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Effect of Treating *Candida utilis* with Acid or Alkali, To Remove Nucleic Acids, on the Quality of the Protein

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Dried *Candida utilis* cells were variously extracted with acidic and alkaline solutions to maximize the removal of nucleic acids but to minimize the damage to proteins. Response surface methodology procedures were used to examine effects of yeast concentration, time, temperature, and pH on protein yield, nucleic acid removal, available lysine content, lysinoalanine formation, racemization, and amino acid destruction. At increased cell concentrations, time, temperature, and pH, alkaline extractions were more efficient in removing nucleic acids but caused decreased availability of lysine, amino acid destruction, increased racemization, and formation of lysinoalanine. A 0% nucleic acid content, predicted for an alkaline isolate, would cause extensive damage to proteins. However, an isolate was obtained that could be consumed by an adult to essentially meet the daily recommended protein requirement without risking the danger of elevated uric acid levels. Low available lysine was the most serious detectable defect in the acid-extracted products.

At present single-cell protein (SCP) is mainly used for animal feed. The economics of yeast as a source of human protein have been discussed (Cartwright, 1958), but the use of yeast proteins in human food on a large scale has been limited by its high nucleic acid (NA) content. Nucleic acid is metabolized into uric acid, but man, unlike the lower forms of life, has no uricase enzyme to convert uric acid into allantoin which is soluble and easily excreted. Thus, high levels of uric acid can crystallize in tissues and organs to cause gout, stones in the urinary tract, and tophi in the soft tissues. Daily intake of NA should not exceed 2 g because higher quantities increase the uric acid content of blood plasma to abnormal levels (Edozien et al., 1970).

Rogozhin et al. (1970) used various concentrations of sodium hydroxide to extract protein from disintegrated yeast cells to obtain a product that was 96% protein and contained less than 2% NA. Ayukawa (1971) heated SCP in anhydrous liquid ammonia to obtain a product with increased protein content, improved odor, color, taste, and texture, and decreased NA content. Hedenskog and Ebbinghaus (1972) used alkaline conditions to extract proteins from SCP and reduced their NA content to 1–2% but observed that the availability of lysine was reduced after extraction at pH 12. Vananuvat and Kinsella (1975) homogenized a cell suspension in sodium hydroxide solution in the presence of glass beads to reduce the NA content of the cells to less than 5%. Zee and Simard (1975) reduced the NA content of *Rhodotorula glutinis* from 6.5 to 1.5% by heating the cells at 100 °C for 40 min at pH 11.0. Viikari and Linko (1977) reported that the NA content of *Paecilomyces varioti* was easily reduced from the initial 9% to less than 2% with diluted sodium hydroxide solutions. Newell et al. (1975) developed processes to prepare isolated protein products from yeast with reduced NA content. Sinskey and Tannenbaum (1975) have reviewed some methods for reducing the NA content of SCP using exogenous and endogenous enzymes.

Tumura et al. (1972) dehydrated wet yeast cells with methanol and esterified the proteins with methanolic hydrochloric acid and observed that the RNA was almost completely removed by the esterification process. Akin and Chao (1972) reduced the NA content of *Candida utilis* cells from 10.5 to 3.3% by treating the cells with phos-

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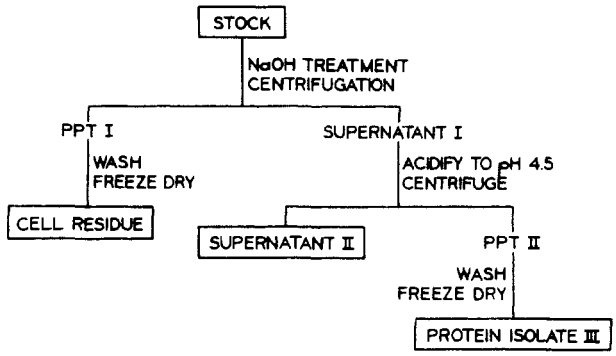

Figure 1. Scheme for NaOH treatments of disintegrated yeast cells.

phoric acid at pH 1.9 for 10 min at 121 °C. Also, Akin and Chao (1974) heated *C. utilis* cells with aqueous ethanolic mineral acids such as hydrochloric, phosphoric, or sulfuric acids to reduce the NA content of the SCP to less than 2%. Zee and Simard (1975) reduced the NA content of yeast cells to 1.2% by heating them at 90 °C and pH 2 in hydrochloric acid.

