Supplementary Materials

Rendered-Protein Hydrolysates as a Low-Cost Nitrogen Source for the Fungal Biotransformation of 5-Hydroxymethylfurfural

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METHODS

Macrocomponents and elementary analysis methods

The percentage of moisture, fat, ash, and nitrogen of the *fines* fractions obtained were determined following standard methods (AOAC 2000). The moisture content was measured using an oven (934.01). The ash content was determined using a muffle furnace (942.05). The total protein was analyzed by the Dumas method with a nitrogen-to-protein conversion factor of 6.25 (968.06). Lipid content was obtained by Soxhlet extraction with hexane. Measurements were performed, at least, in triplicate.

Elementary analysis (Na, Mg, P, S, K, Ca, Mn, Fe, Cu, and Zn) was performed by inductively coupled plasma hyphenated with mass spectrometry (ICP-MS) using an Agilent 7700 Series (Agilent Technologies, Santa Clara, USA). The samples were analyzed by Scientific and Technical Services (SCT) from the University of Lleida (Lleida, Spain).

Amino acid composition

The amino acid content of samples was determined on freeze-dried and pulverized tissue. Hydrolysis of the samples (50 mg) was carried out by acidic hydrolysis using 5 mL of 6 M HCl (110 °C, overnight, under N_2) [1,2]. Hydrolysis tubes were cooled and centrifuged at 3,000 *g* for 30 min to remove particulate matter. Aliquots of 25 μL of hydrolysate were evaporated using SpeedVac™ SPD131DDA (Thermo Electron Corporation) and reconstituted in 500 μL of water:acetonitrile (20:80, *v*/*v*). Samples were filtered through a 0.22 μm hydrophilic polytetrafluoroethylene (PTFE) membrane prior to injection. The injection volume was 5 μL.

Quantitation of individual amino acids was performed using a method described by Guo et al. [3] with modifications. UHPLC was performed using a Waters Acquity system (Waters, Milford, MA, USA) equipped with a BEH Amide column (2.1 x 150 mm; 1.7 μm) (Waters,

Manchester, UK). The mobile phase consisted of solvent A (10 mM ammonium formate in water with 0.15% formic acid) and solvent B (ammonium formate-saturated acetonitrile with 0.15% formic acid). The gradient elution followed was 15% A and 85% B maintained for 3 min at 0.5 mL/min. Then, from 15% to 20% A in 3 min; from 20% to 24% A in 1.5 min; from 24% to 60% A at 0.6 mL/min in 1.5 min and maintained for 3 min. Finally, initial conditions were regained in 2 min. The flow rate of the phase was 0.5 mL/min, and the column temperature was maintained at 30 °C. The column was cleaned with weak (20% acetonitrile) and strong (80% acetonitrile) washing solvents between injections.

Detection and quantitation of amino acids in the hydrolysate were performed using a multiple reaction monitoring method (MRM) in a Waters Triple Quadrupole Detector (TQD) mass spectrometer (Micromass MS Technologies, Manchester, UK). The system was equipped with an electrospray ionization (ESI) source operated in positive ion mode. Parameters in the source were set as described in the bibliography by Guo et al., (2013). MRM transitions were tested successfully and optimized in our conditions for phenylalanine (Phe), leucine (Leu), isoleucine (Ile), methionine (Met), valine (Val), proline (Pro), hydroxyproline (Hyp), tyrosine (Tyr), alanine (Ala), threonine (Thr), glycine (Gly), glutamic acid (Gln/Glx), serine (Ser), aspartic acid (Asp/Asx), histidine (His), arginine (Arg), lysine (Lys), and cysteine (Cys). Tryptophan (Trp) was not determined as it was totally degraded during the hydrolysis conditions. Cone voltage and collision energy were optimized for each individual amino acid. A stock solution containing a commercial amino acid standard mixture and Hyp were serially diluted in water:acetonitrile (20:80, *v*/*v*) to prepare a 7-point standard curve. Data were processed using QuanLynx software. Amino acids were quantified from absolute response without internal standard and results were expressed as mg amino acid/g sample.

