Enzymatic Saccharification of Pretreated Rice Straw and Biomass Production

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Accepted for publication November 25, 1985

A comparative study on the saccharification of pretreated rice straw was brought about by using cellulase enzyme produced by *Aspergillus terreus* ATCC 52430 and its mutant strain UNGI-40. The effect of enzyme and substrate concentrations on the saccharification rate at 24 and 48 were studied. A syrup with 7% sugar concentration was obtained with a 10% substrate concentration for the mutant case, whereas a syrup with 6.8% sugar concentration was obtained with 3.5 times concentrated enzyme from the wild strain. A high saccharification value was obtained with low substrate concentration; the higher the substrate concentration used, the lower the percent saccharification. The glucose content in the hydrolysate comprised 80–82% of total reducing sugars; the remainder was cellobiose and xylose together. The hydrolysate supported the growth of yeasts *Candida utilis* and *Saccharomyces cerevisiae* ATCC 52431. A biomass with a 48% protein content was obtained. The essential amino acid composition of yeast biomass was determined.

INTRODUCTION

Conversion of cellulosic wastes into utilizable materials, such as single cell protein (SCP), alcohol, and a number of other useful products, has gained much importance in recent years and opened new vistas in the fields of food, energy, and chemicals to augument and conserve current world energy sources.[1–3] Substantial studies have been done on utilizing cellulose either by hydrolyzing it chemically or enzymatically to reducing sugars. The hydrolysis of cellulosics by chemical methods, such as acids, has so far not been commercially feasible because the drastic conditions results in degradation of sugars to undesirable products.[4,5] As a result, enzymatic hydrolysis has received increasing attention. Although extensive studies have been done on bioconversion of cellulose, only a few investigations deal with the cultivation of noncellulolytic microorganisms, generally yeasts on enzymatic hydrolysate of cellulosics for SCP production.[6–8]

In the present investigation, comparative saccharification of agricultural wastes was brought about by enzymes produced by *A. terreus* ATCC 52430 and its mutant UNGI-40; the hydrolysate was utilized for the cultivation of yeast.

MATERIALS AND METHODS

Micoorganisms

The cellulolytic fungus *A. terreus* ATCC 52430[9] and its mutant UNGI-40 were used for cellulase production. The fungi were maintained on modified *Trichoderma viride* medium[10] with 2% agar and Whatman filter paper No. 1 as the sole source of carbon while the yeasts *Candida utilis* and *Saccharomyces cerevisiae* ATCC 52431[11] were maintained on Wickerham's medium.[12]

Mutant Isolation

The mutant UNGI-40 was isolated by a combined UV-irradiation and nitrosoguanidine (NTG) treatment of spores of the wild strain. *A. terreus* ATCC 52430. Five milliliters of spore suspension (spore count of 10^6 mL) was exposed to ultraviolet light in a sterilized Petri dish for 60 s at a height of 30 cm from the source of irradiation. The treated spores were plated using a 10-fold serial dilution on modified *Trichoderma viride* medium with 2% agar, 0.5% Walseth cellulose, and 5% glycerol. The seeded agar plates were incubated at 30°C for 2.5 days. The plates with grown colonies were further incubated at 50°C for 18–20 h to accelerate the action of extracellular cellulases and thus rapidly develop clearing zones around the cellulose producing colonies. The colonies with the greatest diameter of clear zone were isolated and tested for cellulase production in shake flask. One mutant strain showed high cellulase activity. It was designated as UV3-69, and

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CCC 0006-3592/86/101503-07$04.00

was further mutated with NTG in the concentration of 500 μg/mL spore suspension. The method of application was similar to method used by Adelberg and coworkers.[13] The treated spores were plated on Walseth cellulose agar and mutants were picked up by adopting the plate-clearing assay method of Montenecourt and Eveleigh[14] described above; mutant UNGI-40 was selected for further studies.

Enzyme Source

The cellulases were produced in 1 L broth culture by growing the above mentioned organisms on 1% microcrystalline cellulose powder in modified *Trichoderma viride* medium with 0.01 mL Tween 80. The media were sterilized by autoclaving at 15 lb for 15 min. The pH of medium was found to be 5.5. The flasks were then inoculated with 5 mL spores [(3–3.5)] $\times$ 10^8 mL] of *A. terreus* ATCC 52430 or its mutant. The seeded flasks were incubated at 28°C on a rotary shaker running at 180 strokes/min. At the end of fifth day the culture fluid was separated from mycelia through cheesecloth; the crude enzyme thus obtained was centrifuged at 6000 *g* at 30°C for 20 min. The cellulase activity of the centrifuged broth was determined by carrying out the cellulase assays. The protein content of the filtrate was determined using the method of Lowry et al.[15] with crystalline bovine serum albumin as a reference standard. The enzyme obtained from wild strain was further concentrated to about fourfold in a rotary evaporator at 40°C.

