum activity of lipases produced by *Y. lipolytica* is noted at a temperature between 30°C and 40°C and pH 5.0 [43]. Moreover, these culture conditions significantly influence *Y. lipolytica* lipid accumulation during the primary anabolic growth when cultivated on fatty substrates [36–38].

The standard laboratory YPD medium, used in this study as supplementary, contains yeast extract with only 0.10%-0.15% of fatty acids. Yeast extract is mainly added to the medium as a nitrogen source; however, it also contains several biocomponents, including sufficient amounts of carnitine, to

complement the carnitine requirements of yeast [4, 7]. It was reported that the effect of yeast extract on L-carnitine biosynthesis was dissimilar among tested fungal strains. Yeast extract had a slight effect on the increasing in the L-carnitine concentration in *Aspergillus oryzae* and *Rhizopus oryzae* biomasses but did not influence L-carnitine production in *Neurospora intermedia*. Moreover, it was found that, as long as glucose is present in the medium, it serves as the primary energy and carbon source, preventing L-carnitine consumption. However, after glucose exhaustion, although the biomass weight kept increasing, the concentration of free L-carnitine in the biomass started to decrease. Therefore, the prolongation of the cultivation time caused the fungus to consume glucose entirely and then L-carnitine was utilized as a carbon source [7]. In our study, endogenous free L-carnitine in both tested *Y. lipolytica* strains was detected when they were cultivated in YPD medium. The highest levels of L-carnitine in this medium in the industrial *Y. lipolytica* A-101 biomass were obtained when it was grown at a temperature of 30°C and pH 7.0, although promising results were also obtained at pH 5.0 (Fig. 1A). The reference ATCC 9793 strain cultivated in YPD at a temperature of 20°C and pH 6.0 contained two times more L-carnitine than the industrial A-101 strain (Fig. 1B). However, a similar amount of L-carnitine in the ATCC 9793 biomass was obtained when the strain was cultivated at the temperature of 30°C and pH 5.0 (Fig. 1A). We showed previously that these conditions were optimal to obtain protein-enriched biomass of *Y. lipolytica* cultured in biofuel waste [21, 35]. Thus, the ability to produce nutritional elements is not a static property, and it can be considerably affected by fermentation process parameters. However, as shown in Fig. 2, reference *Y. lipolytica* ATCC 9793 grown in YPD medium, irrespective of the culture parameters, proved to be a better producer of free L-carnitine than another investigated strain.

In the presented work, the effect of precursors for L-carnitine biosynthesis was observed as well (Fig. 2B). Strijbis et al. [3, 9] reported that trimethyllysine (TML) is a main precursor to synthesize carnitine. TML is a component of the first enzyme of the carnitine biosynthesis pathway, namely TML dioxygenase (TMLD), which requires the presence of ascorbate (vitamin C) and iron(II) for its enzymatic activity. The results confirmed that the addition of L-carnitine precursors increased the growth and quantity of yeast biomass growing in the fat-acid-rich medium, i.e. biofuel waste. This confirms the previous reports that the growth of such oleaginous yeasts as *C. albicans*, *Y. lipolytica*, or engineered *S. cerevisiae* strains on non-fermentable fat-rich carbon sources is possible only in the presence of L-carnitine biosynthesis intermediates [3, 6, 12]. In turn, in wild *S. cerevisiae*, peroxisomal membranes are impermeable to acetyl-CoA, when the yeast is cultivated on fatty acids as carbon source. Therefore, wild *S. cerevisiae* are not able to grow in fat-rich waste substrates [42] in contrast to *Y. lipolytica*. In this respect, production of nutritional yeast biomass by oleaginous species on available inexpensive wastes used as carbon and energy sources (e.g. biofuel waste) is desired by industry in the broad sense.