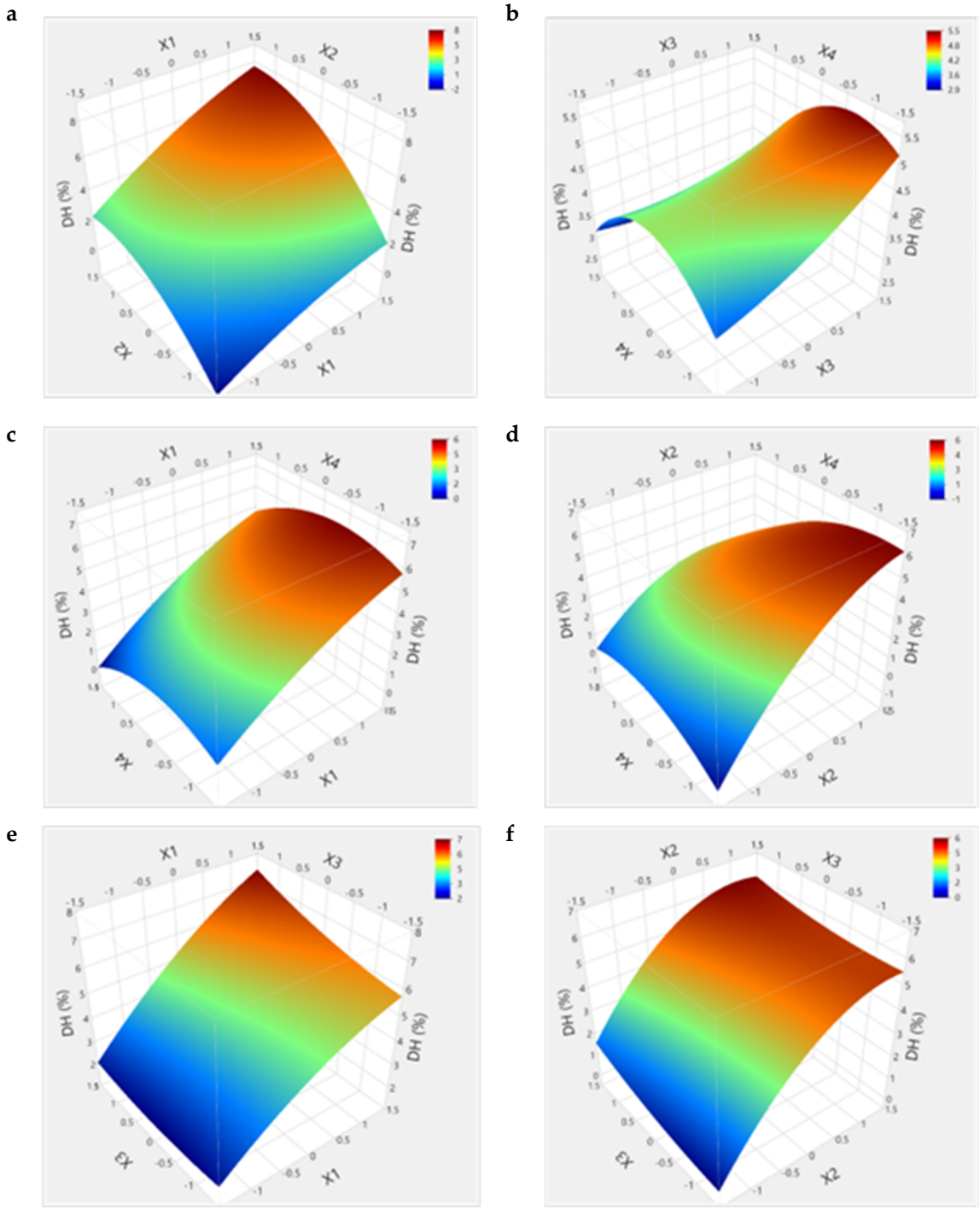


Figure S1. Response surface plots showing the effects of independent variables E/S ratio (X_1), time (X_2), initial pH (X_3), and temperature (X_4) on DH in enzymatic hydrolysis of PDF with enzyme Neutrase 0.8L: **a**) E/S ratio vs. time; **b**) temperature vs. initial pH; **c**) temperature vs. E/S ratio; **d**) temperature vs. time; **e**) initial pH vs. E/S ratio, and **f**) initial pH vs. time.

