Lipid Production from Amino Acid Wastes by the Oleaginous Yeast *Rhodosporidium toruloides*

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Abstract: Microbial lipids have been considered as promising resources for the production of renewable biofuels and oleochemicals. Various feedstocks, including sugars, crude glycerol, and volatile fatty acids, have been used as substrates for microbial lipid production, yet amino acid (AA) wastes remain to be evaluated. Here, we describe the potential to use AA wastes for lipid production with a two-stage culture mode by an oleaginous yeast strain *Rhodosporidium toruloides* CGMCC 2.1389. Each of the 20 proteinogenic AAs was evaluated individually as sole carbon source, with 8 showing capability to facilitate cellular lipid contents of more than 20%. It was found that L-proline was the most favored AA, with which cells accumulated lipids to a cellular lipid content of 37.3%. When blends with AA profiles corresponding to those of meat industry by-products and sheep viscera were used, the cellular lipid contents reached 27.0% and 28.7%, respectively. The fatty acid compositional analysis of these lipid products revealed similar profiles to those of vegetable oils. These results, thus, demonstrate a potential route to convert AA wastes into lipids, which is of great importance for waste management and biofuel production.

Keywords: amino acid wastes; biofuels; microbial lipids; *Rhodosporidium toruloides*; two-stage culture

1. Introduction

Biodiesel has emerged as one of the most promising energy sources for the renewable biofuel market, owing to its excellent compatibility with the current fuel infrastructure systems [1]. Lipids produced by oleaginous microorganisms have been exploited as alternative feedstocks for biodiesel production [2]. Some oleaginous yeasts are attractive due to their high growth rates and high cellular lipid contents [3]. Specifically, the oleaginous yeast *Rhodosporidium toruloides*, recently reclassified as *Rhodotorola toruloides*, has been demonstrated as an excellent lipid producer because it can accumulate large amounts of lipids under high cell-density culture conditions [4–7]. More importantly, *R. toruloides* uses diverse substrates for lipid production and can naturally tolerate inhibitory compounds found in hydrolysates of lignocellulosic biomass [8]. Although various raw materials have been utilized for microbial lipid production, including carbohydrates from various sources, waste glycerol, and volatile fatty acids [9], the costs of feedstocks remain remarkably high to ensure economic competitiveness of microbial lipids to vegetable oils [10]. Therefore, efforts are devoted continuously to exploring innovative processes, new feedstocks, and valuable co-products in order to improve the techno-economics of microbial lipid technology.

Amino acid (AA) wastes have been implicated as possible feedstocks for the production of biochemicals and biofuels, such as biobutanol from engineered microorganisms [11,12]. In fact, AAs can be envisioned as organic amines; once the nitrogen atom is released from the AA, the residual carbon skeleton can be readily converted into pyruvate or an intermediate of the citric acid cycle, thus fueling cellular metabolism [13]. Previously, food waste hydrolysates were evaluated for lipid and protein production by *R. toruloides* Y2 [14]. So far, AA wastes have not been used alone for microbial lipid production, likely because oleaginous microorganisms normally accumulate lipids under nitrogen limitation [15], while the catabolism of AA naturally generates a relatively nitrogen-rich environment. Interestingly, early studies indicated that either phosphate limitation or a two-stage culture mode could be used to achieve lipid production under nitrogen-rich conditions [16,17] or with nitrogen-containing substrates, such as chitin degradation products [18]. The meat wastes generated from abattoirs and meat processing industries correspond up to 50% of the total slaughtered animal weight, which is costly in terms of ecological disposal [19]. Specifically, huge amounts of fish and sheep wastes are produced [20,21], and these protein wastes can be further converted into AA mixtures [22]. Unlawful disposal of these wastes is known to cause serious environmental problems [23]. Therefore, the conversion of AA wastes into lipids merits some efforts in terms of investigating meat industry profitability, wastes management, and biofuel production.