Losses of amino acids have been reported on alkali treatment of proteins (Whiting, 1971; Provansal et al., 1975). Some of these losses have resulted from the formation of new and unusual amino acids such as lysinoalanine (LAL) (Whiting, 1971; Sternberg et al., 1975); lanthionine (Watanabe and Klostermeyer, 1977), and ornithinoalanine (Asquith et al., 1969). Extremes in pH, acidic or alkaline, can lead to racemization in amino acids, destroying their biological activity (Tannenbaum et al., 1970).

It was the purpose of this study to establish optimum conditions for maximum removal of NA from SCP, using alkali or acid solutions, with minimum damage to the proteins.

MATERIALS AND METHODS

Spray-dried *C. utilis* type B, produced on sulfite waste liquors, was purchased from Lake State Division, St. Regis Paper Co.

Experimental Design. The design was based on response surface methodology procedures (Davies, 1956). A "cube plus star plus five center points" rotatable composite design was used to examine the four predictor variables (factors), namely, yeast concentration, time, temperature, and pH of treatments. There were a total of 29 treatment combinations of the variables consisting of 16 factorial, 5 center, and 8 axial points. The basic method of analysis was to fit, to the response data obtained, a polynominal of first or second order (whichever was adequate). The fitted equations were obtained by the method of least squares, and the usual tests of lack of fit and significance of the regression were carried out by using the method of Draper and Smith (1966). Canonical analysis was done on the second-order fitted surfaces to aid in their practical interpretations.

Disruption of Yeast Cell Wall. Whole dried cells of *C. utilis*, 9.6% (w/v), were suspended in water and disintegrated by using glass beads in an Eppenback Colloid Mill, under the following conditions: volume of glass beads = 600 mL/L; gap setting of the Colloid Mill = 0.030 in.; rotations per minute (rpm) = 8500; temperature = 3-5 °C; time = 30 min.

Approximately 90% disintegration of the cells was achieved as determined by the Gram stain method (Nickerson, 1963). The disintegrated yeast suspension was decanted, was freeze-dried, and constituted the stock material for the acid and alkaline treatments.

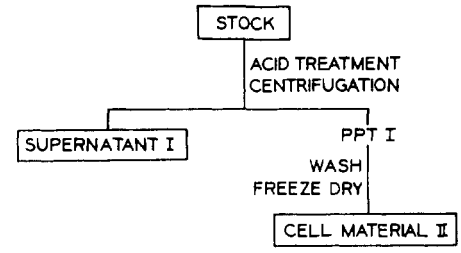

Figure 2. Scheme for acid treatment of disintegrated yeast cells.

Alkali and Acid Treatments of Stock Material. The schemes used are shown in Figures 1 and 2, respectively. Centrifugation was at 3000*g* for 40 min.

Yield. Protein isolate III (Figure 1) and cell material (Figure 2) were weighed on a torsion balance to determine the dry weight yield.

Total Nitrogen. This was determined by the Kjeldahl method (AOAC, 1975). The percentage of total or Kjeldahl protein was obtained by multiplying the percentage of total nitrogen by 6.25.

Measurement of Nucleic Acid. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were determined by pentose analysis using the orcinol (Aldrich) and diphenylalanine (Aldrich) reactions (Munro and Fleck, 1966) after the extraction of phosphorous compounds with trichloroacetic acid and of lipids with ethanol (Schmidt and Thannhauser, 1945).

Measurement of Available Lysine. The reagent 1-fluoro-2,4-dinitrobenzene (FDNB) (Eastman) was used to react with the free ϵ-amino group of lysine according to the method of Holm (1971). ϵ-DNP-lysine was measured colorimetrically in a Beckman spectrophotometer at 435 nm using pure N^{ϵ}-DNP-L-lysine as a standard.

Racemization. The method of Larson et al. (1971) was used to determine the extent of racemization in acid- or alkali-treated material. After incubation, sulfosalicylic acid (3.5%) (Fisher) (Chen et al., 1975) was used to precipitate the D-amino acid oxidase and catalase (Worthington).

Amino Acid Composition. Samples were hydrolyzed with 6 N HCl (5 mg of sample to 1 mL of acid) (Savoy et al., 1975) at 110 °C for 22 h in flasks closed with a ground glass stopper fitted with an acid resistant o-ring and Teflon stopcock (Stahmann and Woldegiorgis, 1975). Amino acids and lysinoalanine were determined with a Beckman Model 118B automatic amino acid analyzer (Moore et al., 1958). Tryptophan was determined by the method of Gaitonde and Dovey (1970). Cystine was estimated by first reducing it to cysteine with aqueous dithiothreitol solution (Cleland, 1964). The resulting cysteine was measured by the method of Gaitonde (1967).