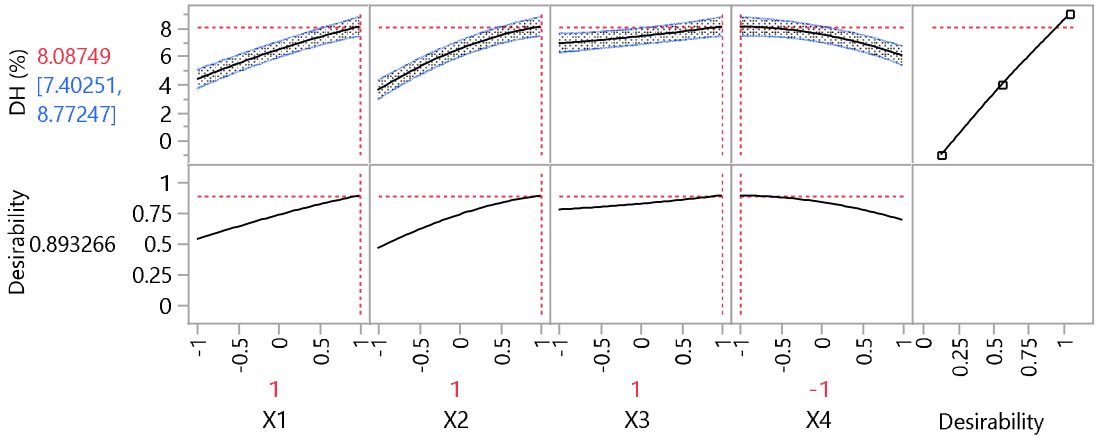


Figure S2. Profiles for the predicted DH and the desirability level for different factors (X_1 – E/S ratio; X_2 – time; X_3 – initial pH, and X_4 – temperature) for optimum DH for hydrolysis of PDF with Neutrase 0.8L.

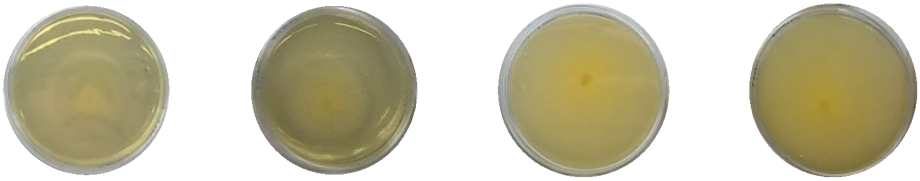
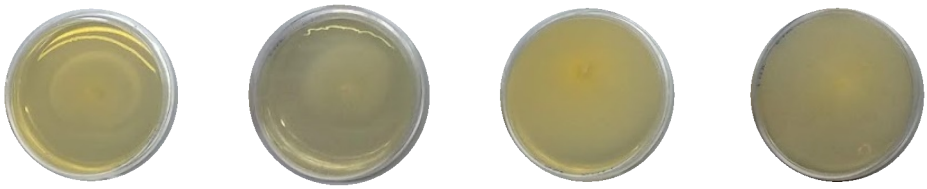
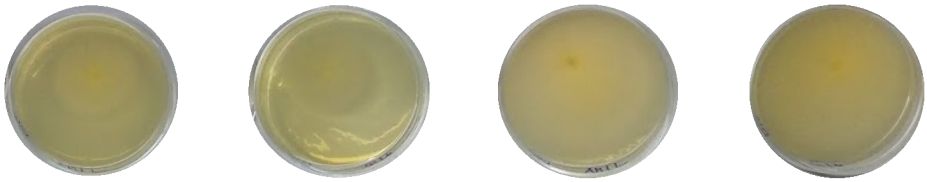
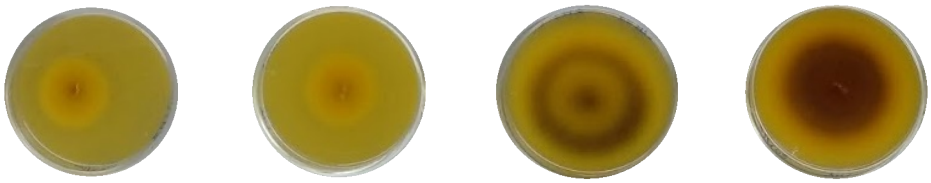
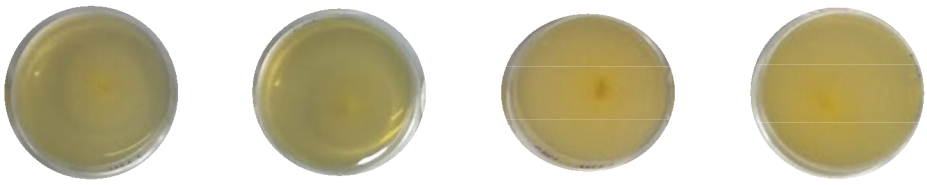
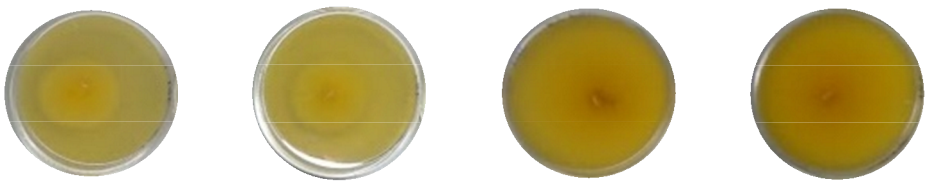
Culture medium	3 days		6 days	
	Light	Darkness	Light	Darkness
MEA				
MEA-WP				
MEA-50WP				
MEA-HA				
MEA-50HA				
MEA-HN				
MEA-50HN				