The aim of this study was to explore the potential to use AA wastes for lipid production by the oleaginous yeast *Rhodosporidium toruloides* CGMCC 2.1389. The carbon sources of the lipid production cultures were made with each proteinogenic AA alone, or with designated blends with AA profiles corresponding to those of fish muscle (FM) [24], meat industry by-products (MI) [25], or sheep viscera (SV) [21], as these are the major meat wastes with abundant proteins and AA contents [20]. Results showed that *R. toruloides* CGMCC 2.1389 could accumulate lipids to more than 20% when cultivated with some AA alone or blends as sole carbon sources by using a two-stage culture mode [26]. Further analysis indicated that those neutral lipid products comprised mainly long chain fatty acids with 16 or 18 carbon atoms, which may be used to make biodiesel and other related oleochemicals. This study demonstrates that AA and related nitrogen-rich wastes can be explored to produce microbial lipids, which fits well with the protein-based biorefinery concept [12].

2. Materials and Methods

2.1. Microorganism, Media, and Growth Conditions

The yeast strain *R. toruloides* CGMCC 2.1389, originally obtained from China General Microbiology Collection Center, was maintained at 4 °C on yeast extract-peptone-dextrose (YEPD) agar plate contained (g/L) glucose·H_2O 20, peptone 10, yeast extract 10, and agar 20, and was sub-cultured twice a month. The peptone (total nitrogen 14.5%) and yeast extract (total nitrogen 9.0%) were obtained from Aoboxing Biotech. Co. Ltd. (Beijing, China).

The medium used for seed culture contained (g/L) glucose·H_2O 20, yeast extract 10, and peptone 10 (pH 6.0). For lipid production experiments, media with single AA or AA blends in 500 mM 2-(N-morpholino) ethanesulfonate (MES) buffer (pH 5.5) were used, and the concentrations of AAs were adjusted such that the media contained a total carbon at 16 or 28 g/L (unless otherwise specified). Accordingly, media with a single AA contained (g/L) L-asparagine (Asn) 50, L-aspartic acid (Asp) 44.66, L-valine (Val) 31.23, L-isoleucine (Ile) 29.15, L-arginine (Arg) 46.81, L-methionine (Met) 39.79, L-glutamine (Gln) 38.97, L-histindine (His) 42.58, L-glutamic acid (Glu) 39.2, L-proline (Pro) 30.6, L-alanine (Ala) 69.26, L-serine (Ser) 46.7, L-threonine (Thr) 39.7, L-glycine (Gly) 50, L-phenylalanine (Phe) 24.46, L-cysteine (Cys) 78, L-tryptophan (Trp) 24.75, L-lysine (Lys) 40.59, L-tyosine (Tyr) 26.8, or L-leucine (Leu) 29.15. Media with AA blends contained mixtures, with their compositions shown in Table 1. All the media were sterilized at 121 °C for 20 min.

Table 1. Compositional profiles of amino acid (AA) blends used for lipid production.

AA	Initial Concentration (g/L)		
	SV Blends	**FM Blends**	**MI Blends**
L-Aspartic acid (Asp)	3.22	1.14	5.02
DL-Asparagine (Asn)	3.21	-	-
L-Isoleucine (Ile)	2.62	2.54	2.32
L-Valine (Val)	3.45	3.07	2.90
L-Methionine (Met)	0.82	2.51	1.36
L-Arginine (Arg)	-	3.47	4.15
L-Histidine (His)	1.46	1.16	1.64
L-Glutamine (Gln)	3.82	-	-
L-Proline (Pro)	4.56	0.43	8.77
L-Glutamic acid (Glu)	7.30	6.50	8.96
L-Alanine (Ala)	5.39	3.12	3.96
L-Threonine (Thr)	2.69	6.54	2.51
L-Glycine (Gly)	7.95	1.20	6.17
L-Serine (Ser)	3.18	2.01	2.90
L-Cysteine (Cys)	0.82	1.49	0.66
L-Phenylalanine (Phe)	3.65	6.46	2.22
L-Tryptophan (Trp)	-	2.13	0.65
L-Tyrosine (Tyr)	0.73	5.91	1.64
L-Lysine (Lys)	5.06	5.85	4.73
L-Leucine (Leu)	5.28	4.37	3.95
Total	65.23	59.88	64.52

All amino acids were of analytical grade from Sangon Biotech (Beijing, China), with analytical grade reagents and chemicals purchased locally.