RESULTS AND DISCUSSION

Yield of Dry Material. Alkaline extraction resulted in the solubilization of protein from the disrupted cells, thus yielding a protein isolate from the solubilized material, whereas the acidic extraction did not solubilize much protein and left a residue containing substantial quantities of cellular debris. Table I gives data on the yield of isolates from alkaline extraction resulting from selected treatments of disrupted *C. utilis* cells. The entire experiment consists of 29 experimental "runs" or "trials", numbered sequentially in the first column. The second to sixth columns show the experimental conditions for each run. Tables II–VI are all results from identically corresponding experimental conditions and, for that reason, only the response measurements are shown, along with the sequential numbering for easy identification. All response data are averages of two determinations. These two are not true

Table I. Yield of Isolates from Alkaline Extraction or of Dry Cell Material from Acidic Extraction Resulting from Various Treatments of Dried *C. utilis* Cells

	experimental conditions					response values			
				pH		yield, % dry wt (w/w)			
trial no.	cell concn, % w/v	time, min	temp, °C	NaOH	acid	NaOH[a]	HCl[b]	PCA[c]	TCA[d]
1	6	20	40	9	2	0.7	76.7	82.0	81.7
2	6	20	40	11	4	10.8	76.7	79.3	75.0
3	10	20	40	9	2	5.2	75.2	83.2	79.6
4	10	20	40	11	4	7.4	77.2	76.4	76.2
5	6	40	40	9	2	1.5	76.0	80.7	77.3
6	6	40	40	11	4	11.3	76.0	77.3	78.7
7	10	40	40	9	2	2.4	77.6	81.6	80.4
8	10	40	40	11	4	23.0	76.0	77.2	74.4
9	6	20	80	9	2	5.0	74.0	82.7	80.0
10	6	20	80	11	4	37.7	78.0	79.3	76.3
11	10	20	80	9	2	4.8	74.8	82.8	80.6
12	10	20	80	11	4	35.4	74.4	78.8	77.4
13	6	40	80	9	2	6.4	76.0	78.7	79.0
14	6	40	80	11	4	37.7	71.3	80.7	76.7
15	10	40	80	9	2	4.6	73.6	80.8	80.4
16	10	40	80	11	4	36.0	73.2	78.0	77.4
17	8	30	60	10	3	10.8	74.5	81.0	76.0
18	8	30	60	10	3	11.3	76.0	79.5	75.0
19	8	30	60	10	3	15.8	74.0	83.5	76.8
20	8	30	60	10	3	14.5	72.0	81.0	76.0
21	8	30	60	10	3	11.0	75.5	78.5	78.5
22	8	30	20	10	3	1.8	75.5	79.0	81.5
23	8	30	100	10	3	22.5	70.5	76.5	77.3
24	8	10	60	10	3	4.4	76.0	83.0	77.8
25	8	50	60	10	3	13.5	73.5	83.0	77.8
26	4	30	60	10	3	9.0	74.0	83.0	76.5
27	12	30	60	10	3	10.8	74.7	83.3	82.6
28	8	30	60	8	1	1.3	76.0	84.5	78.3
29	8	30	60	12	5	37.3	71.1	72.5	74.8

[a] Sodium hydroxide. [b] Hydrochloric acid. [c] Perchloric acid. [d] Trichloroacetic acid.

repeat runs, cannot be used for pure error purposes (Draper and Smith, 1966, p 29), and so are not shown individually. From a statistical point of view, the experiment is a "cube" (a 2^4 factorial design, trials 1–16) plus a "star" (eight "axial points", trials 22–29) plus five "center points" (trials 17–21) which permit estimation of the pure error. This "design" is a standard and excellent type of experimental arrangement for determining the shape of a response surface. For statistical details see, for example, Myers (1971), especially Chapters 7 and 8.

The standard analysis of such data involves fitting a planar or quadratic response function, as needed by the data, and predicting on the basis of this fitted function. Techniques of canonical reduction (Myers, 1971, Chapter 5) and/or ridge analysis (Draper, 1963) enable identification of regions of the space of experimental conditions where desirable responses exist, and the conclusions reached below are based, in part, on the interpretation of such analyses.

The Table I data indicate that an increase in yeast concentration, time, temperature, and pH of the alkaline treatments tended to increase yields of protein isolates and that the optimum conditions for maximizing the yield of protein isolate were to heat a 4% (w/v) suspension of disrupted *C. utilis* cells in NaOH solution for 31.9 min at 81.9 °C and pH 11.7. The predicted yield of protein isolate there is 51%.

