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The Consumption of Amino Acids and Production of Volatile Aroma Compounds by *Yarrowia lipolytica* in Brewers' Wort

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Abstract: The yeast *Yarrowia lipolytica* is well known for its versatile production of metabolites from various substrates, but, although isolated from, e.g., wild-fermented Belgian Sour beers, it is rarely considered a starter culture in fermented beverages. In this study, we aimed to elucidate the ability of *Y. lipolytica* to ferment brewers' wort containing iso-α-acid for 7 days at low and high aeration and at 20 °C and 30 °C, with a special focus on amino acid consumption and production of volatile aroma compounds. *Y. lipolytica* was able to grow in the wort under all four conditions, although the growth was inhibited. Furthermore, it only consumed glucose and fructose, and no ethanol was formed. Moreover, under high aeration conditions, *Y. lipolytica* consumed 75–80% of the amino acids in the wort. Interestingly, no esters were produced during the fermentations, and only five higher alcohols (1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 3-methyl-3-buten-1-ol, and 2-phenylethanol), two aldehydes (3-methylbutanal and (E)-2-nonenal), two ketones (cyclopentanone and 9-oxabicyclo [6.1.0]nonan-4-one), one fatty acid (3-methyl-butanoic acid), and one benzene derivate (1,2,4-trimethyl-benzene) were produced. These results may contribute to the potential use of *Y. lipolytica* in a traditional brewery for the production of novel beers; e.g., alcohol-free beer.

Keywords: non-conventional yeast; *Yarrowia lipolytica*; beer; amino acids; aroma

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1. Introduction

The non-conventional, non-pathogenic, dimorphic yeast *Yarrowia lipolytica* has been extensively researched since it was identified and described in 1972 by David Yarrow [1–6]. *Y. lipolytica* is being used as a model organism for non-conventional yeast, and it harbours severe physiological differences compared to other model yeasts, such as *Saccharomyces cerevisiae* [7,8]. *Y. lipolytica* has been isolated from various, often lipid-containing, food sources, such as cheese and meat products [5]. It has, however, also been isolated from traditional Belgian sour beer [9,10].

It is well established that *Y. lipolytica* is an obligate aerobic yeast [2,8]. Additionally, it is Crabtree-negative, thus unable to produce ethanol under aerobic conditions [11]. Furthermore, wild-type strains of *Y. lipolytica* are from natural side able to assimilate a variety of carbon sources. They range from simple sugars (glucose and fructose) over various alcohol types to more complex substrates, such as organic acids, free fatty acids, animal-derived fats and multiple forms of hydrocarbons [12–14]. There is general consent that the optimal growth temperature for strains of *Y. lipolytica* is somewhere in the range between 25 °C and 30 °C, and growth is generally not seen above 35 °C [2].

Y. lipolytica can synthesise and secrete a great variety of compounds, including organic acids, sugar alcohols and interesting aroma compounds such as 2-phenylethanol and γ-decalactone [3,15–21]. How external factors affect the physiology, metabolism, and gene expression of *Y. lipolytica* has been studied in various substrates [14,15,20–22]. Braga and Belo [20], e.g., showed that it was possible to increase the production of γ-decalactone by increasing the agitation and aeration of their medium. Until today, most focus has been

on optimising the production of one or more metabolites, e.g., citrate, 2-phenylethanol, sugar alcohols and lipids, in various substrates to purify the metabolites later for further usage. Only a few examples, where *Y. lipolytica* has been used as a starter culture in food, have been seen, mainly in dairy and meat production. Here, the catabolism of amino acids, various lipids, sugars and organic acids by *Y. lipolytica* has been examined. It was found that *Y. lipolytica* can contribute significantly to the flavour of cheese and dried fermented sausages through the production of volatile compounds, such as alcohols, acids, aldehydes, ketones, terpenes, alkanes and cyclic hexane as well as free fatty acids and some organic acids [5,14,23,24]. Spitaels et al. (2015) isolated and identified *Y. lipolytica* from spontaneous fermented Belgian sour beers and suggested that *Y. lipolytica* may play a vital role in producing some Belgian sour beers [10]. However, it seems that no further studies have been published on the effect of *Y. lipolytica* on sour beer.

Amino acid consumption by *Y. lipolytica* in complex media has only been the subject of very few published scientific papers. Mansour et al. (2008) found that the specific consumption of different amino acids by *Y. lipolytica* in a defined cheese-like medium containing high and low concentrations of amino acids varied depending on factors, such as amino acid concentration and the presence of lactate [14]. In common for all treatments, *Y. lipolytica* consumed most proline, followed by glutamine [14]. This contrasts sharply with what is typically found for *S. cerevisiae* in aerobic fermentations of beer wort, where proline is the last amino acid consumed [25].

In this paper, we report the use of *Y. lipolytica* as a starter for fermenting brewers' wort, as a proof-of-concept study for the production of novel beers. We have investigated the growth and fermentation properties, including consumption of amino acids and production of volatile aroma compounds, of an in-house strain of *Y. lipolytica* in brewers' wort at low and high aeration and at 20 °C and 30 °C.

