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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Urea and urine are a viable and cost-effective nitrogen source for *Yarrowia lipolytica* biomass and lipid accumulation

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Abstract

Yarrowia lipolytica is an industrial yeast that has been used in the sustainable production of fatty acid-derived and lipid compounds due to its high growth capacity, genetic tractability, and oleaginous properties. This investigation examines the possibility of utilizing urea or urine as an alternative to ammonium sulfate as a nitrogen source to culture *Y. lipolytica*. The use of a stoichiometrically equivalent concentration of urea in lieu of ammonium sulfate significantly increased cell growth when glucose was used as the carbon source. Furthermore, *Y. lipolytica* growth was equally improved when grown with synthetic urine and real human urine. Equivalent or better lipid production was achieved when cells are grown on urea or urine. The successful use of urea and urine as nitrogen sources for *Y. lipolytica* growth highlights the potential of using cheaper media components as well as exploiting and recycling non-treated human waste streams for biotechnology processes.

Keywords *Yarrowia lipolytica* · Urea · Urine · Nitrogen · Metabolism

Introduction

Yarrowia lipolytica is a non-conventional yeast that has been used for the industrial production of organic acids (Liu et al. 2015; Sarris et al. 2011) and single-cell proteins (Zhao et al. 2013). Its extracellular secretory capabilities have made it a promising industrial host for the production of secreted lipases (Brigida et al. 2014; Fickers et al. 2004; Moftah et al. 2013). *Yarrowia lipolytica* has been engineered to produce lipids with high titer and productivity (Blazeck et al. 2014; Papanikolaou and Aggelis 2002), free fatty acids (FAs) (Ledesma-Amaro et al. 2016), omega-3-rich triacylglycerides (Xue et al. 2013), fatty alcohols (Wang et al. 2016), polyhydroxyalkonates (Haddouche et al. 2010), pentane (Blazeck et al. 2013), itaconic acid (Blazeck et al. 2015), and carotenoids (Schwartz et al. 2017). In addition, the ability of *Y. lipolytica* to grow on and tolerate a wide range of substrates enables its utilization as a production host for a diverse assortment of biochemical products (Bankar et al. 2009; Goncalves et al. 2014).

One of the main advantages of *Y. lipolytica* is its ability to efficiently utilize alternative carbon sources (Shabbir Hussain et al. 2016b). Furthermore, the growing range of genetic engineering tools available for *Y. lipolytica* (Blazeck et al. 2011; Curran et al. 2014; et al. 2016; Shabbir Hussain et al. 2016) enables engineering metabolism of non-native substrates (Rodriguez et al. 2016; Shaw et al. 2016). Currently, *Y. lipolytica* is naturally capable of metabolizing hydrophobic substrates including *n*-alkane, fats, and oils (Fickers et al. 2005). Studies have also exploited various waste streams as potential carbon sources to grow *Y. lipolytica* including olive mill wastewater (D'Annibale et al. 2006; Lanciotti et al. 2005), palm oil mill effluent (Oswal et al. 2002), fish waste (Yano et al. 2008), stearin (Papanikolaou et al. 2007), pineapple waste (Imandi et al. 2008), and sugarcane bagasse hydrolysate (Tsigie et al. 2011). Using these substrates is not only economical but also simultaneously allows for waste disposal and the creation of value-added products.

In addition to carbon, there are several other nutrients essential for microbial growth and use as a biochemical production platform. Among these, nitrogen is one of the most important components of microbial media as it is required for important metabolic processes such as nucleotide and amino

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acid biosynthesis. The source and concentration of nitrogen can influence the morphology and production of metabolites by the microorganism (Szabo 1999). For example, lipase production has been shown to be improved using tryptone as a sole nitrogen source compared to yeast extract and ammonium sulfate (Fickers et al. 2004). Furthermore, the nitrogen source is known to influence triacylglyceride accumulation in some yeast (Evans and Ratledge 1984). While ammonium sulfate is commonly used for *Y. lipolytica* growth in defined media, alternative nitrogen sources have been investigated ranging from simple nitrogen sources such as ammonium chloride and urea to the more complex forms such as peptone and yeast extract (Evans and Ratledge 1984). Among these, urea holds great potential as a low-cost nitrogen source for *Y. lipolytica* biomass accumulation. Furthermore, urea is abundant in human waste such as urine, which typically contains 10–20 g/L urea (~ 1–2% *w*/*v*), and is the most abundant compound in urine after water (Bouatra et al. 2013). In isolated conditions and resource-poor settings, the ability to use natural waste to produce biochemicals is highly desirable.

Here, we investigate the use of urea as a nitrogen source for *Y. lipolytica* biomass and lipid accumulation. When normalized to total nitrogen content, urea outperforms other nitrogen sources providing higher growth rates and biomass accumulation. For optimal growth, urea concentrations as high as 20 g/L can be utilized although increasing concentrations beyond this level cause significant growth inhibition. We also investigated a synthetic urine formulation as a nitrogen source for *Y. lipolytica* growth. Again, high cell densities were achieved although the rate of growth was slightly reduced. Increasing the C/N ratio by either increasing glucose concentration or lowering nitrogen content in synthetic urine improved lipid accumulation, similar to ammonium sulfate. Finally, we demonstrated that human urine could also be an effective nitrogen source without significant growth inhibition. Together, this work shows that urea and urine are both effective nitrogen sources for *Y. lipolytica* growth. Since urea is cheaper than ammonium sulfate on an equivalent nitrogen basis, a significant cost benefit may be realized. Similarly, urine experiments demonstrate the potential to grow *Y. lipolytica* on human waste.