Although the isolates from the alkaline extractions were mainly proteinaceous, their actual amount (quantities) of protein were lower than for the cellular residue after acid treatments. The values in Table II were obtained by multiplying values of dry materials from acid and alkaline treatments by their respective Kjeldahl protein values and dividing by 100. The differences in the total protein contents within the acid treatments were slight, but the values for HCl were consistently lower. Possibly, these lower values reflect better extraction of NA nitrogen with this acid compared with PCA and TCA. This reasoning is supported by the lower values for NA content obtained after HCl treatments. From a nutritional standpoint, it is likely that the alkaline isolates would be more readily digested compared to the acid-treated products containing cellular debris. It remains to be seen how the biological values of the two protein products compare.

Nucleic Acid Content. The nucleic acid content of isolates obtained by extracting disrupted *C. utilis* cells with NaOH solution ranged from 0.43 to 11.97%, whereas those for the acid-treated residues varied from 2.37 to 6.90% (Table III). The nucleic acid content of untreated disrupted *C. utilis* cells was 12.10%. The more severe alkaline treatments yielded products with the lowest NA content. Among the acids, HCl was most effective in reducing the NA content of the cells. For the acid treatment, heating low yeast concentrations at low pH and elevated time and temperature resulted in lower NA content. The predicted conditions for maximum removal of NA from alkaline protein isolate was to heat an 8.3% (w/v) disrupted cell in NaOH solution at 92.6 °C and pH 11 for 32.3 min. Under these conditions, the residual NA content would be zero, but from consideration of subsequent data on damage to proteins as a result of alkaline extractions, it would seem that the aforementioned conditions to effect complete removal of NA would be too damaging to the proteins.

Availability of Lysine. The data in Table IV present the availability of lysine (not the absolute lysine content) in the isolates after treatments. It is expressed as percent (weight/weight). For example, if lysine in yeast is a% available and if after either acid or alkaline treatment the lysine is b% available, the lysine is $(b/a \times 100)$% available. The acidic treatments seemed most detrimental to the

Table II. Yield of Crude Protein in the Isolates from Alkaline Extraction or in the Dry Well Cell Material from Acidic Extraction Resulting from Various Treatments of Dried *C. utilis* Cells

trial no.	yield of protein, % dry wt (w/w)			
	NaOH[a]	HCl[b]	PCA[c]	TCA[d]
1	0.4	30.1	32.4	33.4
2	6.1	30.7	31.8	31.1
3	3.0	28.6	32.3	31.8
4	4.2	30.1	30.8	31.2
5	0.8	29.3	34.1	32.6
6	6.5	30.0	31.2	31.8
7	1.4	28.4	32.3	33.4
8	13.0	30.1	31.4	31.4
9	2.9	28.2	31.7	32.3
10	21.5	30.8	31.4	30.9
11	2.7	28.6	31.6	32.0
12	19.3	29.7	32.2	32.4
13	3.5	28.7	30.2	32.1
14	20.5	28.0	32.5	32.2
15	2.6	27.7	31.6	33.0
16	19.8	28.5	32.1	32.9
17	6.2	27.9	31.1	31.5
18	6.4	30.0	33.0	30.9
19	9.4	27.2	33.5	31.6
20	8.2	26.9	32.6	31.7
21	6.3	29.2	31.9	31.2
22	0.8	30.1	30.4	33.7
23	12.6	26.7	28.8	30.8
24	2.4	29.3	33.4	31.5
25	7.7	27.3	33.9	32.1
26	4.8	28.3	33.4	31.9
27	6.3	28.6	32.5	33.9
28	0.8	27.8	30.8	30.3
29	20.3	29.2	28.9	31.9

[a] Sodium hydroxide. [b] Hydrochloric acid. [c] Perchloric acid. [d] Trichloroacetic acid.

Table III. Nucleic Acid Content of the Products from NaOH- and Acid-Treated *C. utilis* Cells

trial no.	nucleic acid content, % (w/w)			
	NaOH[a]	HCl[b]	PCA[c]	TCA[d]
1	6.98	4.09	4.80	5.74
2	4.70	4.32	6.43	6.34
3	9.81	4.10	5.48	6.29
4	4.89	4.66	6.73	6.88
5	6.84	3.64	4.17	5.68
6	4.43	4.20	6.15	6.31
7	8.84	3.87	5.12	5.76
8	4.82	4.44	6.26	6.34
9	5.82	3.53	4.17	5.46
10	1.50	3.97	4.79	5.71
11	7.64	3.73	4.25	5.70
12	1.62	4.18	5.08	5.98
13	5.53	3.37	2.73	5.19
14	0.93	3.90	4.62	5.65
15	5.94	3.57	4.51	5.44
16	0.49	4.12	4.67	5.82
17	5.75	3.65	3.94	5.60
18	4.77	3.67	4.37	5.33
19	4.69	3.43	3.83	5.64
20	5.34	3.86	5.21	5.34
21	4.84	3.87	4.50	5.85
22	3.21	4.48	4.88	6.38
23	1.14	3.43	2.95	4.85
24	6.78	4.00	4.75	6.30
25	4.38	3.40	4.31	6.02
26	3.09	3.35	3.69	5.88
27	5.85	3.46	5.66	6.73
28	11.97	2.37	2.74	3.80
29	0.43	5.06	6.88	6.90