2.2. Culture Conditions

R. toruloides CGMCC 2.1389 cells were cultivated in YEPD media at 30 °C, 200 rpm for 24 h, then harvested by centrifugation at 5000 rpm for 5 min and washed twice with distilled water. To produce lipids, cells were resuspended in AA media in 500 mL shake flasks at an initial cell density of 4.0 g/L, and incubated at 30 °C and 200 rpm for 108 h, unless otherwise specified.

All culture experiments for lipid production were done in triplicate, and error bars shown in figures are standard deviations.

2.3. Analytical Methods

To determine dry cell mass, cells in 30 mL of culture broth were centrifuged at 8000 rpm for 5 min, washed twice with distilled water, dried at 105 °C for 12 h to constant weight, and determined gravimetrically [27]. Lipid was extracted with methanol/chloroform (1:2, v/v) according to a known method [5]. Cellular lipid contents were obtained by dividing lipid with dry cell weight.

Lipid products were transmethylated and analyzed by using a gas chromatography (GC) method [5]. Briefly, lipid samples (70 mg) were stirred with 5% KOH methanol solution (0.5 mL) at 65 °C for 50 min, then 0.7 mL of BF_3 diethyl etherate and methanol solution (4:6) were added, refluxed for 10 min, cooled, diluted with distilled water, and extracted with n-hexane. The organic layer was washed twice with distilled water and used for analysis. Finally, the compositional profiling of fatty acids was measured by using a 7890F GC system (Techcomp Scientific Instrument Co. Ltd., Shanghai, China), equipped with a cross-linked capillary free fatty acid phase (FFAP) column (30 mm × 0.25 mm × 0.25 mm) and a flame ionization detector. The flow rates for N_2, H_2, and air were 720 mL/min, 30 mL/min, and 100 mL/min, respectively. The temperatures of the injection port, oven, and detector were set at 250, 190, and 280 °C, respectively. The injection volume was 0.5 uL. Fatty acids were identified

by comparing them with the retention time of standards and quantifying them by the respective peak areas.

AAs were analyzed at 30 °C using a Dionex ICS-5000 ion chromatography system (Thermo-Fisher Scientific, MA, USA). The AminoPac PA10 column set consisting of a guard column (4 mm × 50 mm) and an analytical column (4 mm × 250 mm) was used to separate individual AAs. Gradient elution was performed at a flow-rate of 0.25 mL/min, with water, sodium hydroxide, and sodium acetate as mobile phases. The gradient conditions and the standard chromatogram used to analyze 20 proteinogenic AAs are shown in Supplementary Materials (Table S1 and Figure S1). AAs were quantified based on standard curves obtained under the same chromatographic conditions. Individual standard curves were established based on the correspondence between the AA concentration and peak area.

2.4. Statistical Analysis

SPSS Statistics 23 (IBM Software, Inc., California, USA) was used for statistical analysis. Two-way ANOVA with Tukey's multiple comparison test was conducted to compare different groups. Degrees of freedom, sum of squares, mean square, and distribution of the ratio among p-0.05 were taken into consideration, the results of which are shown in Tables S2–S5. Data with $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Evaluating Individual AAs as Carbon Sources for Lipid Production