Materials and methods

Strains and chemicals *Y. lipolytica* strain PO1f (ATCC MYA-2613; *MATa leu2-270 ura3-302 xpr2-322 axp*) was used for all studies unless otherwise stated. Since this strain is a leucine auxotroph, cells were transformed with a skeletal vector (pSL16) containing the *LEU2* gene and centromeric and autonomous replicating sequence (CEN1/ARS) prior to all growth studies (Shabbir Hussain et al. 2016a). Transformation was performed using the LiAc protocol that has been described previously (Barth 1996). Yeast complete synthetic media deficient in leucine (YSC-LEU) was prepared using 6.7 g/L yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories) and 0.69 g/L complete synthetic media (CSM-LEU) (Sunrise Science Products). Our rationale for this was that we wanted to use conditions similar to future experiments that will use these plasmids to overexpress genes to produce useful products. The dry cell weight of this strain is known to be linearly correlated with optical density (Fig. S1). The pH remained roughly constant at 6.5–7.5 throughout the experiments. The Δ*pex10* strain used in this study was created using CRISPR-Cas9-mediated genome editing (Schwartz et al. 2016). A 6 bp deletion was made in *pex10* from + 271 to + 277 bp, causing a loss of function mutation. It was also transformed using the abovementioned pSL16 vector. Various concentrations of glucose (*w*/*v*) were used as the sole carbon source for all experiments. For comparison between nitrogen sources, equimolar concentrations of nitrogen (g/L) were supplied into the media using either ammonium sulfate (Amresco) or urea (Amresco). To find an optimal concentration of urea, we increased the concentration until deleterious effects were observed. Human synthetic urine was formulated based on the most abundant metabolites commonly found in urine, including urea, creatine (Sigma-Aldrich), citric acid (Sigma-Aldrich), glycine (Sigma-Aldrich), sodium nitrate (Fisher Scientific), hippuric acid (Fisher Scientific), and L-cysteine (Fisher Scientific). Data from the human urine metabolome project report average values for the main components of human urine are 300 mM urea, 15 mM creatine, 7.5 mM hippuric acid, 7.5 mM citric acid, 3.8 mM L-cysteine, and 3.8 mM glycine (Bouatra et al. 2013). Pooled human urine that was not treated was obtained from Lee Biosolutions (991-03-P-1). Urine urea concentration was quantified using a urea assay kit (Sigma-Aldrich). Urine was stored at 4 °C and was handled within a biosafety level 2 cabinet. Urine was characterized immediately prior to use and stored for no longer than 3 weeks.

Cell culture Transformed *Y. lipolytica* from YSC-LEU agar plates were inoculated into 14-mL culture tubes containing 2 mL of YSC-LEU supplemented with 2% (*w*/*v*) glucose. Cultures were allowed to grow for 48 h at 28 °C while shaking at 215 rpm. Cells in late exponential phase were centrifuged at 8000 rpm for 2 min and resuspended in 1.5 mL of sterile deionized water. Cells were then inoculated at final OD_{600} of 0.2 into either fresh 2 mL cultures in 14-mL culture tubes or 15 mL cultures in 50-mL baffled flasks, in triplicate. Cultures were allowed to grow for either 96 or 120 h at 28 °C while shaking at 215 rpm. Cell densities were measured every 24 h using a NanoDrop 2000c from Thermo Scientific.

Dry cell weight quantification Dry cell weight was determined by washing 5 mL of cell culture with distilled deionized water three times. The cell pellet was then resuspended in 0.5 mL of water, placed in an aluminum dish, and dried for 24 h at 100 °C prior to measuring the dry cell weight. Three replicates were measured and used to determine average dry cell weight and standard error.

Lipid extraction and quantification Lipids were extracted, methylated, and analyzed by gas chromatography with flame ionization detection (GC-FID) exactly as described previously (Rodriguez et al. 2016). Briefly, 1 mL of cells was harvested and washed with distilled deionized water. In order to classify and measure fatty acids in the cell biomass, lipids were transesterified to fatty acid methyl esters (FAMEs). Prior to transesterification, 100 μL glyceryl triheptadecanoate (0.2 mg/μL hexane) was added to the cell pellet as an internal standard along with 100 μL of pentadecanoic acid (0.2 mg/μL hexane) for FAME quantification purposes. Five hundred microliters of 0.5 M sodium methoxide (20 g/L sodium hydroxide in methanol) was added for alkaline transesterification of lipids to FAMEs. The solution was vortexed at room temperature for 40 min at 2000 rpm and then neutralized with 40 μL sulfuric acid. Eight hundred fifty microliters hexane was added to extract the FAMEs and vortexed for 20 min at 2000 rpm. Finally, the mixture was then centrifuged for 1 min at 8000 rpm, and 750 μL of the hexane layer was collected for GC analysis. GC was performed using a 7890B GC system from Agilent Technologies. Samples of 2 μL were injected with split ratio of 10 and injector temperature of 250 °C. FAME species were separated on an Agilent J&W DB-23 capillary column (30 m × 0.25 mm × 0.15 μm), with helium carrier gas at a flow rate of 1 mL/min. The temperature of the oven started at 175 °C, and the temperature was ramped with a gradient of 5 °C/min until 200 °C. The FID was operated at a temperature of 280 °C with a helium make up gas flow of 25 mL/min, hydrogen flow of 30 mL/min, and air flow of 300 mL/min.