Measurement of Cellulase Activity

Enzyme activity was measured by filter-paper activity; CM–cellulase activity was measured as described by Mandels and co-workers,[16] and β-glucosidase was measured according to Andreotti and co-workers.[17] Filter-paper activity (FPA) was carried out as follows: 50 mg Whatman No. 1 filter paper cut into strip (1 $\times$ 6 cm), 1 mL of 0.2*M* acetate buffer, pH 5.6, 0.5 mL distilled water, and 0.5 mL enzyme solution were placed in a test tube and incubated at 50°C in a shaker water bath for 60 min.

CM-Cellulase Activity

The assay system consisted of 5 mg carboxy-methyl cellulose in 1 mL of 0.2*M* acetate buffer, pH 5.6, and 0.5 mL enzyme solution. The reaction mixture was incubated at 50°C for 30 min.

β-Glucosidase Activity

Five milligrams salicin in 1 mL acetate buffer, 0.2*M*, pH 5.6, and 0.5 mL enzyme filtrate was incubated at 50°C for 30 min. The reducing sugars formed in all the above assays were measured by the 2,4-dinitrosalicylic acid method.[18] One unit of FPA, CM-cellulase, and β-glucosidase activity equals to 1 μmol glucose produced/min under the test conditions.

Saccharification of Rice Straw

The dried rice straw was reduced to a size of ca. 3 cm by chopping and then pulverised in a hammermill to a 10–20-mesh size. It was then delignified by boiling with 1% sodium hydroxide solution in the liquor ratio of 1:6 for 1 h. The treated straw was expressed free of alkali, thoroughly washed with water and sun dried.

Preliminary experiments on hydrolysis of treated rice straw were carried out to study the effect of enzyme and substrate concentration on the saccharification rate at 24 and 48 h. Treated rice straw (125 mg, 250 mg, 375, and 500 mg) was taken in test tubes and 2.5 mL 0.2*M* acetate buffer, pH 5.6, was added to each tube. Three sets were prepared and different amounts of enzymes were added: 0.5 mL to the first set, 1 mL to the second, and 1.5 mL to the third. The hydrolysis was carried out at 45°C for 48 h with concentrated enzyme of *A. terreus* ATCC 52430 and the unconcentrated enzyme from mutant UNG1-40. The former was used at activities of 0.98, 1.13, and 18.55 U/mL of filter paper, β-glucosidase, and CM–cullulase activities, while the later enzyme was at 0.97, 1.53, and 17.5 U/mL of filter paper, β-glucosidase, and CM–cullulase activities, respectively. The amount of reducing sugars formed were estimated by the DNS method[18] at the end of 24 and 48 h.

Subsequently, the hydrolysis of treated rice straw was carried out in 250-mL Erlenmeyer flasks with a substrate concentration at 5 and 10% (w/v) with 5 mL enzyme from mutant UNG1-40 and concentrated enzyme from *A. terreus* ATCC 52430 in a 50-mL volume on a shaker water bath at 50°C. The amount of reducing sugars and glucose were estimated at different time intervals up to 24 h. Percent saccharification was found out by using the formula adopted by Mandels and Sternberg.[19] For calculating the saccharification value of various cellulosic substrates,

$$\text{Percent saccharification} = \frac{\text{glucose (mg/mL) (0.9)}}{\text{substrate (mg/mL)}} \times 100$$

Biomass Production

The syrup obtained was separated and the amount of glucose was estimated by the glucose–oxidase method described by Dalquist,[20] using a modified Tris–glucose reagent. Furthermore, the syrup was concentrated and added to carbon-free Czapek–Dox medium in a 250-mL Erlenmeyer flask where the sugar concentration was adjusted to 3% with the enzymatic hydrolysate. It was supplemented with 0.1% peptone and traces of

Table I. Cellulase production by *A. terreus* ATCC 52430 and its mutant UNGI-40 on cellulose in shake flask.