[a] Sodium hydroxide. [b] Hydrochloric acid. [c] Perchloric acid. [d] Trichloroacetic acid.

lysine content of the products (Table IV). Possibly, acidic treatments catalyzed carbonyl–amine reactions involving the ϵ-amino group of lysine resulting in decreased availability of lysine. Increase in time, temperature, and pH of treatment resulted in decreased available lysine in the isolates from alkali-treated material. For the acid treatments, increased time and temperature but decreased pH seemed to decrease the availability of lysine. The best conditions for maximum retention of available lysine were to heat an 8.4% (w/v) cell in NaOH solution at 58.3 °C and pH 8.6 for 15.2 min. Under these conditions, the lysine would be 104.4% available; however, it is likely that insufficient NA would be removed from the cells.

Table IV. Availability of Lysine in the Products from Alkali- and Acid-Treated *C. utilis* Cells

trial no.	available lysine, % (w/w)			
	NaOH[a]	HCl[b]	PCA[c]	TCA[d]
1	100.0	82.9	76.9	68.8
2	87.5	93.6	72.7	61.8
3	100.0	77.6	72.7	70.1
4	80.4	82.9	79.7	84.1
5	100.0	80.9	64.0	70.1
6	80.1	86.3	65.2	84.8
7	100.0	78.9	72.2	68.8
8	79.8	88.3	74.5	77.8
9	100.0	80.7	71.0	52.7
10	79.7	80.9	62.9	65.0
11	97.6	80.3	69.3	66.3
12	79.0	71.6	74.5	74.6
13	94.7	70.9	68.7	71.4
14	71.7	85.6	61.8	73.9
15	89.0	74.9	61.1	61.2
16	68.9	75.3	65.2	72.1
17	96.1	84.3	74.5	80.3
18	96.4	85.6	76.9	76.5
19	91.8	82.9	69.9	80.3
20	91.8	78.3	74.5	65.0
21	91.1	80.3	75.7	71.0
22	91.8	94.3	78.0	78.4
23	73.8	74.4	70.8	65.5
24	100.0	80.3	76.9	72.7
25	89.0	80.3	72.4	67.0
26	89.7	80.3	66.4	79.0
27	90.8	77.6	77.4	71.4
28	97.3	68.1	56.4	60.2
29	68.2	102.0	84.4	87.3

[a] Sodium hydroxide. [b] Hydrochloric acid. [c] Perchloric acid. [d] Trichloroacetic acid.

Racemization of Amino Acids. The extent of racemization increased as the time, temperature, and pH of the alkaline treatments were increased (Table V). The extent of racemization of lysine, a limiting amino acid in many cereals, could not be estimated by the method used because D-lysine is not destroyed by D-amino acid oxidase (Larson et al., 1971). Hydrochloric acid caused negligible racemization, while TCA and PCA were most detrimental in causing racemization, especially at low pH values.

Lysinoalanine (LAL) Formation. Heating disrupted yeast cells at high temperatures and pH resulted in the formation of LAL. The treatment combinations that were most effective in reducing the NA content of the cells were also those that resulted in the formation of large quantities of LAL (Table VI). These trial combinations also gave the highest values for racemization in amino acids. Heating a 4% yeast suspension in sodium hydroxide solution at 60 °C and pH 10 for 30 min gave a product in which no LAL was detectable and the NA content was low (3.09%). Even under these conditions the extent of racemization in amino acids was high. It thus was not easy to achieve a substantial reduction in the NA content of alkaline-extracted protein isolate from yeast cells without causing the formation of LAL and giving rise to racemization of the amino acids.