Results

Urea is a preferred nitrogen source for *Y. lipolytica* growth Ammonium sulfate is a commonly used nitrogen source for yeast media formulations, with 5 g/L as the standard concentration in yeast complete synthetic formulations. We wanted to determine if the nitrogen sources abundant in urine could be suitable replacements for ammonium sulfate in defined media. Tested alongside ammonium sulfate were alterative nitrogen sources used at equivalent total nitrogen concentrations of 1.06 g/L (Fig. 1). At the start of stationary phase after 48 h of growth, cultures containing urea showed dramatically higher cell densities reaching an OD_{600} of 35 in shake flasks.

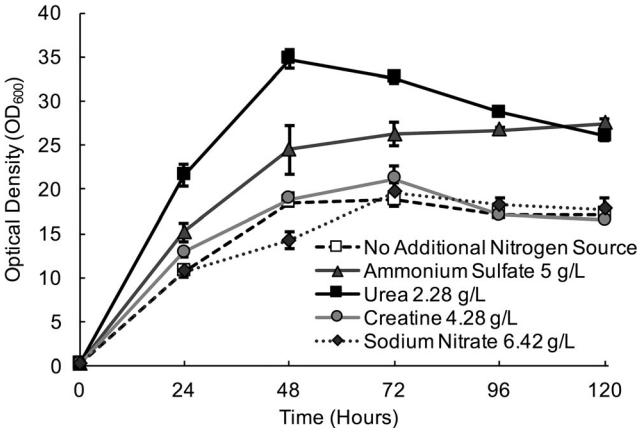

Fig. 1 *Yarrowia lipolytica* grown on different nitrogen sources. Five grams per liter ammonium sulfate was used as a baseline amount of nitrogen. All other sources were used at a stoichiometrically equivalent ratio. Ammonium sulfate data is shown with triangles, urea with shaded squares, creatine with circles, sodium nitrate with diamonds, and no additional nitrogen source control with open squares. Strain PO1f-pSL16 was run in triplicate. Error bars represent one standard error

Furthermore, the urea containing culture also demonstrated faster growth than the other cultures. The cell culture containing ammonium sulfate had comparably slower growth rates and reached lower cell densities after 5 days of growth. Creatine or sodium nitrate containing cell cultures performed similar to culture containing no additional nitrogen and therefore are not good nitrogen sources for biomass accumulation. To optimize the cell culture process, urea is the more desired nitrogen source due to the improved growth rates and the ability to reach high cell densities.

Optimizing urea concentrations Given that urea can effectively be used as a nitrogen source, the next goal was to determine an optimal concentration of urea to attain high cell densities. Urea was added to the media at varying concentrations ranging from 2.56 to 40 g/L (Fig. 2). Urea concentrations as low as 0.29 g/L

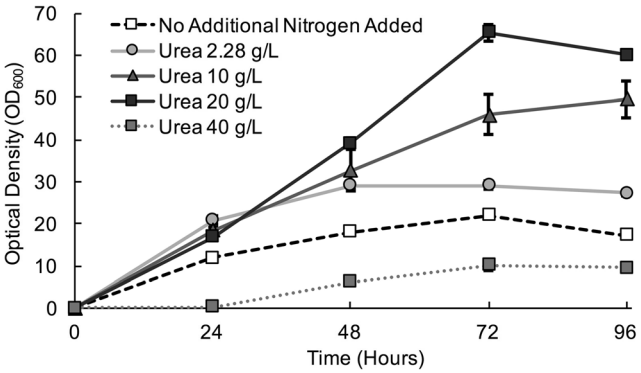

Fig. 2 *Yarrowia lipolytica* grown on different concentrations of urea as the only added nitrogen source. Growth curves using urea concentrations of 2.28 g/L (stoichiometrically equivalent to 5 g/L ammonium sulfate) to 40 g/L. Strain PO1f-pSL16 was run in triplicate. Error bars represent one standard error

still elicit significantly better growth when compared to a no additional nitrogen control (Fig. S2). Urea supplied at a concentration of 10 and 20 g/L leads to significantly higher growth after 96 h of culture when compared to all other tested concentrations (Fig. 2). Concentrations of up to 20 g/L of urea showed the most pronounced improvements in biomass accumulation reaching an OD_{600} of ~70 after 72 h of growth in shake flasks (Fig. 2). This improved growth rate might result from the increased flux of carbon into cell biosynthesis instead of storage molecules due to the better utilization of nitrogen. Increasing the urea concentration beyond 20 g/L urea elicited growth inhibition as shown for cultures containing 40 g/L urea (Fig. 2).

Synthetic urine is a feasible nitrogen source for biomass accumulation Synthetic human urine contains approximately 18 g/L urea along with relatively low concentrations of other substances including creatine, hippuric acid, citric acid, L-cysteine, and glycine. Given that *Y. lipolytica* accumulates significant biomass with 20 g/L urea, synthetic urine was tested as a viable nitrogen source for optimal growth (Fig. 3). Here, we observed that high cell densities were still achieved with synthetic urine (18 g/L urea), with a fourfold improvement in cell density relative to the no additional nitrogen source culture, although the initial growth rate was somewhat diminished relative to cultures using urea alone as a nitrogen source (20 g/L in Fig. 2). This demonstrates the ability of *Y. lipolytica* to utilize urea and to tolerate the other chemicals in synthetic urine. Interestingly, cell growth was inhibited by the ammonium sulfate concentration of equivalent nitrogen content (Fig. 3). Together, these results demonstrate the potential for *Y. lipolytica* growth and bioproduction on nitrogen-rich human or animal waste streams.