Organism	Protein (mg/mL)	β-glucosidase activity (U/mg)	Specific activity (U/mg)	Filter paper activity (U/mL)	Specific activity (U/mg)	CM–cellulase activity (U/mL)	Specific activity (U/mg)
A. terreus ATCC 52430	0.45	0.33	0.73	0.28	0.62	5.30	11.77
Mutant UNGI-40	0.64	1.53	2.39	0.97	1.51	17.0	26.56

malt extract. The medium was sterilized at 10 lb for 15 min and inoculated with yeast cells, *Candida utilis*, and *Saccharomyces cerevisiae* ATCC 52431 for the production of biomass. Five milliliters yeast suspension (yeast count of 10^6/mL) was added to 95 mL of the above medium in each flask and incubated at 30°C for two days on a rotary shaker (180 rpm). At the end of incubation the cell mass was separated by centrifugation, and the unutilized sugar concentration in the centrifuged broth was determined. The cell mass was washed with water and dried at 80°C for 8–10 h. The nitrogen present in the cell mass was estimated by a modification of the Kjeldahl–Nesslerization method. The amount of protein was then calculated by multiplying the nitrogen value by 6.25.

Determination of Amino Acid Content of Yeast Biomass

The essential amino acid content of the biomass was found in a automatic amino acid analyzer, a LKB alpha Biochrome 4150 model, according to the procedure described by Moore.[21] In this method, a sample containing 10 mg protein (N × 6.25) was hydrolyzed with 10 mL of 6*N* HCl containing 0.01% phenol and 0.11% β-mercaptoethanol at 110 ± 1°C for 24 h in evacuated tubes. The acid was evaporated under reduced pressure after hydrolysis in a rotary evaporator. The residue was dissolved in 0.2*M* sodium citrate buffer, pH 2.2, and amino acid analysis was carried out with a buffer sequence of pH 3.20, 4.25, and 6.45. Cochromatography of standard amino acids was also carried out.

RESULTS AND DISCUSSION

The results of the enzyme yield and activities of the wild strain *A. terreus* ATCC 52430 and its mutant UNG1-40 is summarized in Table I. It was observed that the mutant UNG1-40 showed a 4.6-fold increase in β-glucosidase activity, and 3.46- and 3.3-fold increases in filter-paper activity and CM–cellulase activity, respectively, compared to the wild strain. Furthermore, it was also noted that the activities of β-glucosidase, filter paper, and CM–cellulase were not increased proportionately in the mutant strain. This might be due to the fact that the genes or genomes controlling the production of cellulase complex is not under coordinate control in this organism as in case of mutants of *T. viride*[22] and *Sclerotium rolfsii*.[23]

In the preliminary studies on saccharification it was observed that 1.66–7.1% sugar solution was obtained

Table II. Effect of concentrated enzyme from *A. terreus* ATCC 52430 and substrate concentration on hydrolysis of treated straw.

Substrate concentration	Concentrated enzyme volume (mL)	Temperature (°C)	Reducing sugars at 24 h (mg/mL)	Reducing sugars at 48 h (mg/mL)	Saccharification at 24 h (%)	Saccharification at 48 h (%)
2.5	0.5	45	17	19.4	61.2	69.8
5.0	0.5	45	24.5	28	44.1	50.4
7.5	0.5	45	31.5	36	37.8	43.2
10.0	0.5	45	36	42	32.4	37.8
2.5	1.0	45	23.5	23.5	84.24	84.6
5.0	1.0	45	41.6	42	74.6	75.6
7.5	1.0	45	57.5	62	69	74.4
10.0	1.0	45	61	66.2	54.9	59.58
2.5	1.5	45	23.8	24	85.6	86.4
5.0	1.5	45	42.6	42	76.6	75.6
7.5	1.5	45	61.2	64.6	75.8	80.16
10.0	1.5	45	70	75	63	67.5

Note: percent saccharification = [glucose (mg/mL) × 0.9/substrate (mg/mL)] × 100.

Table III. Effect of enzyme from UNG1-40 and substrate concentration on hydrolysis of treated straw.