Table V. Racemization of Amino Acids in the Product from Sodium Hydroxide Treated *C. utilis* Cells

	racemization, % (w/w)					
trial no.	Thr[a]	Val[b]	Met[c]	Ile[d]	Leu[e]	Phe[f]
1	10.1	10.2	12.8	9.5	11.9	10.6
2	25.1	16.2	23.9	18.5	22.1	21.3
3	7.9	4.9	17.4	4.5	8.0	6.2
4	21.2	17.8	25.8	19.8	19.6	23.2
5	12.8	13.8	15.9	10.4	14.5	14.9
6	27.3	17.7	26.4	20.1	23.2	27.4
7	9.0	5.8	19.7	5.0	8.1	6.9
8	22.4	18.8	27.3	20.9	19.7	24.6
9	14.1	15.1	17.6	12.8	15.1	16.4
10	28.9	19.3	28.1	24.2	23.8	28.7
11	9.4	6.5	10.7	5.9	9.0	8.8
12	23.9	21.9	30.1	23.7	23.2	26.8
13	15.8	15.2	18.4	13.4	15.0	16.7
14	30.4	22.4	30.8	25.0	23.7	28.8
15	9.7	6.7	21.9	6.7	9.1	11.1
16	26.8	22.2	29.5	24.5	23.1	26.9
17	8.6	7.5	12.8	7.2	9.7	14.3
18	9.0	7.6	13.0	7.6	9.9	15.0
19	9.6	7.7	13.8	8.0	19.5	17.3
20	9.7	7.7	12.6	7.9	10.3	16.2
21	9.0	7.3	13.1	7.3	9.8	14.9
22	7.5	6.8	6.8	6.0	8.8	12.0
23	12.2	8.6	15.6	9.5	11.7	20.3
24	8.2	7.6	12.9	6.6	8.3	14.6
25	9.4	7.3	13.3	7.0	10.2	16.4
26	33.2	8.3	16.9	8.0	10.5	16.3
27	5.9	14.6	23.4	14.8	16.2	20.7
28	0	0.8	6.7	1.1	6.2	2.7
29	14.9	18.7	36.3	21.4	22.0	30.1

[a] Threonine. [b] Valine. [c] Methionine. [d] Isoleucine. [e] Leucine. [f] Phenylalanine.

Table VI. Formation of Lysinoalanine (LAL) and the Removal of Nucleic Acids (NA) from Protein Isolates of *C. utilis* Cells Heated with Sodium Hydroxide Solution

trial no.	LAL, nmol/mg	NA, % (w/w)
1	0	6.98
2	0	4.70
3	0	9.81
4	0	4.89
5	0	6.84
6	0	4.43
7	0	8.84
8	0	4.82
9	0	5.82
10	18.5	1.50
11	0	7.64
12	20.5	1.62
13	0	5.53
14	28.7	0.93
15	2.1	5.74
16	49.3	0.49
17	0	5.75
18	0	4.77
19	0	4.69
20	0	5.34
21	0	4.84
22	0	3.21
23	24.6	1.14
24	0	6.78
25	0	4.38
26	0	3.09
27	0	5.85
28	0	11.97
29	20.5	0.43

Amino Acid Profile. Heating of disrupted yeast cells under alkaline conditions did not cause much decomposition of the amino acids except lysine which under alkaline pH and heat was converted to compounds such as LAL. Also, the heating of yeast cells under acidic conditions did not result in extensive damage to the amino acids of the residual material. However, amino acid contents of acid-treated products were lower than those of alkaline-extracted isolates. This may be due to the fact that acid solutions can catalyze reactions between constituent amino acids (Ikawa and Snell, 1961; Smyth and Elliott, 1964). Tryptophan and cystine are substantially destroyed by heating in acidic solutions.

The data, therefore, indicate that prolonged heating of disrupted *C. utilis* cells in NaOH solution at high temperature and pH resulted in high yields of isolate, and more NA was removed. Such NaOH treatments, however, caused pronounced racemization in essential amino acids and formed substantial amounts of LAL. Although neither protein-bound nor free LAL has been conclusively shown to be harmful when consumed in large amounts, its formation leads to decreased availability and utilization of lysine, because LAL is not utilized.

The nucleic acid content was also reduced by extraction of cells with acid solutions, although none of the acids tested was as efficient as NaOH solution in removing NA. Extraction with NaOH gave residual NA content of as low as 0.43%. Lysine was 68% available and extensive racemization was caused. On the other hand, the lowest residual NA content after acid extractions was 2.37% for HCl and lysine was 68% available (Tables III and IV). Presumably, more extensive removal of NA with more exhaustive acid extractions would result in unacceptable levels of available lysine. Zee and Simard (1975) reduced the NA content of yeast cells to 1.2%, and although they stated that the amino acid yields were high, they did not report on the effect of their procedure on the availability of lysine. Also, the total protein ($N \times 6.25$) and amino acid contents were lower after all the acid extractions. This could be due to the fact that the isolates obtained after NaOH extractions were purer and therefore contained more nitrogen and amino acids in a given weight than the cellular residue after acid treatments.