Lipid accumulation in oleaginous yeast is induced by nutrient limitation, most commonly the C/N ratio. We first altered the C/N ratio using a constant 2 *w/v*% glucose concentration and increasingly lowered the concentrations of the nitrogen source. We compared lipid titers and lipid profiles using either ammonium sulfate or synthetic urine as the nitrogen source. We observed general improvements in lipid accumulation in the PO1f strain using synthetic urine as the sole nitrogen source compared with ammonium sulfate (Fig. 4a). Based on the results presented here, the best conditions to accumulate lipids with urea as a nitrogen source are a C/N ratio of 120:1. The fatty acid profiles remain nearly identical between the two nitrogen sources demonstrating that the nitrogen source may not be a major contributor to FA diversity in *Y. lipolytica* (Fig. 4b). We can further conclude that the use of urea does not induce additional membrane stress that would normally be reflected in a change in the fatty acid profile (Rodriguez-Vargas et al. 2007). We alternatively used a higher glucose concentration to accommodate a higher nitrogen concentration (Fig. 4c). This approach was not successful in improving lipid concentrations with 8% (*w/v*) glucose. The 60:1 ratio demonstrated poor lipid accumulation in 8% (*w/v*) glucose, while at 120:1 and 240:1, 8% glucose resulted in similar lipid percentage with expected increases in cell biomass and lipid titer (Fig. 4c and Table 1). It was interesting to note that there is a shift towards a more saturated fatty acid profile in the 60:1 ratio containing 8% (*w/v*) glucose when compared to the same ratio containing 2% (*w/v*) glucose (Fig. 4d). Lipid profiles for all C/N ratios are summarized in Table 1.

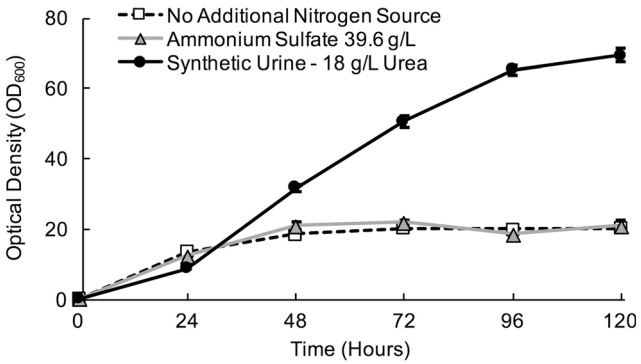

Fig. 3 *Yarrowia lipolytica* grown with synthetic urine as its only added nitrogen source. *Yarrowia lipolytica* was also grown in synthetic urine (circles) and compared with 39.6 g/L ammonium sulfate (stoichiometrically equivalent to 18 g/L urea; triangles) as its only added nitrogen, and a no additional nitrogen source control (squares). Strain PO1f-pSL16 was run in triplicate. Error bars represent one standard error

Synthetic urine is a feasible nitrogen source for lipid accumulation Being able to accumulate significant amounts of lipids is one of the more desirable traits of *Y. lipolytica*. A common method to obtain increased lipid yields is to eliminate competing pathways, such as beta oxidation, while shifting the flux towards triacylglyceride accumulation by using a higher C/N ratio (Sarris et al. 2011). Here, we knocked out *pex10*, implicated in peroxisomal biogenesis required for fatty acid metabolism (Xue et al. 2013) and used synthetic urine as a nitrogen source in comparison to the more commonly used ammonium sulfate. By gradually increasing the C/N ratios from 60 to 240, we observed that cultures containing synthetic urine as a nitrogen source were able to accumulate significantly more lipids compared with the cultures utilizing ammonium sulfate (Fig. 5a). The FA profile for cells grown in these two conditions were similar, with higher proportions of oleic acid and lower proportions of stearic acid found when using synthetic urine as a nitrogen source (Fig. 5b and Table 2).

Human urine is a good nitrogen source for *Y. lipolytica* growth Finally, we purchased fresh untreated pooled human urine and used it as a replacement for ammonium sulfate in defined media. We measured 13.5 g/L urea in the pooled urine and set up a control culture with the same concentration of urea as the nitrogen source. Controls containing human urine