Substrate concentration (%)	Enzyme volume (mL)	Temperature (°C)	Reducing sugars at 24 h (mg/mL)	Reducing sugars at 48 h (mg/mL)	Saccharification at 24 h (%)	Saccharification at 48 h (%)
2.5	0.5	45	16	19	59.7	68.4
5.0	0.5	45	25	29.5	45	53.1
7.5	0.5	45	34	37	40.8	44.4
10.0	0.5	45	38	42	34.2	37.8
2.5	1.0	45	21.4	23	77	87.8
5.0	1.0	45	42	43	75.86	77.4
7.5	1.0	45	55.48	61.8	66.9	74.1
10.0	1.0	45	61	67	54.9	60.3
2.5	1.5	45	23	23.2	82.8	83.5
5.0	1.5	45	43.4	43	78.1	77.4
7.5	1.5	45	62.2	65.6	74.6	78.7
10.0	1.5	45	71	76.2	63.9	68.4

after 24 h hydrolysis at 45°C, while at 48 h, 1.9–7.6% sugar was obtained, as can be seen from the results presented in Tables II and III. As the substrate concentration is increased, the amount of sugars formed increases but the percent conversion reduces. Similarly, as the enzyme concentration is increased, saccharification increases but the amount of sugar produced is less per unit enzyme. Ten-percent substrate concentration and 1 mL enzyme from culture filtrate of mutant UNG1-40 gave 6.1% sugar in 24 h hydrolysis, and 6.7% sugar in 48 h, while concentrated enzyme from *Aspergillus terreus* ATCC 52430 produced 6.1 and 6.62% in 24 and 48 h, respectively. From the results, it was observed that the rate of hydrolysis decreases considerably after 24 h. The drastic reduction in the hydrolysis rate was not due to the lack of substrate but rather from other causes, such as the transformation of insoluble cellulose into a less accessible

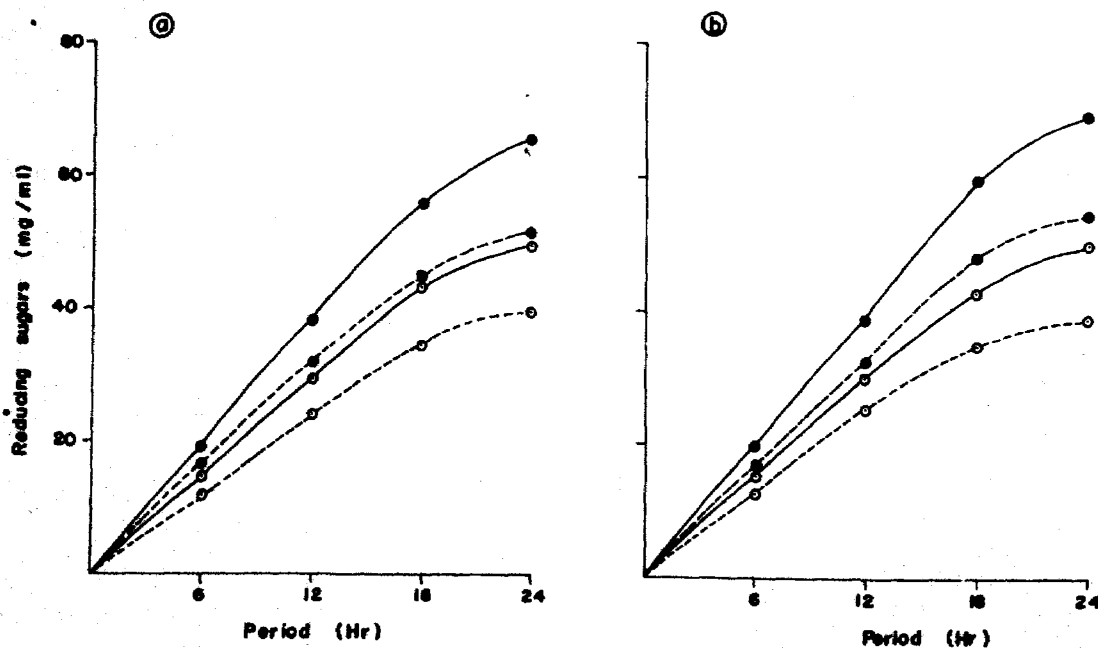

Figure 1. Hydrolysis of treated rice straw by (a) concentrated enzyme from *A. terreus* ATCC 52430 and (b) enzyme from UNG1-40: (⊙——⊙) reducing sugars with 5% substrate concentration, (●——●) reducing sugars with 10% substrate concentration, (⊙——⊙) Glucose with 5% substrate concentration, and (●——●) glucose with 10% substrate concentration.