Figure S3. *Fusarium striatum* growth in Petri dishes containing different culture media after 3 and 6 days. MEA: malt extract agar medium; MEA-WP: malt extract agar medium without peptone; MEA-50WP: malt extract agar medium without peptone and 50% of malt extract; MEA-HA: malt extract agar medium containing Alcalase 2.4L hydrolysate; MEA-50HA: malt extract agar medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; MEA-HN: malt extract agar medium containing Neutrase 0.8L hydrolysate; MEA-50HN: malt extract agar medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate.

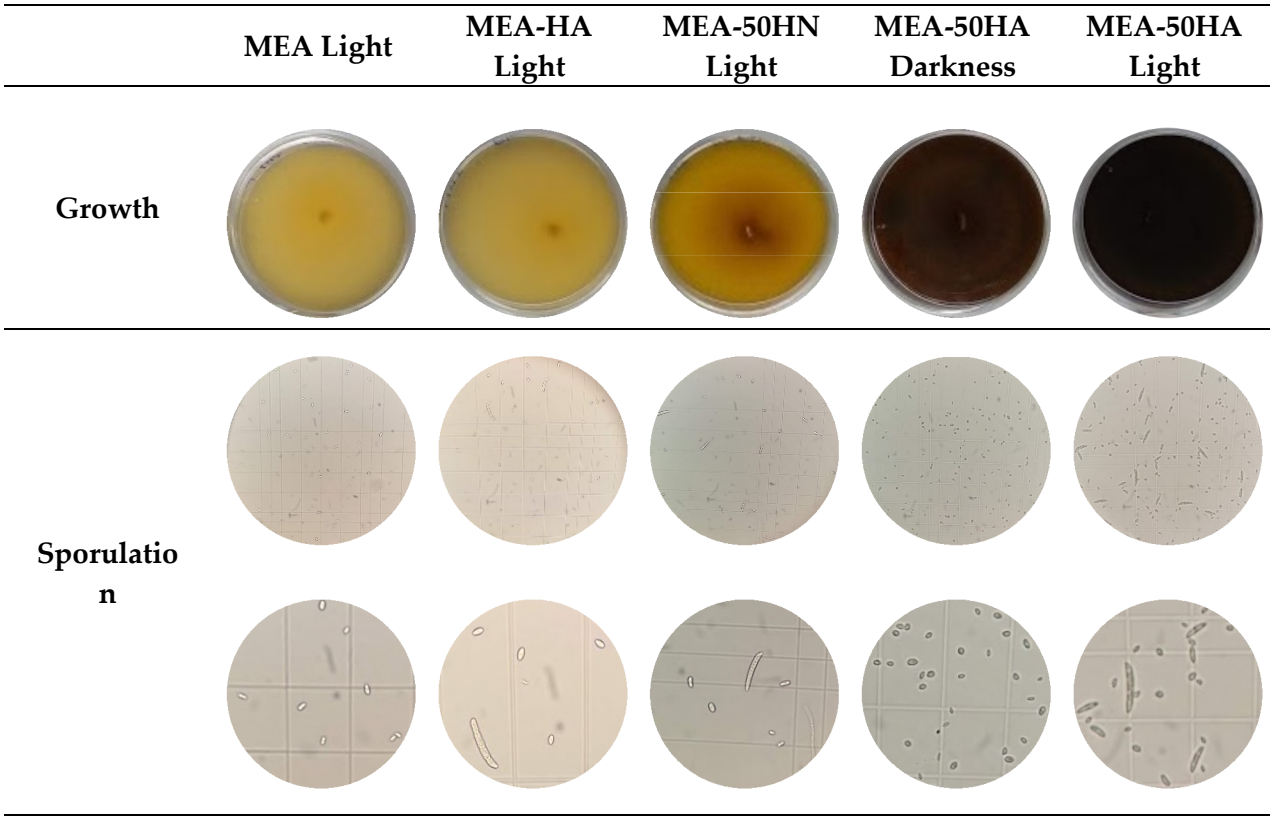


Figure S4. Cultural and morphological characters of *F. striatum* after 10 days in MEA, MEA-HA, MEA-50HN, and MEA-50HA. MEA: malt extract agar medium; MEA-HA: malt extract agar medium containing Alcalase 2.4L hydrolysate; MEA-50HN: malt extract agar medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate; and MEA-50HA: malt extract agar medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate.

Table S1. CCD for the enzymatic hydrolysis of PDF fraction with enzyme Neutrase 0.8L.

	Factors				Response	
Run	**X_1**	**X_2**	**X_3**	**X_4**	**$DH_{experimental}$ (%)**	**$DH_{predicted}$ (%)**