2. Materials and Methods

2.1. Microorganisms and Media

Yarrowia lipolytica FNC0006 was obtained from the inhouse Carlsberg Research Laboratory (J.C. Jacobsens Gade 4, DK-1799 Copenhagen V, Denmark, CRL) yeast collection. The yeast was stored in a −80 °C freezer in 20% (*v*/*v*) glycerol until used. Yeast cells were prepared by spreading the content of a 1 µL inoculation needle of the yeast/glycerol mix onto a regular YDP agar plate (Yeast extract 10 g, Peptone 20 g, Dextrose 20 g) and incubating for 96 h at 30 °C. Subsequently, the yeast was tested for microbial contamination using internal methods.

2.2. Fermentation Medium

In these experiments, the medium was standard brewers' wort from the same 258 L batch (produced after a standard protocol at Carlsberg Research Laboratory). Raw materials for the brew were: 100% pilsner malt 39.25 kg (Viking malt batch: 3727). Additives: Enzyme: Ultraflo Max 5.89 g (Novozymes, Bagsværd, Denmark), salts: $CaCl_2$ 41.62 g and $ZnSO_4$ 1.32 g, acid for pH adjustment: H_3PO_4 290 mL of a 25% solution (Brenntag Nordic A/S) to reach pH 5.2, and pre-isomerised α-acid 27 mL of 30% solution (Barth-HassGroup, IsoHop®, Nürnberg, Germany), corresponding to 28 IBUs in the wort, to mimic hop-addition to the wort.

2.3. Fermentation Conditions

Fermentations were carried out in a Sartorius Biostat A Bioreactor system (Sartorius, Guxhagen, Germany). The working volumes in the fermenters were one litre. The wort was pre-fermentation, sterilised in 5 L blue cap bottles by autoclaving at 120 °C for 45 min before it was homogenised and transferred into the autoclaved fermenters. The density in the autoclaved wort was 11.2 °Plato.

The fermentations were carried out at 20 °C and low aeration (L20), 30 °C and low aeration (L30), 20 °C and high aeration (H20), and 30 °C and high aeration (H30). Low and

high aeration levels were obtained by having two different agitation rates in the fermenters, 400 rpm (rounds per minute) and 1400 rpm, respectively. To create an environment that is possible to recreate in a traditional brewery, the air was dosed at 0.4 vvm into the headspace of the fermenters. All parameters were controlled by the Sartorius system and Lucullus software (Ramcon, Birkerød, Denmark). pH and temperature data were measured by an Endress + Hauser (Ceragel CPS71D) pH probe in the fermenters and logged continuously using Lucullus software. All fermentations were conducted over seven days in biological triplicates. Samples were taken at an interval of 24 h. All samples were centrifuged at 6000× *g* for 10 min, and the supernatants were subsequently filtered through a 0.2 µm syringe filter and stored at −21 °C until analysis.

2.4. Preculture and Inoculation

Precultures for inoculation were prepared by dosing 1 µL of cells from several colonies on the YPD plate, using a 1 µL–inoculation loop, into 100 mL of 10 °Plato brewers' wort. The inoculum was cultivated for 16 h in Erlenmeyer flasks on a shaking table (120 rpm) at 23 °C to a cell density of 1.0×10^7 cells/mL; 10 mL of inoculum were transferred to each fermenter, equivalent to an inoculation concentration of 1.0×10^5 cells/mL.

2.5. Analytical Methods

2.5.1. Analyses of Growth and Sugars

At each sampling point, 5 mL of the culture broth was withdrawn from each fermenter and diluted to an appropriate level for optical density at 600 nm (OD_{600}) measurement using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany).

Six sugars were measured (glucose, fructose, sucrose, maltose, maltotriose, maltotetraose) using a high-pressure ion chromatography system (HPLC, Thermo Fisher Scientific, Glostrup, Denmark). The system was a Dionex ICS-6000 DC connected to a Dionex ICS-6000 HPAEC-PAD, equipped with a CarboPac PA-200 (3 × 250 mm) BioLC column and a Pulsed Amperometric Detector with a disposable Au electrode. Two eluents were used. Eluents A (50 mM potassiumhydroxide) and B (50 mM potassiumhydroxide + 100 mM potassium acetate) were graduated with 5% gradient curve from 100% eluent A to 100% eluent B over the time of 58 min. The injection volume was 10 µL, and the flow was 0.5 mL/min. The temperatures of the column and detector were 40 °C and 30 °C, respectively. The system was controlled using Chromeleon software (Thermo Fisher Scientific, Glostrup, Denmark).

2.5.2. Analysis of Ethanol

Gas Chromatograph Mass Spectrometry SIM mode (GC-MS SIM) was used to detect and quantify ethanol levels. The system used was Agilent technologies 7890B GC (Agilent Technologies Denmark ApS, Glostrup, Denmark) fitted with an Agilent J&W GC DB-wax column, coupled with a computer with the analytical software MassHunter (version B.08.00). The analytical conditions were as follows: constant helium flow of 1.5 mL/min, transfer line temp. 250 °C, ion source temperature 230 °C, MS quad temp. 150 °C, injection temp. 250 °C. Samples were incubated for 10 min at 60 °C with agitation of 500 rpm. The injection was performed with static headspace with a gas-tight syringe. The injection volume was 100 µL. The column temperature programme was: 7 min at 50 °C, from 50 °C to 240 °C at 30 °C·min^{-1}. The programme was finished upon reaching 240 °C.