form with enzyme action thereby changing structural features, such as an increase in crystallinity, as the crystalline regions of cellulose remain unattacked by the enzymes and reduction in surface area.[24] It was also observed that, as crystallinity increases, the cellulose becomes increasingly resistant to further hydrolysis.[25,26]

Thus, it is evident from the results in Figure 1 that the reducing sugar concentration increases rapidly in the early stages but the rate of this increase was substantially reduced at later stages. Hydrolysis almost ceases even when a substantial amount of undigested or residual cellulose still remains in cellulose suspension. A syrup with 7% sugar concentration was obtained with a 10% substrate concentration and 5 mL enzyme from mutant UNG1-40 in a 50-mL hydrolysis volume in 24 h of subsidence; with the concentrated enzyme from parent strain *A. terreus* ATCC 52430, a syrup with 6.8% sugar concentration was obtained. Similarly 5–10% sugar was obtained using various cellulosic wastes in 24 h with cellulase from *Trichoderma viride*.[27] Toyama and Ogawa[28] also obtained a sugar solution of ca. 6–10% in 48 h by using a commercial cellulase preparation. During the enzymatic hydrolysis of pretreated sawdust, a sugar concentration of 6.5–7.5% was obtained after 24 h hydrolysis of 15% suspension of saw dust with cellulase from *T. koninghii*.[29] Furthermore, they also observed that the rate of hydrolysis was highest during the initial 12 h and decreased thereafter. Likewise, in the present studies, it was also observed that the rate of hydrolysis was highest during the initial 18 h and then, subsequently, there was gradual increase in sugar concentration from 6.2 to 7% with enzyme from mutant UNG1-40 and from 5.65 to 6.8% with the enzyme from *A. terreus* ATCC 52430. Thus, it was concluded from the results discussed above that the enzyme from the mutant could hydrolyze rice straw and produce a syrup with about the same concentration of reducing sugars as compared to hydrolysis with 3.5 times concentrated enzyme from *A. terreus* ATCC 52430. The glucose content alone in the hydrolysate comprised 80–82% total reducing sugars and the remainder, cellobiose and xylose together. The glucose concentration was more in the hydrolysate due to the high amount of β-glucosidase in the hydrolysis system. A similar observation was made with cellulase from *Pestalotiopsis versicolor* which had a high amount of β-glucosidase compared to other cellulase components.[30] Figure 2 shows the percent saccharification of treated straw with enzyme from parent strain and mutant. It was observed that the saccharification value varied inversely with the substrate concentration. The results are in accordance with Mandels and Sternberg,[19] who also obtained high saccharification values with low substrate concentrations after carrying hydrolysis studies with a variety of substrates, using cellulase from *T. viride* mutant. In the present studies, ca. 90% saccharification was obtained with 5% substrate concentration, whereas ca. 62% saccharification was obtained with 10% substrate concentration in 24 h hydrolysis time. During the enhanced enzymatic hydrolysis by simultaneous attrition with 2% substrate