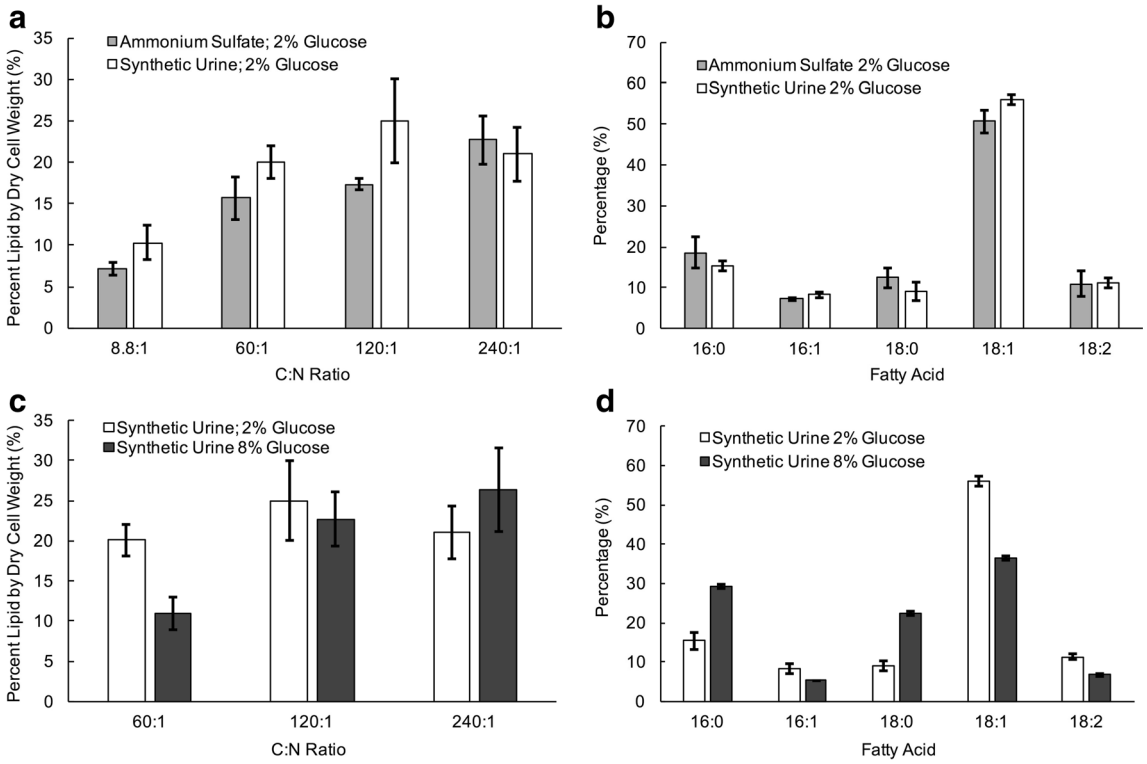

Fig. 4 *Yarrowia lipolytica* grown on different nitrogen and carbon conditions. **a** Percentage of lipids by dry cell weight when grown on varying nitrogen sources. **b** Sample lipid profile using a C/N ratio of 60:1. **c** Percentage of lipids by dry cell weight when grown with varying concentrations of carbon. **d** Sample lipid profile using a C/N ratio of 60:1. Strain PO1f-pSL16 was run in triplicate. Error bars represent one standard error. All data was collected at 120 h

that were not inoculated with yeast showed no detectable change in OD_{600} (data not shown). Wild-type *Y. lipolytica* grows faster on untreated pooled human urine compared to urea alone (Fig. 6a). Nearly equivalent dry cell weights were

Table 1 *Yarrowia lipolytica* grown with either ammonium sulfate of human synthetic urine as a nitrogen source. Strain PO1f-pSL16 was run in triplicate

C/N ratio	Conc. (g/L)		Cell conc. (g/L)	Lipid conc. (g/L)	Fatty acids (%)				
	$(NH_4)_2 SO_4$	Urea[a]			C16:0	C16:1	C18:0	C18:1	C18:2
8.8:1	5	0	2.30 ± 0.14	0.166 ± 0.016	18.6 ± 0.5	7.4 ± 1.2	9.9 ± 0.9	52.5 ± 1.8	11.6 ± 0.8
30:1	1.47	0	2.93 ± 0.03	0.414 ± 0.014	17.6 ± 0.1	4.9 ± 0.7	16.2 ± 0.4	49.5 ± 0.4	11.7 ± 0.9
60:1	0.735	0	3.76 ± 0.39	0.588 ± 0.077	18.6 ± 3.7	7.2 ± 0.3	12.5 ± 2.5	50.6 ± 2.7	11.0 ± 3.1
120:1	0.368	0	3.39 ± 0.08	0.621 ± 0.019	17.1 ± 0.4	6.0 ± 1.4	10.2 ± 0.1	52.9 ± 1.0	13.7 ± 0.0
240:1	0.184	0	2.59 ± 0.03	0.586 ± 0.074	18.4 ± 1.1	7.5 ± 0.5	13.3 ± 0.8	46.5 ± 2.7	14.2 ± 1.4
1.1:1	0	18	5.47 ± 0.14	0.362 ± 0.051	21.7 ± 0.8	5.3 ± 0.8	20.7 ± 3.1	29.2 ± 1.1	23.1 ± 2.5
8.8:1	0	2.26	1.54 ± 0.19	0.159 ± 0.024	14.3 ± 0.5	5.6 ± 0.0	10.2 ± 0.2	47.1 ± 0.8	22.8 ± 0.8
30:1	0	0.667	2.27 ± 0.15	0.197 ± 0.012	11.1 ± 0.5	5.2 ± 0.9	7.4 ± 1.1	61.6 ± 4.1	14.6 ± 1.6
60:1	0	0.333	2.81 ± 0.12	0.566 ± 0.050	15.4 ± 1.2	8.4 ± 0.7	9.0 ± 2.1	60.0 ± 1.3	11.2 ± 1.4
60:1	0	1.333	3.53 ± 0.30	0.387 ± 0.062	29.2 ± 0.5	5.3 ± 0.1	22.4 ± 0.6	36.4 ± 0.6	6.6 ± 0.3
120:1	0	0.167	1.65 ± 0.14	0.420 ± 0.074	19.4 ± 0.2	6.9 ± 0.5	13.1 ± 0.9	48.1 ± 1.8	12.6 ± 1.6
120:1	0	0.667	4.30 ± 0.03	0.978 ± 0.146	33.5 ± 0.3	4.0 ± 1.1	22.8 ± 0.5	35.2 ± 1.4	4.5 ± 0.2
240:1	0	0.084	2.08 ± 0.14	0.441 ± 0.061	17.5 ± 1.2	8.1 ± 0.2	11.4 ± 0.6	48.5 ± 3.1	14.6 ± 1.4
240:1	0	0.333	4.45 ± 0.10	1.160 ± 0.231	24.4 ± 1.1	6.0 ± 0.8	17.1 ± 0.8	46.4 ± 0.9	6.2 ± 0.2