2.5.3. Analysis of Amino Acids

Samples were degassed for 10 min in an ultrasonic bath and centrifuged for 10 min at 4000× *g* 400 µL sample + 40 µL 30% sulfosalicylic acid were mixed by a vortex mixer for 10 s and allowed to stand for 15 min. The samples were then centrifuged for four minutes at 14,000× *g*; 300 µL of the supernatant of the sample was extracted and mixed with 30 µL 1 M sodium hydroxide. The mixtures were vortex mixed for 10 s. Samples were diluted from undiluted to 10 × in Milli-Q water as necessary. AccQ-Tag Ultra reagent powder was reconstituted according to producers' guidelines (Waters, Milford, MA, USA); 20 µL

of the reconstituted AccQ-Tag Ultra reagent was added to the sample mixture and vortex mixed for 5 s immediately after. When all samples were ready, they were heated in vials for 15 min at 55 °C in a preheated oven.

Samples were analysed using a UPLC from Waters (Waters, Milford, MA, USA) with a Photo Diode Array detector and an ACCQ-Tag Ultra (2.1 × 100 mm, 1.7 µm) column (Waters, Milford, MA, USA). Two eluents were used; eluents A (50 mM potassium hydroxide) and B (50 mM potassium hydroxide + 100 mM potassium acetate) were graduated with a 6% gradient curve from 99% eluent A to 40% eluent A and 60% eluent B over the time of 10 min. The column temperature was 51 °C, and the sample temperature was kept at 8 °C. Data were collected using MassLynx version 4.2 software (Waters, Milford, MA, USA), where concentrations were calculated based on internal standard curves.

2.5.4. Analysis of Volatile Compounds

Trapping and separation of volatile aroma compounds by Dynamic Headspace Gas Chromatography/Mass Spectrometry (GC–MS) were performed as previously described by Liu et al. [26] using Tenax-TA traps (Markes International, Llantrisant, UK) in a dynamic headspace sampling system and a ZB-Wax capillary column (30 m × 0.25 × 0.50 µm) (Phenomenex, Værløse, Denmark), respectively; 1 mL internal standard (5 ppm 4-methyl-1-pentanol (Aldrich, Steinheim, Germany) in water) was added to each sample.

Peak areas and mass spectra were extracted from the chromatograms using the PARAFAC2-based software PARADISe (University of Copenhagen, Copenhagen, Denmark), as described by Johnsen et al. [27]. The NIST05 database was used for the identification of the mass spectra of the volatiles. Peak areas, normalized using the internal standard, were used as relative measures of concentration. Volatile compound identification was confirmed by comparison with retention indices (RI) of authentic reference compounds or retention indices reported in the literature. The results were presented as peak areas of the identified compounds.

2.6. Statistics

A one-way ANOVA with Tukey's HSD test was used for each volatile compound to test if there was a significant difference (95% level) between the sampling points; day 0, day 4, and day 7. All statistics were conducted using Microsoft Excel with the add-in XLSTAT (Addinsoft, Paris, France).

3. Results

3.1. Growth, Sugar Consumption, and Ethanol Production

In the two experiments with high aeration, the OD_{600} values of *Y. lipolytica* increased linearly from 1.4 (±0.0) on day 0 of fermentation to 13.2 (±0.1) and 12.5 (±0.2) on day 7 of fermentation in H20 and H30, respectively. Furthermore, in these experiments, only glucose and fructose were consumed from the wort (Figure 1A). The other sugars found in wort (i.e., maltose, maltotriose, maltotetraose and sucrose) were not consumed (Figure S1). Regardless of the temperature, the glucose and fructose concentrations steadily declined from 12.0 g/L (±0.4) and 4.0 (±0.2), respectively, on day 0 to 0.8 g/L (±0.6) and 2.3 g/L (±0.2), respectively, on day 5. From day 5 to day 7, glucose was fully depleted at both temperatures, whereas fructose was completely consumed in H30 and to 1.0 g/L (±0.3) in H20 (Figure 1A).

In the two low aeration experiments, the OD_{600} values of *Y. lipolytica* increased linearly from 1.6 (±0.1) on day 0 of fermentation to 3.1 (±0.0) and 3.9 (±0.1) on day 7 of fermentation in L20 and L30, respectively. Moreover, in these experiments, glucose decreased slightly from day 0 (11.5 g/L (±0.5)) to day 7 (9.3 g/L (±0.5)). No differences were detected between the two temperatures (Figure 1B). Fructose and the other sugars found in the wort (i.e., maltose, maltotriose, maltotetraose and sucrose) were not consumed (Figures 1 and S1).

Ethanol was not produced in any of the experiments (Figure S2).