1	−1	−1	−1	−1	0.00	−0.15
2	−1	−1	−1	1	0.49	0.47
3	−1	−1	1	−1	0.68	0.64
4	−1	−1	1	1	0.85	0.52
5	−1	1	−1	−1	3.92	3.86
6	−1	1	−1	1	2.14	2.43
7	−1	1	1	−1	4.00	4.34
8	−1	1	1	1	2.44	2.17
9	1	−1	−1	−1	1.89	2.08
10	1	−1	−1	1	3.24	2.80
11	1	−1	1	−1	3.95	3.57
12	1	−1	1	1	3.58	3.56
13	1	1	−1	−1	6.66	6.90
14	1	1	−1	1	5.63	5.58
15	1	1	1	−1	8.15	8.09
16	1	1	1	1	5.97	6.03
17	−1.48	0	0	0	1.72	1.80
18	1.48	0	0	0	6.07	6.31
19	0	−1.48	0	0	0.00	0.72
20	0	1.48	0	0	5.93	5.52
21	0	0	−1.48	0	4.41	4.33
22	0	0	1.48	0	4.85	5.25
23	0	0	0	−1.48	4.19	4.05
24	0	0	0	1.48	2.53	2.98
25	0	0	0	0	5.07	4.56
26	0	0	0	0	4.70	4.56
27	0	0	0	0	4.54	4.56
28	0	0	0	0	4.61	4.56
29	0	0	0	0	4.46	4.56

X_1 – E/S ratio; X_2 – time; X_3 – initial pH, and X_4 – temperature

Table S2. Analysis of variance (ANOVA) for the response surface quadratic model.

Source	DF	Sum of squares	Mean square	F value	*P*-value Prob > F
Model	14	120.73680	8.62406	53.0334	<0.001
Residual	14	2.27662	0.16262		
Lack of fit	10	2.0512963	0.205130	3.6416	0.1122
Pure error	4	0.2253200	0.056330		
C. Total	28	123.01341			
R^2=0.9815; $R^2_{adjusted}$=0.9630; $R^2_{predicted}$=0.9064					

Table S3. Effect test of the independent variables and their interactions.