± Represents one standard error

[a] Amount of urea in the human synthetic urine

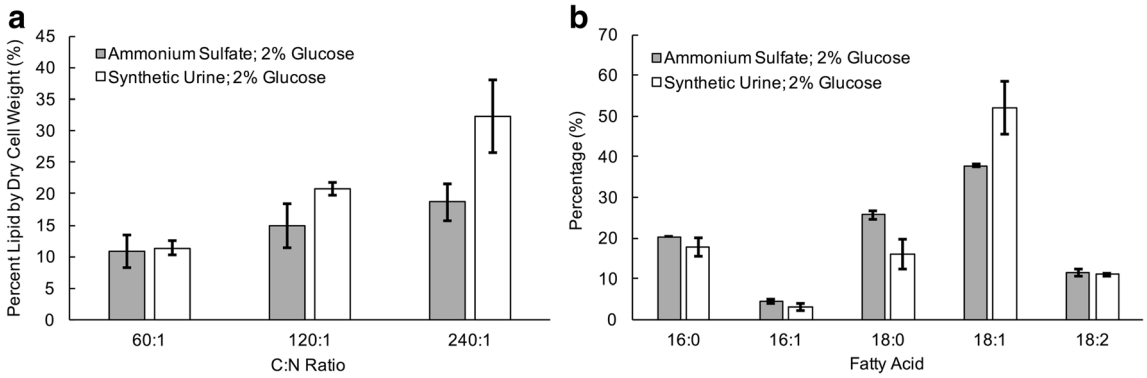

Fig. 5 *Yarrowia lipolytica* Δ*pex10* grown on different nitrogen and conditions. **a** Percentage of lipids by dry cell weight when grown on varying nitrogen sources. **b** Sample lipid profile using a C/N ratio of 60:1. Strain PO1f-Δ*pex10*-pSL16 was run in triplicate. Error bars represent one standard error. Data were collected at 120 h

obtained (Fig. 6b) while fewer lipids accumulated (Fig. 6b) for the human urine samples. The fatty acid distribution was somewhat altered as linoleic acid proportion increased at the expense of palmitic and stearic acids (Fig. 6c and Table 3) for the human urine samples. This demonstrates that real human urine could be used effectively as a nitrogen source for *Y. lipolytica* growth and associated bioproduction.

Lipid accumulation was also quantified when using pooled human urine as the only additional nitrogen source. We found the 120:1 C/N ratio to be optimal for lipid accumulation (Fig. 7a). Comparing these results to Fig. 4a, we see that lipid accumulation and profile (Fig. 7b) are nearly identical between real and synthetic human urine. Interestingly, dry cell weights on real human urine were higher than observed on synthetic urine (Tables 1 and 3).

Discussion

In this work, we have demonstrated the equivalence or benefits of replacing ammonium sulfate with urea for growth of *Y. lipolytica*. On an equivalent nitrogen basis, urea is far more effective for biomass accumulation. Furthermore, urea has a higher nitrogen content on a weight basis compared to ammonium sulfate, making it much more effective on a weight basis. Coupled with the increase in lipid accumulation and titer, urea appears to outcompete ammonium sulfate when using glucose as a carbon source for the production of both biomass and lipids. We went further and examined for the first time the ability to use synthetic and untreated real human urine as a nitrogen source for the growth of oleaginous yeast. To our knowledge, this is the first report considering urine as a feedstock for a microbial bioprocess.

Other studies comparing the effects of nitrogen source on the growth and bioproduct titers in various yeasts have provided inconclusive results. For example, cultures of *Rhodotorula glutinis* grown on olive mill wastewater (OMW) performed better when urea was used as a nitrogen source compared to ammonium sulfate or ammonium nitrate (Karakaya et al. 2012). On the contrary, biomass accumulation was lower compared to ammonium sulfate, ammonium nitrate, and ammonium chloride when grown on plant oils as a carbon source (Darvishi et al. 2009). Lipase production was optimized by the use of urea of a nitrogen source (Corzo and

Table 2 *Yarrowia lipolytica* Δ*pex10* grown with either ammonium sulfate of human synthetic urine as a nitrogen source. Strain PO1f-pSL16-Δ*pex10* was run in triplicate