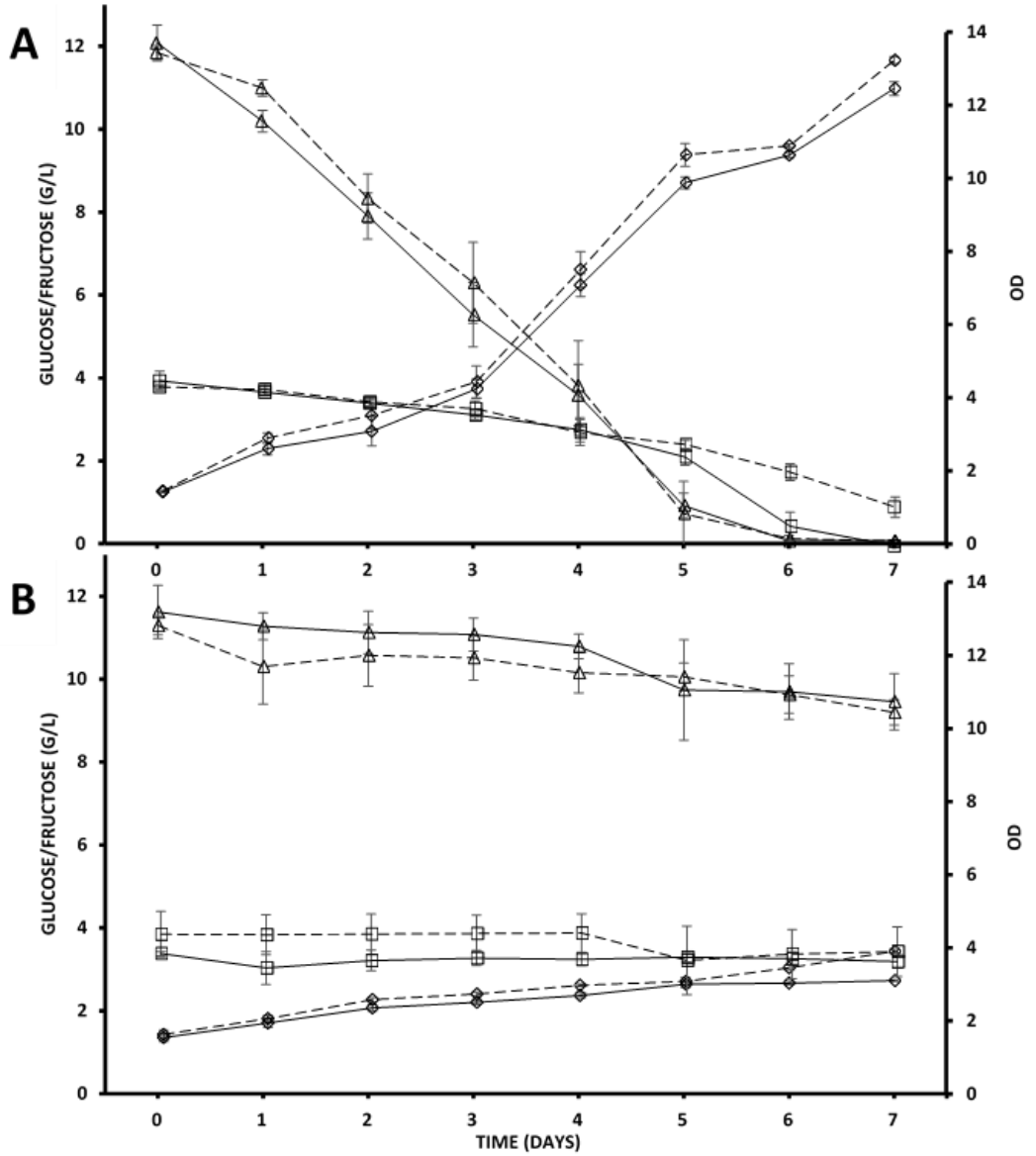

Figure 1. OD values (diamonds) as well as glucose (triangles) and fructose (squares) concentrations during fermentation of brewers' wort with *Y. lipolytica* at (**A**) high aeration and (**B**) low aeration and at 20 °C (dashed curves) and 30 °C (solid curves). Data are average values from triplicate experiments, and error bars indicate standard deviations.

3.2. Consumption of Amino Acids

As for the sugars, more amino acids were consumed by *Y. lipolytica* in the two high-aeration treatments than in the two low-aeration treatments. The consumption of total amino acids on day 4 was 61.1% in H20, 62.6% in H30, 13.2% in L20, and 4.7% in L30, and on day 7 it was 74.9% in H20, 79.7% in H30, 24.9% in L20, and 16.4% in L30 (Table 1).

In the two fermentation experiments with high aeration, most amino acids were consumed by *Y. lipolytica* to a level where 20–100% of the initial concentration of a given amino acid was consumed on day 7 (Figure 2). Threonine and tryptophan, however, were hardly consumed (<20%) (Figure 2 and Table 2). For phenylalanine, 37% of the initial concentration was consumed on day 4 for both temperatures and on day 7 66% (H20) and 69% (H30) of the initial concentration were consumed.

Table 1. Total amino acid uptake (g/L) during 7 days of fermentation of brewer's wort with *Y. lipolytica*.

	High Aeration		Low Aeration	
	20 °C	**30 °C**	**20 °C**	**30 °C**
Day 0	2.40 ± 0.11 (0%)	2.38 ± 0.03 (0%)	2.30 ± 0.10 (0%)	2.32 ± 0.01 (0%)
Day 4	0.93 ± 0.08 (61.1%)	0.89 ± 0.08 (62.6%)	2.00 ± 0.11 (13.2%)	2.21 ± 0.06 (4.7%)
Day 7	0.60 ± 0.04 (74.9%)	0.48 ± 0.04 (79.7%)	1.73 ± 0.04 (24.9%)	1.94 ± 0.05 (16.4%)

Red indicates the highest of the two compared values. If both values are red, no statistically significant different were found. Blue indicates the lowest of the two values. If both red and blue a values are shown on a day the values are significantly different ($p < 0.05$). Values in g/L and uptake in percentage (in brackets) calculated based on initial concentration.