We also observed a stimulatory effect of chromium on the free L-carnitine production. Trivalent chromium, an essential trace element, as diet components improving glucose uptake and fat metabolism was reported [44]. Moreover, the deficiency of trivalent Cr may induce symptoms comparable to those associated with diabetes in mammals [45]. Our experimental results showed that the introduction of water-soluble chromium (Cr (III)) salt as a component of biofuel waste as the culture medium for *Y. lipolytica* resulted in production of a slight amount of free L-carnitine by the yeast. This implies that Cr

supported L-carnitine metabolism in some way in the yeast cells. However, the issue of the chemical dependencies between Cr and L-carnitine in yeast cells needs further investigations. Additionally, we supplemented zinc and selenium into biofuel waste in the pilot plant scale to obtain zinc- or selenium-enriched yeast biomass, but we did not observe a significant increase in the L-carnitine concentrations.

Conclusions

Yarrowia lipolytica growing in fatty acid-enriched substrates, e.g. biofuel waste in strictly aerobic environments probably uses the entire pool of endogenous L-carnitine for growth and production of biomass. Hence, crude biofuel waste, as an inexpensive substrate, can be utilized by *Y. lipolytica* for production of high-value nutritional compounds but at present, it cannot be used for free L-carnitine. However, further studies on process optimization and cultivation medium based on biofuel waste need to be carried out in order to clarify the potential of the industrial *Y. lipolytica* A-101 strain for production of L-carnitine-enriched yeast biomass. The use of L-carnitine precursors contributes to production of greater amounts of L-carnitine in fatty acid-poor medium and can increase the yield of *Y. lipolytica* grown in both fatty acid-poor medium and biofuel waste. Moreover, *Y. lipolytica* growing in fatty acid-poor media or substrates with a small amount of fatty acids, especially various bio-wastes, can be applied as an L-carnitine producer.

Methods

Microbial Strains

In the research, the industrial nutrient productive wild-type yeast *Yarrowia lipolytica* A-101 strain was obtained from Skotan S.A. (Poland) and as the reference yeast *Y. lipolytica* ATCC 9793 strain was obtained from LGC Standards.

Production, harvesting of Y. lipolytica biomass, and yeast growth conditions

Y. lipolytica was cultured in two culture media: an industrial fat-rich SK medium and the chemically defined fatty acid-poor YPD medium (Difco). The SK medium is a waste from biofuel production, which is normally used by Skotan S.A for production of *Y. lipolytica* A-101 biomass rich in nutritional elements for commercial use. Biofuel is made through chemical reaction of vegetable oil with ethanol producing fatty acid esters (long-chain alkyl (methyl, ethyl, or propyl) esters). Crude biofuel waste consists of a mixture of vegetable oils with degumming and glycerol fractions (from 2–7% wt/wt). The degumming fraction contains mainly phosphoric acid derivatives associated with fats and protein as well as free plant fats (up to 10%), protein (up to 10%), ash (up to 5%). The SK medium as a biofuel waste also contains $(NH_4)_2SO_4$ (12.6 g/L), urea (4.0 g/L), $MgSO_4$ (1.0 g/L), KH_2PO_4 (0.5 g/L), and some amount of vitamins B. The mean concentration of these vitamins in 100 ml of the medium is as follows: 0.9 mg of thiamine, 3.65 mg of riboflavin, 3.38 mg of pyridoxine, 138 μg of folic acid, and 6.2 μg of cyanocobalamin. The SK

medium was provided by Skotan S.A. (Poland). The biofuel waste, i.e. a partially refined, desalinated, and methanol-free by-product from biodiesel manufacture, was delivered by Lotos Group Refineries, Poland to Skotan S.A. By contrast, the YPD medium contains a small amount of fatty acids which origin from yeast extract. Yeast extract used for preparation of YPD medium (Difco) contains only 0.10%-0.15% of fatty acids. The biomass of *Y. lipolytica* A-101 was obtained in SK medium in standard culture conditions: i.e. temperature of 30°C and pH 6.0, which were established earlier by Skotan S.A. These industrial culture conditions were considered as reference ones in this study, while two experimental culture conditions were used: 1) variable temperature (from 20°C to 30°C) and constant pH (6.0) values; 2) constant temperature (30°C) and variable pH (from 4.0 to 7.0) values. The pH was adjusted to the required values by adding 1 M NaOH or 1 M HCl, respectively. The sterile media in Erlenmeyer flasks (150 ml) were supplemented with $FeSO_4$, trimethyllysine hydrochloride, and L-ascorbic acid. The following combinations were applied: a) iron(II) sulfate (0.001 g/L), trimethyllysine hydrochloride (0.01 g/L), and L-ascorbic acid (0.002 g/L) with the final pH 6.0; b) iron(II) sulfate (0.01 g/L), trimethyllysine hydrochloride (0.1 g/L), and L-ascorbic acid (0.002 g/L) with the final pH 6.0. *Y. lipolytica* cultivated in the YPD broth at a temperature of 30°C and pH 6.0 was the control culture. *Y. lipolytica* strains were cultured in Erlenmeyer flasks (150 ml) and in a biofermentor (100 L) as a pilot plant scale as previously described (21,26). The sterile SK medium in the biofermentor was prepared with and without chromium (III) chloride (15 mg/L); sodium hydrogen selenite (15 mg/L); or zinc sulfate (1200 mg/L). After 12-hours cultivation, the biomass from the biofermentor was transferred into a tumble dryer and dried at 165–175°C for 1 hour; this yielded dried biomass called *Yarrowia* powder.