C/N ratio	Conc. (g/L)		Cell conc. (g/L)	Lipid conc. (g/L)	Fatty acids (%)				
	$(NH_4)_2$ SO_4	Urea[a]			C16:0	C16:1	C18:0	C18:1	C18:2
60:1	0.735	0	3.24 ± 0.10	0.356 ± 0.083	20.4 ± 0.1	4.5 ± 0.5	25.7 ± 1.0	37.9 ± 0.3	11.5 ± 0.8
120:1	0.368	0	3.79 ± 0.11	0.559 ± 0.127	19.5 ± 4.0	5.7 ± 0.9	11.6 ± 3.1	52.1 ± 3.9	11.1 ± 2.3
240:1	0.184	0	3.23 ± 0.12	0.608 ± 0.091	18.0 ± 0.3	6.9 ± 0.0	9.3 ± 0.2	52.3 ± 0.3	13.4 ± 0.1
60:1	0	0.333	2.46 ± 0.13	0.283 ± 0.024	17.7 ± 2.3	3.1 ± 0.8	16.1 ± 3.6	52.0 ± 6.6	11.0 ± 0.5
120:1	0	0.167	2.80 ± 0.01	0.580 ± 0.028	19.9 ± 0.6	6.1 ± 0.1	11.3 ± 0.9	51.9 ± 0.2	10.9 ± 1.4
240:1	0	0.084	2.06 ± 0.13	0.649 ± 0.113	18.5 ± 0.3	6.5 ± 0.1	9.5 ± 0.2	51.7 ± 0.4	13.3 ± 0.2

± Represents one standard error

[a] Amount of urea in the human synthetic urine

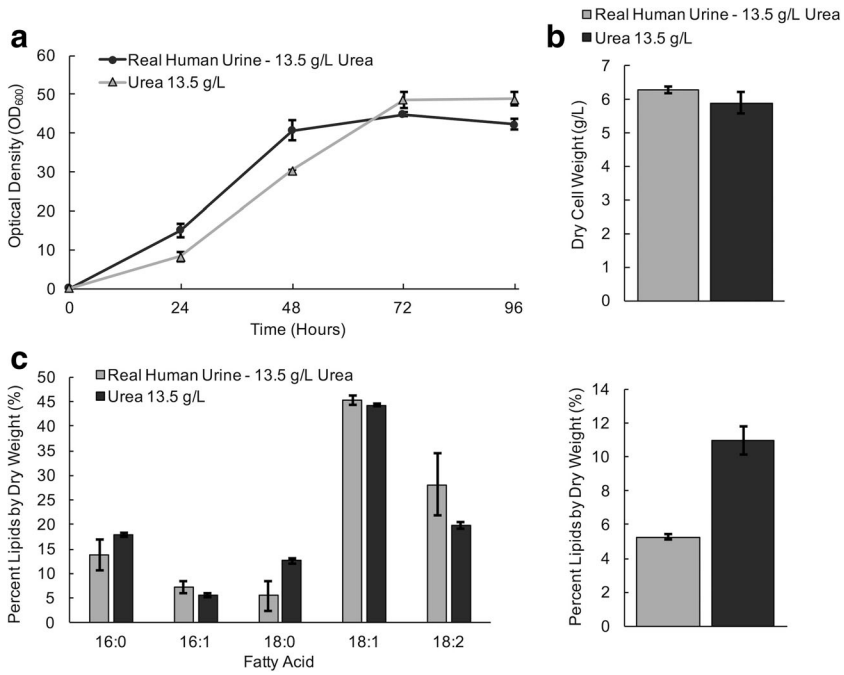

Fig. 6 *Yarrowia lipolytica* grown on different nitrogen conditions with 2% glucose. **a** Growth curve on different nitrogen conditions. **b** Dry cell weights and percentage of lipids by dry cell weight when grown on different nitrogen conditions. **c** Sample lipid profile using nitrogen conditions labeled. Strain PO1f-pSL16 was run in triplicate. Error bars represent one standard error. Data were collected at 96 h

Revah 1999). Noteworthy was that biomass production was optimized by a combination of yeast extract, urea, and ammonium sulfate. The differences observed are likely influenced by changes in carbon source, feedstock complexity, concentration of nitrogen source, and cultivation time, given the central role of nitrogen metabolism. Few differences in fatty acid composition were observed throughout our experiments. One notable exception is the Δpex10 strain grown on ammonium sulfate. In this strain, oleic acid accumulation decreases and stearic acid increases; however, these changes are consistent with the variation seen in other studies (Rigouin et al. 2017; Rodriguez et al. 2016).

To exploit *Y. lipolytica* for industrial bioproduction, it is desirable to be able to either accumulate biomass or lipids in the most cost-effective way. This can be accomplished by either decreasing costs of raw materials or increasing product output. In our experiments, urea accomplishes both. One method to lower costs of raw materials is by decreasing the cost of nitrogen feed stream. Current average market prices suggest that it is more economical to use urea in large-scale bioreactors as compared to ammonium sulfate (Table 4). Although the price per weight for ammonium sulfate is cheaper than urea, the overall cost of urea is less due its higher nitrogen content, and therefore a motivation for using urea as an industrial nitrogen source. We also demonstrated improved lipid accumulation and titer in urea compared to ammonium sulfate, in low nitrogen conditions using a beta oxidation-deficient Δ*pex10* strain. It should be noted that Δ*pex10* strains grown in urea have reduced cell biomass. One possible explanation is that urea and glyoxylate are produced by the breakdown of purines through the degradation of alantoin (Dal) pathway (Prinz et al. 2004). The glyoxylate cycle requires functional peroxisomes and perhaps induces stress in its absence and

Table 3 *Yarrowia lipolytica* grown with either urea or real human urine as a nitrogen source. Strain PO1f-pSL16 was run in triplicate