Table 2. Assimilation order of amino acids from brewers' wort fermented using *Y. lipolytica*.

	High Aeration		Low Aeration	
	20 °C	**30 °C**	**20 °C**	**30 °C**
Group 1 Fast More than 80% on day 4	Proline Alanine Lysine Arginine Asparagine Aspartic Acid Glutamic Acid Glycine	Proline Alanine Arginine Serine Asparagine Aspartic Acid Glutamic Acid Glycine	Aspartic Acid Glutamic Acid	Aspartic Acid
Group 2 Intermediate Between 50% and 80% on day 4	Serine Methionine	Lysine Methionine		Glutamic Acid
Group 3 Slow More than 10% on day 7 and less than 50% on day 4	Leucine Phenylalanine Valine Tyrosine Isoleucine Histidine Tryptophan Glutamine	Leucine Phenylalanine Valine Tyrosine Isoleucine Threonine Histidine Tryptophan Glutamine	Proline Alanine Arginine Serine Asparagine Glutamine	Proline Alanine Arginine Serine Asparagine
Group 4 Little or no absorption Less than 10% on day 7	Threonine	-	Leucine Phenylalanine Valine Lysine Tyrosine Isoleucine Threonine Histidine Tryptophan Glycine Methionine	Leucine Phenylalanine Valine Lysine Tyrosine Isoleucine Threonine Histidine Tryptophan Glycine Methionine Glutamine

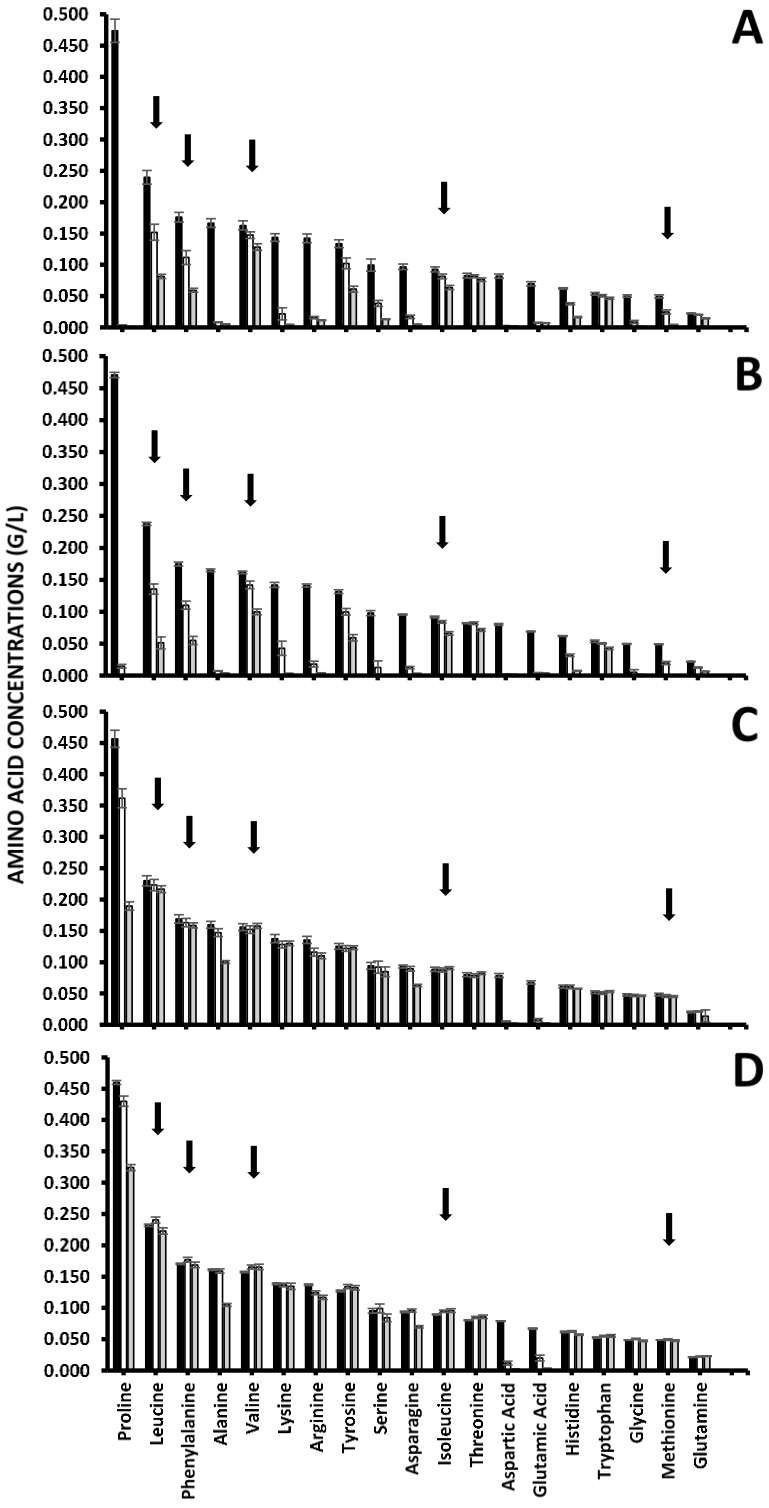

Figure 2. Amino acid concentrations during fermentation of brewers' wort with *Y. lipolytica* at (**A**) high aeration and 20 °C, (**B**) high aeration and 30 °C, (**C**) low aeration and 20 °C, and (**D**) low aeration and 30 °C on day 0 (black), day 4 (white) and day 7 (grey). Arrows indicate amino acids that belong to the group of Strecker aldehyde precursors. Data are average values from triplicate experiments, and error bars indicate standard deviations.