It is well known that the carbon skeletons of AAs upon transamination or deamination can be further converted into metabolites, such as pyruvate, acetyl-CoA, acetoacetyl-CoA, or citric acid cycle intermediates [13,28,29], and those intermediates can be used to synthesize fatty acids and lipids. Two-stage culture mode with an initial cell density of 4.0 g/L was used to evaluate the capability of *R. toruloides* cells to produce lipids on each proteinogenic AA. To make a reasonable comparison, initial AA concentration was set at a total carbon concentration of 16 g/L. It was found that there were cell mass increments for 11 AAs, and most of these cases also had cellular lipid contents higher than 20% (Figure 1). Specifically, lipid contents were 27.3%, 26.3%, 25.3%, 23.5%, 21.7%, and 22.0% for the cultures with Glu, Pro, Ala, Asp, Ser, and Gln, respectively. There were small cell mass changes for Gly and Tyr, and significantly reduced cell mass was observed for Met, His, Arg, Thr, Trp, Tyr, Lys, and Leu ($p < 0.05$).
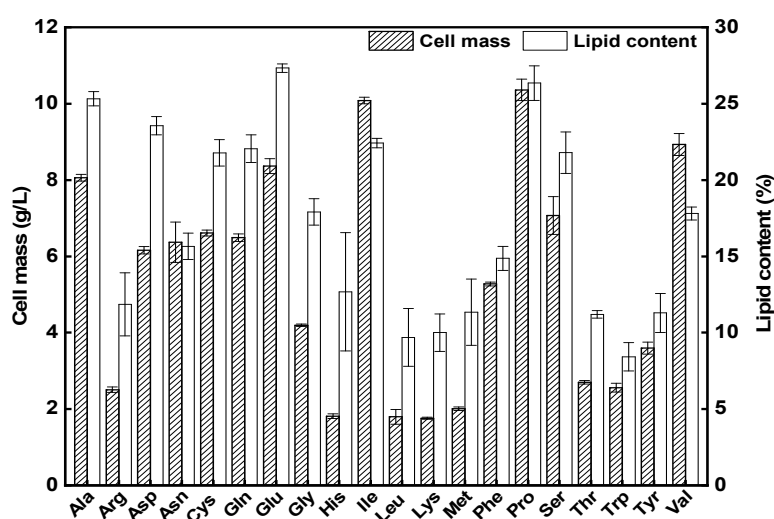

Figure 1. Lipid production results for *R. toruloides* CGMCC 2.1389 with each AA.

Literally, AAs can be classified via their catabolic precursors into ketogenic AAs, glucogenic AAs, or both. Similarly, they can be categorized on the basis of their structures and the chemical characteristics of their side chains into aliphatic, hydroxyl, aromatic, acidic, basic, or neutral AAs [28]. So far, substantial differences have been found, even within the same group, in terms of their efficacy as substrates for lipid production by *R. toruloides*. For instance, while aliphatic AAs such as Ala, Ile and Val were favored for lipid production, Leu was disfavored. For hydroxyl-group-containing AAs, Ser was favored, while Thr was disfavored. These results further suggest complex regulation mechanisms for lipid accumulation beyond the carbon sources of catabolism. While detailed discussions about the metabolism of each AA are beyond the scope of this work, these data are valuable references for further engineering of the yeast *R. toruloides* with genetic tools [30].

3.2. Lipid Production on AA Blends

Next, we used AA blends as carbon sources for lipid production by *R. toruloides*. Three AA blends were used, which had AA compositions similar to FM, MI, and SV, respectively (Table 1). The amounts of each AA were added such that the total carbon concentration was 28 g/L. Results showed that cells accumulated lipids close to or more than 20% after 108 h (Figure 2a). The lipid contents were 28% and 27%, and cell mass contents were 8.37 g/L and 6.50 g/L, in amino acids form sheep viscera (SVAA) and amino acids form meat industry by-products (MIAA), respectively. In terms of the culture with amino acids form fish muscle (FMAA), the lipid content and cell mass were 19% and 4.45 g/L, respectively. The compositional profiles of these AA blends may have major contributions to the lipid production results. For FMAA media, relatively high contents of Met, His, Arg, Thr, Cys, Trp, Tyr, Lys, and Leu were found, which failed to support lipid accumulation by *R. toruloides* cells when used as sole carbon sources in the media (vide ante). On the other hand, SVAA and MI media contain high amounts of AAs that support lipid accumulation, such as Asp, Pro, Ala, Glu, and Ser, yet low amounts of those disfavoring AAs, such as Met, Thr, Trp, and Tyr (Figure 1).