Another reason could be that aqueous acid solutions catalyzed reactions between constituent amino acids. Lysine was more available in the isolates after NaOH treatment than in the acid-extracted residues. Possibly acid solutions catalyzed carbonyl–amino reactions involving the ϵ-amino groups of lysine. However, alkaline treatments resulted in more racemization than the acidic extractions.

Among the acids, HCl was the most effective in removing NA. Lysine was more available after this acid treatment, and unlike TCA and PCA it did not cause any detectable racemization.

Generally, therefore, the major problems associated with alkaline treatments were the racemization of amino acids and formation of LAL, whereas with the acid extractions it was the destruction of lysine. But since NaOH was more effective in removing NA, it might be preferred to the acids as a reagent to be used to reduce the NA content of yeast cells.

On the basis of studies by Edozien et al. (1970), the Protein Advisory Group (PAG) of the United Nations established a limit of 2 g of NA daily from SCP sources in addition to the usual diet. The National Research Council (NRC) of the United States has recommended that for the 70-kg "reference man" the protein allowance should be 65 g daily. The Food and Agricultural Organization (FAO) and the World Health Organization (WHO) have recommended a protein intake of 1 g of protein/kg of body weight daily or 70 g of protein for the "reference man". In the present study, NaOH yielded an isolate that was ~50% protein. Thus, an individual requiring 68 g of

Table VII. Content of Amino Acids Essential for Humans in the Products (Containing 1.14 and 2.37% Residual Nucleic Acids) from NaOH- and HCl-Treated Disrupted *C. utilis* Cells, Respectively

	% (w/w) of total protein				
amino acids	FAO-WHO (1973) profile[a]	NaOH extraction		HCl extraction	
leucine	7.0	7.7		8.7	
isoleucine	4.0	6.0		5.8	
valine	5.0	7.1		7.0	
lysine	5.5	7.8		7.2	
methionine	3.5	1.6	2.2	1.5	2.0
(cystine)		0.6		0.5	
phenylalanine		4.7	7.4	4.9	9.6
(tyrosine)	6.0	2.7		4.7	
tryptophan	1.0	1.3		1.2	
threonine	4.0	7.0		6.3	

[a] Energy and protein requirements.

protein (average of 70 and 65) daily and whose only source of protein is from *C. utilis* cells would need ~136 g of isolate daily. Some NaOH extractions gave residual NA values of 0.43–1.14%. Thus, consuming 136 g of these isolates would also mean consuming 0.58–1.55 g of NA daily. At the above residual NA content 4800–11 500 μg/g LAL was formed, 69.2–73.8% of the lysine was available, and racemization was in the order of 20%. Sternberg et al. (1975) have reported LAL values in proteins of home-cooked and commercial foods and ingredients varying from about 50 to 50 000 μg/g with most commercial foods containing about 500 μg/g of protein. Whether an LAL content of 4800–11 500 μg/g in the isolate could be considered excessive or not must await further research in this area. Mottu and Mauron (1967) have reported that lysine was 72.4% available in evaporated milk (a protein-rich product used as food for infants). As a result of the racemization, 136/0.8 = 170 g of isolate, and therefore 1.94 g of NA, would be consumed daily to receive sufficient protein. This level of NA is still below the 2-g daily recommended limit. However, the resultant D-amino acids are not incorporated into proteins, but Clayton et al. (1970) have reported that D-amino acids could be fed to healthy adults without adverse effects. Hence, the isolate containing 1.14% residual NA is not necessarily intolerable since it also contains sufficient lysine (Table VII) to compensate for deficiencies in available lysine.

The lowest residual NA after HCl extraction was 2.37%. Since the protein yield for this acid treatment was ~30%, 68/0.3 = 227 g of the acid-extracted material, containing an unacceptable amount of 5.4 g of NA, must be consumed daily. The low availability of lysine superimposed on this would mean an increased intake of the acid-treated material. The maximum amount of this product that can be consumed would be (227 × 2)/5.4 = 84 g daily. The protein deficit would then necessarily be derived from other sources low in NA.

From the above discussion, the difficulty in making recommendations for an extraction procedure becomes readily evident. However, from the various treatments used in this study, it appears that NaOH extraction of an 8% suspension of disrupted *C. utilis* cells for 30 min at 100 °C and pH 10 would offer the best compromise between residual NA and damage to amino acids. In this case, sufficient isolate could be consumed to offset the damage to the proteins without the ingestion of unacceptable levels of NA. On the other hand, the product from acid-extracted cells would necessarily be consumed at reduced levels because of the higher NA content.