Preparation of yeast disruption in bead mill

Yeast cells were disintegrated with the use of a bead mill (Minilys homogenizer, Bertin Technologies) with the power of 250 V AC / 50–60 Hz, and speed 5 000 rpm. The total working volume of the mill tube was *ca* 2 ml. Zirconium-glass beads (Bertin Technologies) with diameter of 0.5 cm were used in the experiments. For a single homogenization, 50 mg of the *Y. lipolytica* cells were used. The cells were resuspended in 0.5 ml Tris-HCl buffer, pH 7.5. 3D beat-beating was carried out in cycles: 10 x 1 min. of homogenization and 0.5 min. in the ice.

L-carnitine analysis

Total free L-carnitine in the yeast biomass was determined using an L-Carnitine Assay Kit according to the assay procedure (Abnova, Catalog № KA0860).

Statistical analysis of data

All data are expressed as a mean ± SD (standard deviation) of three independent experiments. The differences between the concentrations of L-carnitine in the biomasses of *Y. lipolytica* strains growing at the different conditions were compared to *Y. lipolytica* ATCC A-101 cultured in the YPD medium at the temperature of 30°C and pH 6.0 with two-sided student's *t*-test, using Statistica software version 12.0. The P value < 0.05 was considered statistically significant.

Abbreviations

ATCC - the American Type Culture Collection; CATs - carnitine acetyltransferases; CoA - coenzyme A; EFSA - the European Food and Safety Authority; TCA - tricarboxylic acid; TML trimethyllysine; TMLD–TML dioxygenase.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated for this study are available on request to the corresponding author.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Conceptualization, M.E.J. and A.M.; methodology, M.E.J., K.K. and A.M.; software, M.E.J., T.B. and A.M; validation, M.E.J., K.K. and A.M.; formal analysis, M.E.J. T.B. and A.M.; investigation, K.K.; resources, M.E.J., E.S., A.M; data curation, M.E.J. and K.K.; writing—original draft preparation, M.E.J.; writing—review and editing, M.E.J., E.S., K.K. and A.M; visualization, M.E.J. and T.B.; supervision, A.M.; project administration, M.E.J. and A.M.; funding acquisition, M.E.J. and A.M. All authors have read and agreed to the published version of the manuscript.