The initial C/N molar ratios of the media were determined as 4.43, 3.75, and 3.68 for FM, MI, and SV, respectively, based on their AA compositions. It should be noted that such low C/N molar ratios were inadequate to stimulate lipid accumulation, as C/N molar ratios of more than 70 were suggested for high lipid production by oleaginous yeasts [31]. As no phosphates were included in the media, cells were subjected to strict phosphate limitation. Thus, the fact that *R. toruloides* cells in AA media blends accumulated lipids close to or more than 20% was in agreement with the mechanism of phosphate-limitation-induced lipid production [16,17].

We also traced the residual AA at the end of the culture and the results are shown in Figure 2b–d. It was found that some AAs, such as Asp, Asn, Glu, Pro, Glu, Ala, and Ser, were largely consumed when initially included in the media, while others such as Ile, Val, His, Met, Thr, Gly, Cys, Trp, Tyr, Lys, and Leu were less utilized. Met, His, Trp, and Tyr were among the most disfavored ones, as there was little difference between their corresponding initial and residual data. Interestingly, Ala was found, with less than 50% being utilized in the MI media (Figure 2d), but was exhausted in the other two media (Figure 2b,c). Also, Asp was found, with about 30% leftover in the SV media (Figure 2c), but was exhausted in the other two media (Figure 2b,d). The differences in AA utilization patterns showed that *R. toruloides* cells favor some AAs, such as Pro, Glu, Asp, Ala, Asn, and Ser, while disfavoring some others. It should be noted that *R. toruloides* is a wild-type strain with no AA auxotrophic phenotype, indicating a full competence of AA metabolism. Thus, it is most likely that this yeast lacks an effective importing system for those disfavored AAs. However, the physiochemical properties of AAs in the media in a slightly acidic environment may also play a role in their uptake.
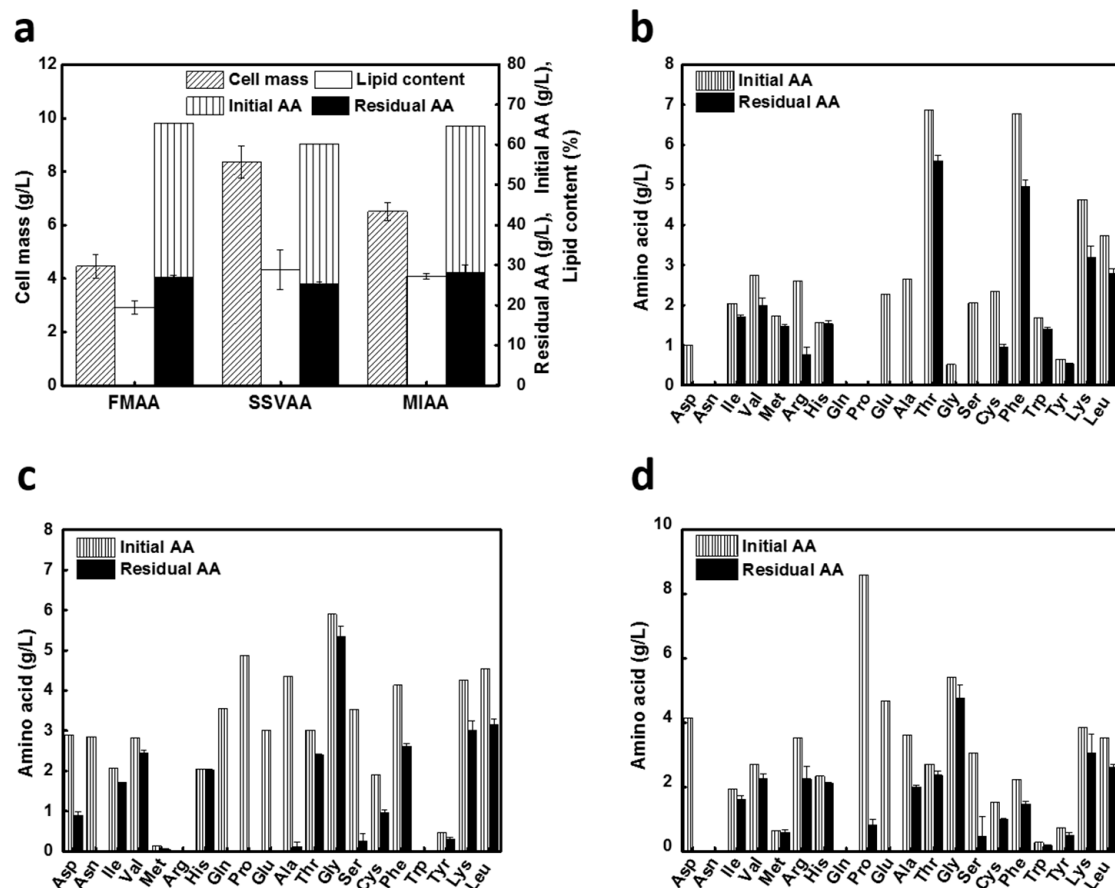
Figure 2. Results of lipid production at 30 °C and 200 rpm for 108 h from AA blends at a total carbon concentration of 28 g/L. (**a**) Lipid production in FMAA, SVAA, and MIAA blends. (**b**) Initial and residual AA profiles of FMAA media blend. (**c**) Initial and residual AA profiles of SVAA media blend. (**d**) Initial and residual AA profiles of MIAA media blend.