Source	DF	Sum of squares	F value	*p*-value Prob > F
X_1	1	47.11452	289.7297	<.0001
X_2	1	53.46283	328.7685	<.0001
X_3	1	1.947405	11.9755	0.0038
X_4	1	2.663886	16.3815	0.0012
X_1X_2	1	0.668306	4.1097	0.0621
X_1X_3	1	0.493506	3.0348	0.1034
X_2X_3	1	0.094556	0.5815	0.4584
X_1X_4	1	0.012656	0.0778	0.7843
X_2X_4	1	4.192256	25.7802	0.0002
X_3X_4	1	0.551306	3.3902	0.0869
X_1^2	1	0.573496	3.5267	0.0814
X_2^2	1	4.600718	28.292	0.0001
X_3^2	1	0.11518	0.7083	0.4142
X_4^2	1	2.419759	14.8803	0.0017

X_1 – E/S ratio; X_2 – time; X_3 – initial pH, and X_4 – temperature

Table S4. Proximate elemental composition of *fines*, PDF, freeze-dried hydrolysates, and precipitates based on DM.

	Samples					
	Fines	**PDF**	**HA**	**HA precipitate**	**HN**	**HN precipitate**
Protein (%)	45.8 ± 0.5	57.7 ± 1.7	69.2 ± 5.1	23.7 ± 2.0	70.5 ± 1.4	46.3 ± 2.6
Ash (%)	12.2 ± 0.2	16.9 ± 1.0	9.8 ± 0.2	42.2 ± 2.9	6.5 ± 0.2	25.2 ± 1.8
Fat (%)	37.2 ± 0.3	18.9	12.9	26.3	2.6	21.1
C/N	6.8 ± 0.1	5.1 ± 0.1	4.2 ± 0.1	10.6 ± 0.5	3.8 ± 0.1	5.8 ± 0.2
^{23}Na (%)	0.58	0.34	3.47	0.78	2.44	0.30
^{24}Mg (%)	0.12	0.16	0.02	0.40	0.07	0.13
^{31}P (%)	2.56	4.09	0.40	9.20	0.69	3.30
^{34}S (%)	0.53	0.61	0.79	0.25	0.73	0.59
^{39}K (%)	0.65	0.30	0.43	0.07	0.81	0.08
^{44}Ca (%)	3.61	5.81	0.07	15.51	0.09	4.80
^{55}Mn (ppm)	2.98	6.98	0.23	14.33	0.19	6.03
^{56}Fe (ppm)	149.81	252.39	23.01	655.51	10.02	351.79
^{63}Cu (ppm)	7.57	10.93	13.19	4.44	11.96	11.70
^{66}Zn (ppm)	89.24	125.30	8.08	293.61	2.54	136.29

Table S5. Amino acid composition (mg amino acid/g sample) and total amino acid content expressed as mean ± SD.