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Figures

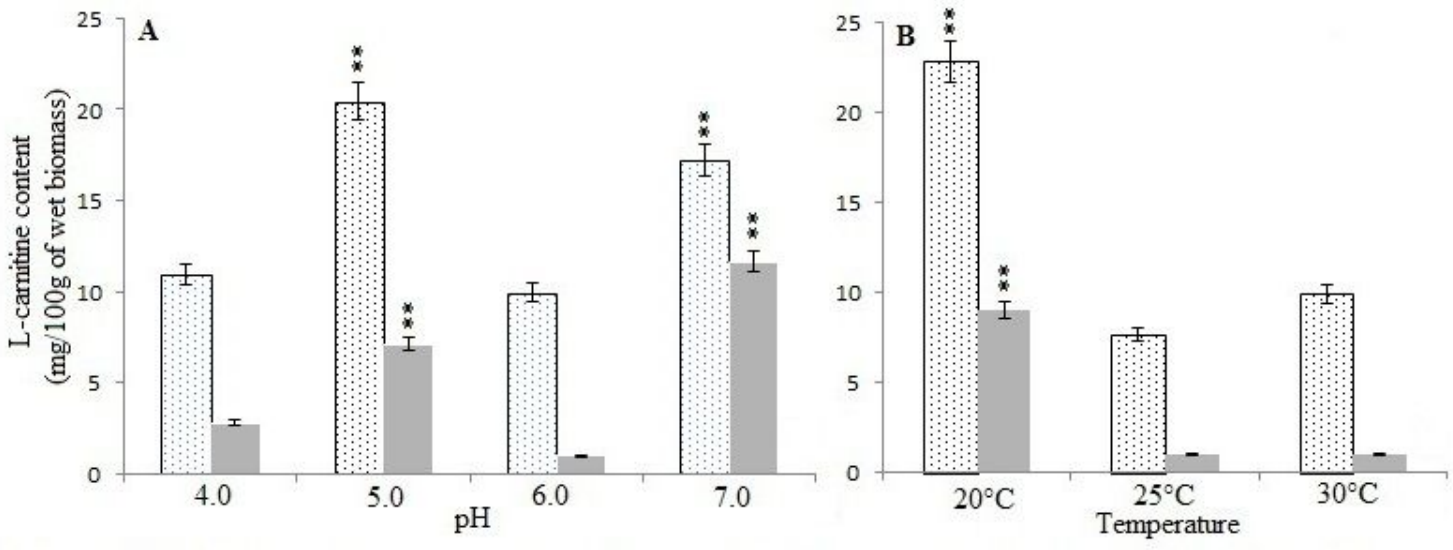

Figure 1

Total free L-carnitine concentration in wet biomass of Y. lipolytica strains cultured in the YPD medium at different conditions. A. constant temperature (30°C) and variable pH values. B. constant pH (6.0) values and variable temperature. Y. lipolytica ATCC 9793 (dotted squares); Y. lipolytica A-101 (filled squares). **P<0.01 indicate significant difference compared to reference cultivation.