3.3. Lipid Production on L-Proline

The above results showed that L-proline was favored by *R. toruloides*, whether used alone or presented in AA blends. Thus, more experiments were designed to assess its capacity as a carbon source for lipid production by using the two-stage culture approach. Here, initial Pro concentrations of 30.6, 53.6, and 76.6 g/L, corresponding to total carbon concentrations of 16 (Pro-16), 28 (Pro-28), and 40 g/L (Pro-40), respectively, were used in the media. Results showed that there were no major differences among these three groups in terms of cellular lipid content (Figure 3). While cell mass was slightly lower (7.0 g/L) for the Pro-40 group, it was essentially identical for the other two groups. Lipid titers were 2.5, 2.6, and 2.1 g/L for Pro-16, Pro-28, and Pro-40, respectively. Thus, there were no significant differences in terms of lipid production, yet proline consumption was increased significantly with an increase in proline concentration ($p < 0.05$). The proline consumption was 19.8, 29.0, and 43.6 g/L for Pro-16, Pro-28, and Pro-40 media, respectively. These data also indicated that Pro at high initial concentrations may exert more osmotic stress [5,27,32], leading to inhibitory effects. For the Pro-28 media, when the culture time increased to 180 h, the cell mass and lipid content were 8.7 g/L and 37.3%, respectively, and there was 22.4 g/L Pro leftover. It seemed that there were limited benefits with prolonged culture time. Nonetheless, the data confirmed that Pro was a relatively good substrate for lipid production for *R. toruloides*.
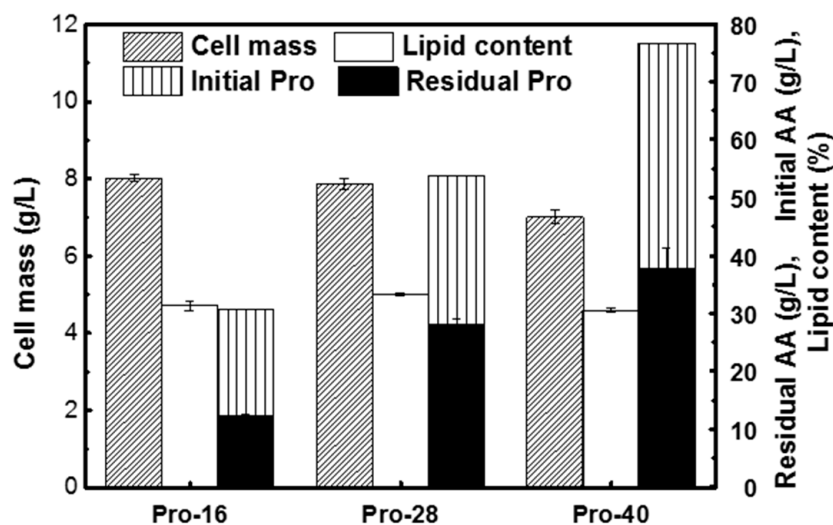
Figure 3. Results of lipid production on Pro at different initial concentrations. Cultures were performed at 30 °C and 200 rpm for 108 h.