In the two experiments with low aeration, only aspartic acid and glutamic acid were consumed close to depletion by *Y. lipolytica* (>96% of initial concentration consumed) on day 7. Regardless of temperature, proline, alanine, and asparagine were consumed too, but

to a lesser degree (>26% of initial concentration consumed on day 7). In the low aeration experiment at 20 °C, 30% of glutamine was also consumed, whereas, in the experiment with low aeration and 30 °C, it was not consumed at all. For all remaining amino acids 0–20% of the initial concentration of a given amino acid was consumed on day 7 (Figure 2).

In Table 2, the amino acids were classified into four groups according to the rate with which they were taken up by *Y. lipolytica* from the wort, that is, group 1; fast (more than 80% of the total of a given amino acid taken up before day 4), group 2; intermediate (more than 50%, but less than 80%, of the total of a given amino acid taken up before day 4), group 3; slow (more than 10% consumed on day 7, but less than 50% on day 4, of the total of a given amino acid), and group 4; little or no absorption (less than 10% of the total of a specific amino acid taken up on day 7).

Regardless of the temperature, proline, alanine, arginine, asparagine, aspartic acid, glutamic acid, and glycine were all classified in group 1 in the two experiments with high aeration. In H20, lysine was classified in group 1 and serine in group 2. In H30, serine was classified in group 1 and lysine in group 2. Methionine was classified in group 2 in both experiments with high aeration.

Regardless of temperature, leucine, phenylalanine, valine, tyrosine, isoleucine, histidine, tryptophan, and glutamine were all placed in group 3 in the two experiments with high aeration. Threonine was placed in groups 3 and 4 in H30 and H20, respectively (Table 2).

A different picture was seen in the two experiments with low aeration than in those with high aeration. In L20, only aspartic acid and glutamic acid were placed in group 1. In L30, only aspartic acid was placed in group 1, and glutamic acid in group 2. No amino acids were placed in group 2, and proline, alanine, arginine, serine, asparagine, and glutamine were placed in group 3 in L20. The same results were observed in L30, except for glutamine. All remaining amino acids were placed in group 4, regardless of the temperature (Table 2).

3.3. Production of Volatile Compounds

Sixty-four volatile compounds were detected in the samples from the four different fermentation experiments with *Y. lipolytica* (Tables 3 and S1). Interestingly no esters were detected (Tables 3 and S1).

Table 3. Volatile compounds (peak areas) during fermentation of brewers' wort with *Y. lipolytica*.

	High Aeration						**Low Aeration**					
	20 °C			**30 °C**			**20 °C**			**30 °C**		
Day	0	4	7	0	4	7	0	4	7	0	4	7
Esters												
Not detected												
Higher Alcohols												
1-propanol												
2-methyl-1-propanol												
3-methyl-1-butanol												
3-methyl-3-buten-1-ol												
1-pentanol												
1-hexanol												
(E)-2-nonen-1-ol												
2-ethyl-1-hexanol												
1-octanol												
propylene glycol												

Table 3. *Cont.*

	High Aeration						Low Aeration					
	20 °C			30 °C			20 °C			30 °C		
Day	0	4	7	0	4	7	0	4	7	0	4	7
α,α-dimethyl-benzenemethanol												
2-phenylethanol												
2-dodecyl 1,3-propanediol												
Phenolic Compounds												
2-methoxy-4-vinylphenol												
2-naphthalenol												
phenol												
Aldehydes												
3-methyl-butanal												
2-methyl-propanal												
2-methyl-butanal												
hexanal												
heptanal												
3-methyl-2-butenal												
octanal												
decanal												
(E)-2-nonenal												
Ketones												
acetone												
2-butanone												
2,3-butanedione												
2,3-pentanedione												
cyclopentanone												
5-methyl-2-hexanone												
acetoin												
1-hydroxy-2-propanone												
9-oxabicyclo [6.1.0]nonan-4-one												
6-methyl-5-hepten-2-one												
4-Cyclopentene-1,3-dione												
Acetophenone												
β-damascenone												
geranylacetone												
Fatty Acids												
acetic acid												
formic acid												
propanoic acid												
2-methyl-propanoic acid												
butanoic acid												
3-methyl-butanoic acid												