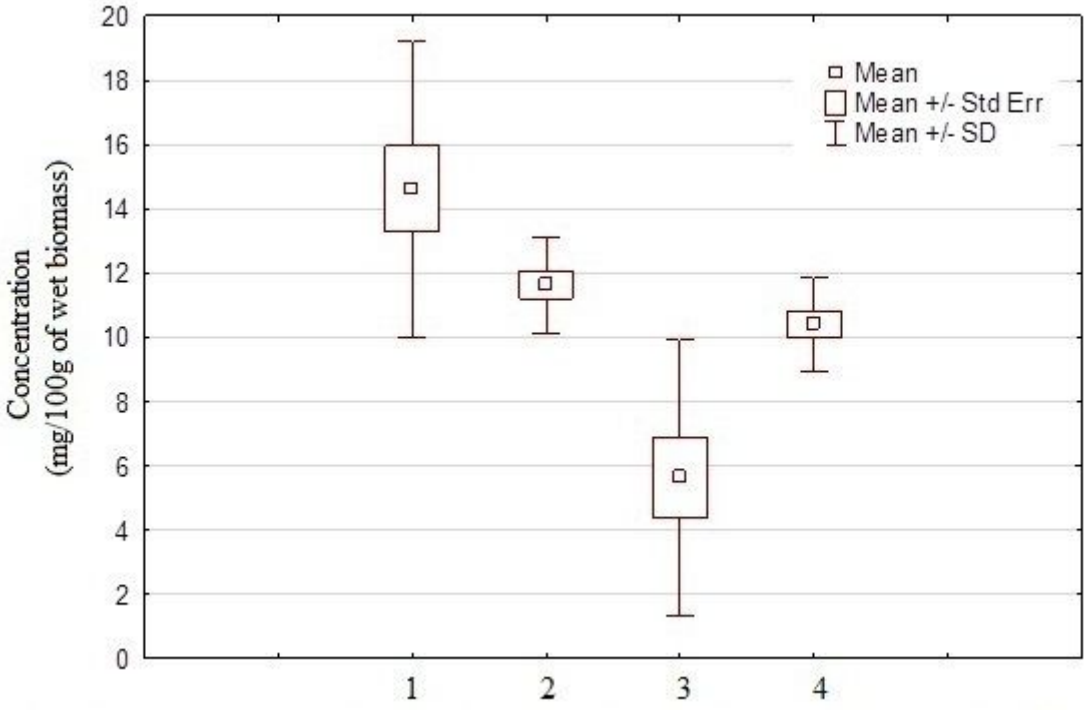

Figure 2

Average values of L-carnitine and protein production by Y. lipolytica A-101 and ATCC 9793 strains growing on YPD medium, shown as irrespective of the culture parameters. 1. L-carnitine concentration in Y. lipolytica ATCC 9793 biomass. 2. Protein content in Y. lipolytica ATCC 9793 biomass [21]. 3. L-carnitine concentration in Y. lipolytica A-101 biomass. 4. Protein content in Y. lipolytica A-101 biomass [21].

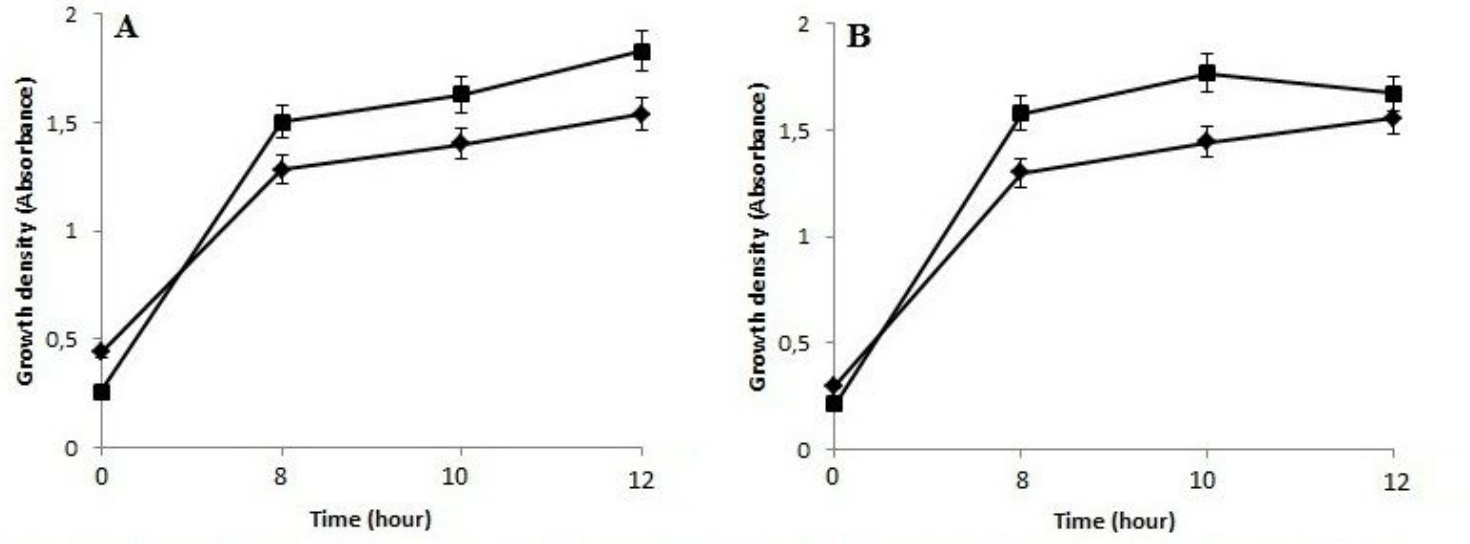

Figure 3

Growth density of Y. lipolytica. A. Y. lipolytica ATCC 9793; B. Y. lipolytica A-101 cultured in SK medium (biofuel waste), (pH 6.0, 30°C) ♦ unsupplemented or ● supplemented with trimethyllysine hydrochloride (0.1 g/L), iron(II) sulfate (0.01 mg/L), and L-ascorbic acid (0.002 g/L).