3.4. Fatty Acid Compositional Profile of the Lipid Products

The lipid samples produced on different substrates were transmethylated into fatty acid methyl esters and analyzed by GC. It was found that palmitic acid (16:0), stearic acid (18:1), and oleic acid (18:0) were the major ones, and that no major fatty acid distributional differences were noticed among those products (Table 2). It should be noted that *R. toruloides* could produce lipids with different fatty acid compositional profiles [5,26,33,34]. As noticed in Table 2, lipid products from AA are more similar to lipids produced from corn stover [34] and palm [35]. Nonetheless, microbial lipids carrying long chain fatty acids with 16 and 18 carbons as the major fractions, which are similar to those of conventional vegetable oils form palm and canola, have been considered as alternative feedstock for biodiesel production [36]. Thus, lipids produced on AA have the potential for biodiesel production.

Table 2. Fatty acid compositional profiles of lipids produced on different substrates by *R. toruloides* and typical vegetable oils.

Media	Lipid Content (%)	Relative Fatty Acid Content (%, w/w)					
		Myristic (14:0)	Palmitic (16:0)	Palmitoleic (16:1)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)
Pro	27.7	3.0	40.9	0.7	15.1	36.9	3.5
FMAA	29.5	2.2	44.6	0.8	16.3	34.4	1.7
MIAA	27.7	3.1	43.0	0.6	16.3	35.6	1.4
SVAA	23.7	2.1	46.1	0.8	14.7	35.0	1.4
Glucose [5]	67.5	1.3	20.0	0.6	14.6	46.9	13.1
Glycerol [26]	35.0	1.4	27.8	0.6	21.8	43.8	2.9
Corn stover [33]	-	2.6	44.6	1.0	15.8	36.0	0.7
Sugarcane juice [34]	45.0	1.0	21.5	0.7	4.6	62.1	7.6
Palm [35]	-	-	42.7	-	2.1	38.4	10.6
Canola [35]	-	-	3.7	0.2	1.9	62.4	20.1

4. Conclusions

Here, we showed that the oleaginous yeast *R. toruloides* CGMCC 2.1389 can use most of the 20 proteinogenic AAs individually or in blends with similar AA compositional profiles to those of meat wastes for lipid production according to a two-stage culture mode. The lipids produced from AA herein showed similar fatty acid compositional profiles to those of microbial lipids produced in sugars and related organic substances. Our results suggests that AA wastes can be used as substrates for lipid production, yet this new route requires further investigation to improve the overall efficiency through the identification of cost-effective protein wastes, more robust oleaginous yeast strains, and advanced bioprocesses.

Supplementary Materials: The following are available online at http://www.mdpi.com/1996-1073/13/7/1576/s1: Table S1: Gradient condition for analyzing all 20 proteinogenic amino acids with IC. Table S2: Analysis of variances for cell mass in Figure 1 (all AAs). Table S3: Analysis of variances for cell mass in Figure 1 (Gly and Tyr). Table S4: Analysis of variances for cell mass in Figure 1 (Met, His, Arg, Thr, Trp, Tyr, Lys, and Leu). Table S5: Analysis of variances in Figure 2. Figure S1: Chromatogram of standard mixture of all 20 proteinogenic amino acids.

Author Contributions: Z.K.Z. conceived the project. Q.L., R.K., and X.Y. designed and performed the experiments. Q.W., Q.L., and R.K. performed ion chromatography analyses. Q.L., R.K., and Z.K.Z. wrote and revised the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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