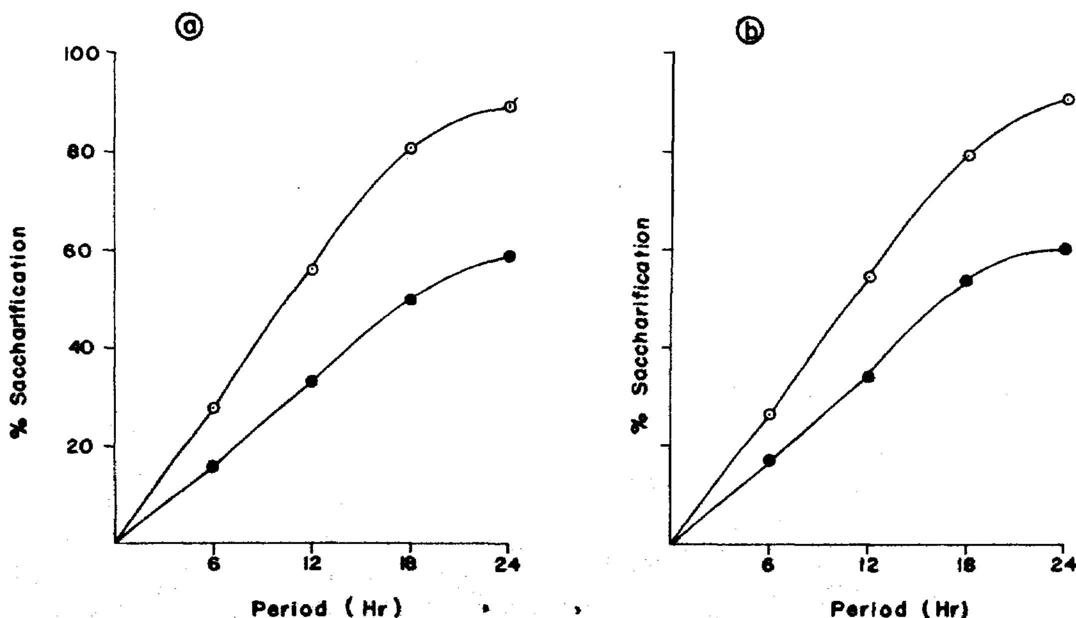

Figure 2. Saccharification of treated rice straw by (a) concentrated enzyme from *A. terreus* ATCC 52430 and (b) by enzyme from UNG1-40: (○———○) with 5% substrate concentration and (●———●) with 10% substrate concentration.

Table IV. Growth of yeasts on hydrolysate of rice straw.

Microorganism	Dry wt cells (g/L)	Concentration of unutilized sugar (g/L)	Protein content, Nx 6.25 (%)
Candida utilis	6.56	2.820	48.1
Saccharomyces cerevisiae ATCC 52431	6.48	2.950	47.97

Yeasts were cultivated in the enzymatic hydrolysate with reducing sugars adjusted to 3% glucose equivalent in carbon-free Czapek-Dox medium. The medium was supplemented with 0.1% peptone and a trace of malt extract as growth factors.

concentration, 89% saccharification was observed after 6 h with enzyme from *T. viride*; 4% substrate concentration gave only 50% conversion.[31] Thus, it was observed that the low saccharification value with the high substrate concentration was due to a higher concentration of sugars to bring about deactivation of enzyme cellulase, minimizing the hydrolysis rate as compared to low substrate concentrations, since sugars are known to be inhibitors of cellulases.

Hydrolysate proved to be a suitable substrate for the cultivation of yeasts *Candida utilis* and *Saccharomyces cerevisiae* ATCC 52431 as 6 g/L dry yeast cell mass was obtained in 48 h (Table IV). The yeast biomass had a protein content of ca. 48%. The amino acid composition of a protein primarily determines its potential nutritional value. Table V presents a profile of the essential amino acid composition of yeast biomass of *C. utilis* used in the present studies, along with other protein products. Table V clearly reveals that the protein nutritional value of *C. utilis* grown on hydrolysate of rice straw is comparable in quality to traditional fodder yeast, soya bean protein concentrate, and the FAO reference. Nelson et al.,[32] after determining the amino acid content of various yeast strains, found the average lysine content to be 7.4 and the average methionine content to be 1.2; hence, the results obtained with *C. utilis* are consistent with this pattern. However, the yeast protein falls short of the FAO reference for sulphur-containing amino acids, although this seems to be a general case with all yeast strains. A similar ob-

Table V. Amino acid composition of yeast protein isolate and other protein products.

Essential amino acid	Composition (g/16 g N)			
	Yeast protein from test isolate *C. utilis*	FAO[a]	Soy bean protein concentrate[b]	Fodder yeast (traditional)[c]
Isoleucine	4.23	4.2	4.9	5.3
Leucine	6.49	4.8	8.0	7.0
Lysine	5.86	4.2	6.6	6.7
Methionine	2.02	2.2	1.3	1.9
Cystine	0.71	2.0	1.6	—
Phenylalamine	3.71	2.8	5.3	4.3
Threonine	5.31	2.8	4.3	5.5
Valine	4.94	4.2	5.0	6.3
Tryptophan	—	1.4	1.4	1.2
Aspartic acid	9.12			
Serene	4.80			
Glutamic acid	12.07			
Proline	5.00			
Glycine	3.47			
Alanine	5.39			
Tyrosine	4.70			
Histidine	1.99			
Arginine	3.16			
Ammonia	0.57			

[a] This is the Food and Agricultural Organization of the United Nations (1957).
[b] Data are from ref. 35.
[c] Data are from ref. 34.

servation was made with *Candida* protein—not only with yeasts but with fungal protein, when the sulphur-containing amino acids were less.[33,34] Hence, the yeast biomass shows great promise for use because of a relatively high concentration of essential amino acids; it could be of potential use as a protein supplement, in particular, to increase the protein quality of animal feed.

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