Amino acid	Concentration (mg amino acid/g sample)					
	Fines	PDF	HA	HA precipitate	HN	HN precipitate
Ala	16.2 ± 0.3^{c}	19.8 ± 0.2^{b}	20.1 ± 0.6^{b}	9.2 ± 1.1^{d}	24.1 ± 1.4^{a}	16.2 ± 0.5^{c}
Arg	36.3 ± 2.9^{c}	41.7 ± 2.4^{c}	49.1 ± 2.7^{a}	20.6 ± 1.7^{c}	51.1 ± 3.3^{a}	34.6 ± 1.4^{c}
Asx	39.8 ± 2.4^{d}	49.7 ± 1.2^{bc}	54.4 ± 1.4^{ab}	22.4 ± 3.4^{e}	56.3 ± 1.4^{a}	43.9 ± 1.2^{cd}
Cys	1.5 ± 0.8^{cd}	5.2 ± 1.0^{ab}	2.7 ± 0.2^{bcd}	3.1 ± 1.1^{bc}	0.5 ± 0.2^{d}	7.4 ± 1.8^{a}
Glx	82.4 ± 2.7^{b}	90.5 ± 1.5^{ab}	96.0 ± 1.8^{a}	39.5 ± 2.3^{d}	94.3 ± 5.8^{a}	61.6 ± 0.1^{c}
Gly	50.8 ± 8.8^{ab}	52.4 ± 10.6^{ab}	51.7 ± 8.6^{ab}	16.7 ± 2.0^{c}	68.5 ± 13.8^{a}	30.2 ± 1.2^{bc}
Hyp	6.1 ± 0.4^{b}	5.6 ± 0.2^{b}	6.2 ± 0.3^{b}	2.3 ± 0.0^{c}	11.2 ± 0.3^{a}	2.4 ± 0.1^{c}
His	15.0 ± 1.4^{ab}	17.3 ± 0.9^{ab}	17.9 ± 0.2^{a}	7.3 ± 0.5^{c}	17.6 ± 1.8^{a}	14.2 ± 0.5^{b}
Ile	12.6 ± 0.7^{bc}	20.4 ± 1.1^{a}	16.8 ± 1.2^{ab}	8.7 ± 2.6^{c}	14.9 ± 2.7^{b}	22.0 ± 0.8^{a}
Leu	49.8 ± 2.2^{b}	65.8 ± 1.1^{a}	68.2 ± 0.6^{a}	24.9 ± 2.1^{c}	62.9 ± 5.8^{a}	46.2 ± 2.0^{b}
Lys	35.5 ± 6.1^{b}	42.3 ± 3.3^{ab}	47.0 ± 3.3^{a}	17.9 ± 3.0^{c}	46.4 ± 4.6^{ab}	38.7 ± 1.3^{ab}
Met	7.3 ± 0.4^{ab}	6.9 ± 4.6^{ab}	11.5 ± 3.2^{a}	2.6 ± 0.7^{b}	9.3 ± 1.6^{ab}	9.3 ± 1.8^{ab}
Phe	11.3 ± 0.7^{b}	15.5 ± 0.7^{a}	16.2 ± 1.2^{a}	6.6 ± 0.5^{c}	15.1 ± 1.3^{a}	12.0 ± 0.4^{b}
Pro	34.5 ± 0.0^{b}	39.1 ± 2.7^{b}	39.5 ± 1.4^{b}	13.8 ± 2.0^{d}	54.3 ± 4.0^{a}	22.9 ± 1.0^{c}
Ser	24.7 ± 1.3^{c}	30.7 ± 1.6^{b}	38.2 ± 0.3^{a}	12.2 ± 1.2^{d}	36.5 ± 2.4^{a}	22.6 ± 0.3^{c}
Thr	29.9 ± 1.0^{a}	31.3 ± 0.6^{a}	31.8 ± 1.2^{a}	14.9 ± 0.1^{c}	31.8 ± 1.4^{a}	19.1 ± 0.9^{b}
Tyr	14.8 ± 0.8^{c}	19.5 ± 1.0^{ab}	22.0 ± 1.2^{a}	9.5 ± 1.5^{d}	19.6 ± 1.4^{ab}	18.2 ± 0.0^{bc}
Val	29.7 ± 1.7^{c}	39.4 ± 1.0^{a}	34.1 ± 1.2^{b}	12.9 ± 1.1^{d}	36.1 ± 2.2^{ab}	26.6 ± 0.7^{c}
Total	498.3 ± 26.5	593.0 ± 23.4	623.5 ± 6.2	244.9 ± 25.2	650.6 ± 51.8	448.4 ± 3.6

Means in the same row not connected by the same letter differ significantly (Tukey HSD test, $\alpha = 0.05$)

PDF: partially defatted *fines*, HA: Alcalase 2.4L hydrolysate, and HN: Neutrase 0.8L hydrolysate

Asx, no separate analysis of Asp/Asn; Glx, no separate analysis of Glu/Gln

References

1. Colgrave, M.L.; Allingham, P.G.; Jones, A. Hydroxyproline Quantification for the Estimation of Collagen in Tissue Using Multiple Reaction Monitoring Mass Spectrometry. *J. Chromatogr. A* **2008**, *1212*, 150–153, doi:10.1016/j.chroma.2008.10.011.
2. Dai, Z.; Wu, Z.; Jia, S.; Wu, G. Analysis of Amino Acid Composition in Proteins of Animal Tissues and Foods as Pre-Column o-Phthaldialdehyde Derivatives by HPLC with Fluorescence Detection. *J. Chromatogr. B* **2014**, *964*, 116–127, doi:10.1016/j.jchromb.2014.03.025.
3. Guo, S.; Duan, J.-A.; Qian, D.; Tang, Y.; Qian, Y.; Wu, D.; Su, S.; Shang, E. Rapid Determination of Amino Acids in Fruits of *Ziziphus Jujuba* by Hydrophilic Interaction Ultra-High-Performance Liquid Chromatography Coupled with Triple-Quadrupole Mass Spectrometry. *J. Agric. Food Chem.* **2013**, *61*, 2709–2719, doi:10.1021/jf305497r.