Table 3. *Cont.*

	High Aeration						Low Aeration					
	20 °C			30 °C			20 °C			30 °C		
Day	0	4	7	0	4	7	0	4	7	0	4	7
pentanoic acid												
hexanoic acid												
heptanoic acid												
octanoic acid												
nonanoic acid												
n-decanoic acid												
benzoic acid												
Benzene Derivates												
(1-methylethyl)-benzene												
1,2,4-trimethyl-benzene												
1,3-bis(1,1-dimethylethyl)-benzene												
benzaldehyde												
benzeneacetaldehyde												
Furans												
2-pentyl-furan												
dihydro-2-methyl-3(2H)-furanone												
furfural												
1-(2-furanyl)-ethanone												
2-furanmethanol												
Others												
methanesulfonyl chloride												
thiazole												
α-methylstyrene												

A one-way ANOVA with Tukey's HSD test was used for each compound to test for significant differences (95% level) between the sampling points; day 0, 4, and 7. Differences in colours presented indicate significant differences ($p < 0.05$) between peak areas on the sampling days. Blue (Tukey letter A) is the lowest value, yellow (Tukey letter C) the intermediate, and red (Tukey letter B) the highest value. Purple (Tukey letters AB) indicates a value that is not significantly different from either of the values in the other treatment.

The measurements on the fermented wort on days 0, 4 and 7 show that most of the volatile compounds, including phenolic compounds, aldehydes, ketones, benzene derivates and furans, found on day 0 were stagnant or degraded over time. It was, in fact, only five higher alcohols (1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 3-methyl-3-buten-1-ol, and 2-phenylethanol), two aldehydes (only in H20, 3-methyl-butanal and (E)-2-nonenal), two ketones (cyclopentanone and 9-oxabicyclo [6.1.0]nonan-4-one), one fatty acid (only in H20, 3-methyl-butanoic acid) and one benzene derivate (only in H20, 1,2,4-trimethyl-benzene) that increased significantly ($p < 0.05$) in the peak area during the seven days of fermentation (Tables 3 and S1).

Regardless of aeration, no marked effect of temperature was observed on the production of volatile compounds (Tables 3 and S1). Regardless of temperature, the same compounds decreased in the peak areas. However, more compounds increased in peak area in H20 (i.e., 11, see above) and H30 (i.e., seven, see above), only two compounds, 3-methyl-3-buten-1-ol and cyclopentanone, were found to increase significantly in L20 and L30 (Tables 3 and S1).

4. Discussion

This work is the first of its kind, presenting the performance of a *Y. lipolytica* strain in the highly complex environment that is brewers' wort. It is essential to consider that our process in the current work is aerobic, whereas normal beer fermentation is anaerobic. Hence, it is not possible to compare the two processes directly.

Hoppy wort can hinder the growth of some non-conventional yeasts, such as *Debaryomyces hansenii* and *Torulaspora delbrueckii*, due to the antimicrobial effect of iso-α-acids and β-acids deriving from hops [28]. Interestingly, we have shown that *Y. lipolytica* can grow in a wort containing iso-α-acid, meaning that it will most likely also be able to grow in a traditional brewers' wort, which typically contains less β-acids than wort used for craft beers due to different hop addition strategies [28]. This trait is, therefore, essential for any further use in a traditional brewery.

The growth of *Y. lipolytica* was almost linear in both the high and low aeration experiments, indicating that it is inhibited under these conditions. The growth-inhibiting factor is most probably a lack of available oxygen in the wort since *Y. lipolytica* is an obligate aerobe [7,8], and since our aeration strategies, even at the high agitation rate, may be unable to dose enough oxygen into the wort. It may, however, also be the iso-α-acid added to the medium, as described above, or a combination of both.

The most typical carbon sources in brewers' wort are fermentable sugars, such as maltose, fructose, sucrose, maltotriose and glucose, of which maltose is the most predominant [29]. In a "normal" beer fermentation, *S. cerevisiae* (brewers' yeast) will assimilate all of these sugars [30]. In our study, the only sugars which were assimilated by *Y. lipolytica* from a typical brewers' wort were the two hexoses: fructose and glucose. This assimilation is expected since *Y. lipolytica* can only degrade hexoses and is considered a maltose-negative yeast [6,12]. Fructose assimilation was considerably slower compared to glucose. These data correspond with previous findings by Moeller et al., suggesting that *Y. lipolytica* favours glucose over fructose uptake [31].

No ethanol was detected during any of the aerated fermentations with *Y. lipolytica*. These data are as expected, since *Y. lipolytica* is a Crabtree-negative yeast [11], and they may suggest the use of *Y. lipolytica* for the production of alcohol-free beer.

Under traditional anaerobic beer fermentation, brewers' yeast will roughly assimilate 50% of the amino nitrogen in wort and utilise all fermentable sugars [29,32]. In our study, *Y. lipolytica* consumed much higher levels of amino acids under the high aeration conditions (i.e., 75–80%), even though it consumed markedly less sugar, as compared to normal beer fermentations. To the best of our knowledge, this trait of a yeast species has yet not been shown in brewers' wort. It could play a key role in producing alternative fermented beverages since low amino acid levels can prolong the flavour stability (less precursors for Strecker Aldehydes) and lower the potential bioactivity of such a beverage [25,32].

Interestingly, *Y. lipolytica* consumed proline and alanine at a fast rate in the experiments with high aeration. These observations are very different from what is typically seen for *S. cerevisiae* under aerobic conditions, where proline and alanine are usually placed in either the slow or no uptake groups [25], suggesting that *Y. lipolytica* may have other amino acid preferences than *S. cerevisiae*. This hypothesis, however, needs to be further elucidated.