C/N ratio	Conc. (g/L)		Cell conc. (g/L)	Lipid conc. (g/L)	Fatty acids (%)				
	Urea	Urea[a]			C16:0	C16:1	C18:0	C18:1	C18:2
1.5:1	13.5	0	5.89 ± 0.33	0.649 ± 0.034	17.9 ± 0.3	5.4 ± 0.4	12.6 ± 0.5	44.3 ± 0.2	19.8 ± 0.6
1.5:1	0	13.5	6.29 ± 0.12	0.330 ± 0.003	13.8 ± 3.1	7.3 ± 1.2	5.5 ± 3.1	45.3 ± 1.0	28.1 ± 6.4
8.8:1	0	2.26	3.22 ± 0.04	0.290 ± 0.026	11.2 ± 0.5	6.5 ± 1.2	4.2 ± 0.6	56.9 ± 0.9	21.2 ± 1.3
60:1	0	0.333	3.18 ± 0.14	0.653 ± 0.023	17.0 ± 0.1	8.1 ± 0.5	8.4 ± 0.2	57.4 ± 2.0	9.1 ± 2.2
120:1	0	0.167	2.45 ± 0.06	0.627 ± 0.040	16.6 ± 0.4	7.7 ± 0.1	8.4 ± 0.0	55.0 ± 0.1	12.2 ± 0.3
240:1	0	0.084	2.79 ± 0.11	0.454 ± 0.066	16.6 ± 0.3	7.6 ± 0.1	8.6 ± 0.2	54.3 ± 0.3	12.8 ± 0.1

± Represents one standard error

[a] Amount of urea in the human urine

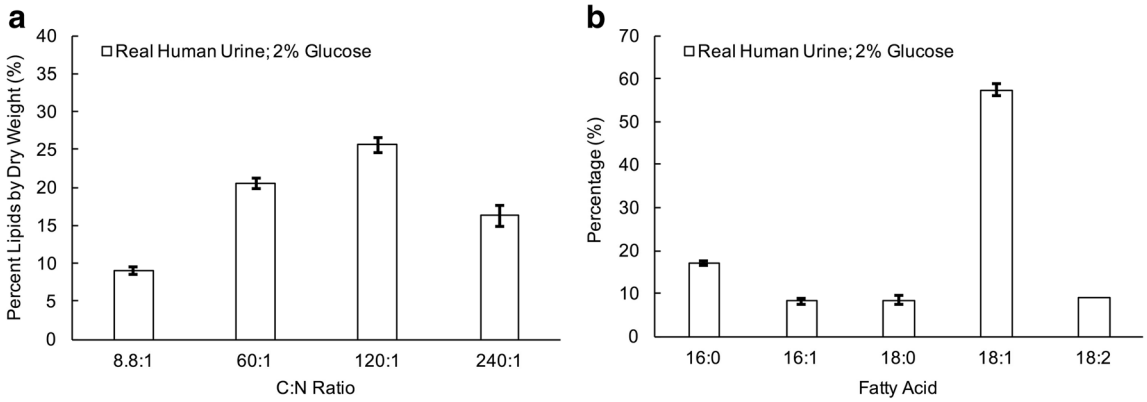

Fig. 7 *Yarrowia lipolytica* grown on real human urine with 2% glucose. **a** Percentage of lipids by dry cell weight at different C/N ratios. **b** Sample lipid profile using a C/N of 60:1. Strain PO1f-pSL16 was run in triplicate. Error bars represent one standard error. Data was collected at 96 h

possible remodeling of the nitrogen metabolism in these conditions (Kunze et al. 2006).

While this single knockout strategy leads to moderate increases in lipid accumulation compared to more modified strains (Blazeck et al. 2014; Qiao et al. 2015), we demonstrate the ability to trigger improved lipid accumulation and titer by simply switching nitrogen source. It is yet to be determined if these improvements are also realized when using higher productivity lipid-accumulating strains.

Another advantage for the use of urea is the relative abundance of this compound. By demonstrating that synthetic human urine can be used as a nitrogen feed source with *Y. lipolytica*, it opens the possibility of potentially using a natural waste product such as human urine as a nitrogen source to achieve maximum biomass accumulation or lipid production. Turning waste into value-added products and creating a more renewable or "closed-loop" system is not only environmentally friendly but may improve process economics. These closed-loop systems are especially important in resource-poor environments such as submarines, remote outposts, and in space travel where there are physical space limitations and issues with storage and removal of waste (Godia et al. 2002; Menezes et al. 2015).

Urea metabolism has been well studied in *S. cerevisiae* and *C. utilis* (Hofman-Bang 1999; Roon et al. 1972). *Yarrowia lipolytica* has two homologs (YALI0E07271p and YALI0E35156p) of the *DUR1,2* urea amidolyase gene from *S. cerevisiae* and two homologs (YALI0B04202p and YALI0C15807p) of the *DUR3* urea transporter. Given the number of duplicated urea metabolism genes, we suggest that *Y. lipolytica* may be better poised to utilize urea than *S. cerevisiae*, which prefers ammonium. However, further studies are needed to clarify the functional relevance of this redundancy and whether it is consistent with the backup hypothesis or if these paralogs have non-overlapping function consistent with the piggyback hypothesis (Qian et al. 2010; Vavouri et al. 2008).

This study shows that urea is a better replacement for ammonium sulfate when growing *Y. lipolytica* on defined glucose containing media. Furthermore, we demonstrated the potential to use human urine as a rich nitrogen source for yeast growth. These findings open the door for bioprocesses in resource-poor environments utilizing human waste for useful products.

Table 4 Nitrogen source cost analysis

Nitrogen source	Average cost ($/ton)	Input required at 1.1:1 C/N (g/L)	Cost per 100,000 L
Ammonium sulfate	$123	39.6	$536.91
Urea (from synthetic urine)	$234	18.0	$464.29

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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