Although *Y. lipolytica* exhibited very slow growth and uptake of sugars in the experiments with low aeration, a substantial part of the amino acids were taken up, and some were even depleted. This indicates that even under low aeration conditions, *Y. lipolytica* has an affinity for amino acid uptake despite nearly no other activity. Additionally, it may indicate that our *Y. lipolytica* strain, under the given conditions, can assimilate amino acids as both carbon and nitrogen source. This trait has earlier been suggested by Mansour et al. in a cheese-like medium [14], but it will need further investigation within a beer context.

Volatile esters play a vital role in the flavour profile of fermented beverages, typically contributing to fruity attributes [33]. No volatile esters were found in our experiments with *Y. lipolytica*. In brewers' yeast, the synthesis of volatile esters is catalysed by the ester synthases alcohol acetyltransferase I and II, encoded by the genes ATF1 and ATF2 [33].

High oxygen levels are known to repress the expression of ATF1 and ATF2 and thus inhibit the synthesis of esters in brewers' yeast in beer fermentations [34,35]. A similar effect in *Y. lipolytica* may explain the missing esters in the present study. This issue, however, needs to be further studied.

2-Phenylethanol is an important flavour compound used in the food and cosmetic industry and is described as having a fresh and rose-like odour [19]. It has previously been reported that *Y. lipolytica* can synthesise 2-phenylethanol [19,36,37], and these data correspond with the findings in this study. It is well established that phenylalanine is a precursor for 2-phenylethanol in brewers' yeast through the Ehrlich pathway [38–40]. This also seems to be the case for *Y. lipolytica*, since the decrease in phenylalanine corresponds with the increase in 2-phenylethanol in the experiments with high aeration, both at 20 °C and 30 °C.

Regardless of aeration, no marked effect of fermentation temperature was found on the amounts of volatile compounds. In contrast, it is reported that a standard beer fermentation, using *S. cerevisiae*, yields higher concentrations of especially higher alcohols and ethyl esters at high, than at low, temperatures [41–44]. This indicates that the production of volatiles by *Y. lipolytica* cannot be controlled by temperature in the same way as for *S. cerevisiae*.

To the best of our knowledge, the literature describing standard beer fermentations does not report the effect of continuous aeration on aroma production. Lehnert et al. [45] did, however, describe the effect of oxygen supply in a continuous alcohol-free beer production using a synthetic media and a strain of *S. cerevisiae*. Here it was reported that increased aeration increases the production of total higher alcohols as well as decreases the production of aldehydes. This increase in higher alcohols and decrease in aldehydes correspond with the findings in the current study, except for two (3-methyl-butanal and (E)-2-nonenal) which were produced in the high aeration experiment at 20 °C but not in the low aeration treatments. Furthermore, research has shown that an increased oxygen transfer rate can boost the production of the interesting aroma compound γ-decalactone by *Y. lipolytica* [20,21,46]. The same effect of oxygen on γ-decalactone was, however, not seen in our study. The reasons for this discrepancy could be due to the different yeast strains and/or different fermentation conditions used in the different studies.

5. Conclusions

In this work, we have shown that *Y. lipolytica* can be used to ferment a normal brewers' wort containing iso-α-acid, which is vital for the possible use of this yeast in a standard brewery. The growth of *Y. lipolytica* is, however, inhibited under the given conditions. Not surprisingly, our strain of *Y. lipolytica* can only utilize glucose and fructose out of the sugars found in a regular brewers' wort, and it does not produce ethanol in the presence of oxygen. For the first time, however, we report that *Y. lipolytica* under high aeration conditions, despite the low sugar uptake, can consume a large part of the amino acids (75–80%) in the wort. Furthermore, we report an assimilation order of amino acids for *Y. lipolytica* in brewers' wort. Interestingly, *Y. lipolytica* can assimilate proline rapidly and almost to depletion. Finally, *Y. lipolytica* does not seem to produce volatile esters under the given conditions, whereas it seems able to produce 2-phenylethanol under high aeration conditions, corresponding with a decrease in phenylalanine in the brewers' wort. This work improves our understanding of how *Y. lipolytica* grows, consumes amino acids, and produces volatile aroma compounds in a traditional brewers' wort, and it may contribute to the potential use of this non-conventional yeast in a traditional brewery for the production of novel beers, e.g., alcohol-free beer.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8110579/s1, Figure S1. Concentrations of maltose (squares), sucrose (crosses), maltotriose (diamond), m and malitotetraose (triangles) during fermentation of brewer's wort with *Y. lipolytica* at high aeration and 20 °C (solid curves) high aeration and 30 °C (short dotted curves), low aeration and 20 °C (inter mediate dotted curves), and low aeration and 30 °C (long dotted curves). Data are average values from triplicate experiments, and error bars indicate standars deviations. Figure S2. Ethanol concentrations during fermentation of brewer's wort with *Y. lipolytica* at (A) high aeration and (B) low aeration and 20 °C (dotted curves) and 30 °C (solid curves). Data are average values from triplicate experiments, and error bars indicate standard deviations. Table S1. Development of volatile compounds (peak areas/10.000) during fermentation of brewer's wort with *Y. lipolytica*.

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