It is true that a computer search of the response surfaces after setting desirable constraints on yield, lysine availability, LAL content, NA concentrations, and racemization could be performed. But the choices for setting the parameters are difficult because of mutual exclusivity. For example, in the alkaline extraction, a desirable LAL content would yield a product with unacceptable NA content. Likewise, in the acid extraction, acceptable NA would result in low lysine availability. However, full inclusion of the data in Tables I–VI would allow anyone to take this approach.

The data in this study indicate (a) that NaOH was more efficient in reducing the NA content of *C. utilis* cells than any of the acids tested and (b) that although an alkaline extraction process yielded an isolate that could be consumed to essentially meet the daily recommended protein and NA intake, extensive damage to proteins can occur as a result of acidic or alkaline treatments of disrupted *C. utilis* cells in order to reduce their NA content. In this regard, therefore, consideration should be given to the heat-shock activation of endogenous nucleases to reduce the levels of NA in SCP (Maul et al., 1970).

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Preharvest Changes in Polyphenols, Peroxidase, and Polyphenol Oxidase in Sorghum Grain

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At various stages of preharvest development, "bird-resistant" sorghum [*Sorghum bicolor* (L.) Moench] grain was analyzed for tannin content and polyphenol oxidizing enzymes. Tannins appeared first at the milk stage, reached a maximum at the hard dough stage, and thereafter declined by 25%. Polyphenol oxidase (PPO) and peroxidase activities were detected during the flowering stage. PPO activity declined rapidly as the grain began to develop while the decline of peroxidase was slower. Neither activity could be detected in mature grain. Both enzymes were characterized and PPO behaved as a catechol oxidase. It appeared that these enzymes were not responsible for tannin deposition in sorghum grain. The decrease in extractable tannin was attributed to the high molecular weight tannin complexing with cellular components to become insoluble.

Sorghum grain polyphenols, in the form of condensed tannins, protect the grain against depredation by birds but can reduce the nutritional quality of the grain (Chavan et al., 1979). In southern Africa where the grain is used for brewing sorghum beer, the tannins inhibit enzymes required during brewing (Daiber, 1975).

Davis and Hoseney (1979) determined the preharvest changes in the tannin content of bird-resistant sorghum grain using its ability to inhibit α-amylase. They found that enzyme inhibition increased gradually until ~6 weeks before harvest. After that, inhibition levels decreased until the time of harvest. In another study, in which tannin was measured by a modified vanillin assay, a similar pattern was found (Price et al., 1979). The amount of extractable tannin increased to a maximum and then decreased. There was wide variation in the decline of tannin in the nine different cultivars studied.

A similar pattern occurs in ripening fruit (bananas, plums, and peaches) where there is a loss of astringency as the fruit ripens. Goldstein and Swain (1963) suggested that this loss of astringency was probably connected with the polymerization of the tannins to increased molecular weight. Two theories which account for tannin polymerization involve either an enzymatic or a nonenzymatic process (Brown, 1964). Polyphenol oxidases were associated with the aerobic oxidation of gallocatechin to form tannins in the cambrium layer of oak trees (Hathway and Seakins, 1957; Hathway, 1958). In sorghum it has been proposed that tannin polymerization is nonenzymatic (Jacques et al., 1977; Gupta and Haslam, 1979). This nonenzymatic reaction would involve a spontaneous condensation via a carbocation.

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This investigation is concerned with the preharvest changes in tannins in the testa of bird-resistant sorghum grain as well as two enzymes which could be associated with polyphenol polymerization: polyphenol oxidase (PPO) (EC 1.14.18.1) and peroxidase (EC 1.11.1.7). This study was carried out to see what interrelation existed between enzyme activity and tannin deposition.

PPO has been well reviewed (Mayer and Harel, 1979). Sorghum seedlings yielded three fractions of PPO activity when extracts were chromatographed on Sephadex G-100 (Stafford and Dresler, 1972), and a minimum of five bands of peroxidase activity were obtained by electrophoretic separation (Stafford and Bravinder-Bree, 1972). In another study, sorghum seedling peroxidase carried out the aliphatic hydroxylation of (*p*-hydroxyphenyl)acetonitrile which is an intermediate in dhurrin biosynthesis (Liljegren, 1978). From sorghum grain, peroxidase has been isolated but no polyphenol oxidase activity has been reported (Sae et al., 1971).

EXPERIMENTAL SECTION

Bird-resistant sorghum [*Sorghum bicolor* (L.) Moench], cultivar SSK 52, was used to study the preharvest changes while bird-resistant cultivar NK 300 was used to study the properties of the enzymes. Both cultivars were grown at the Plant and Seed Control Division, Horticultural Research Centre, Roodeplaat, South Africa. Samples of SSK 52 were harvested at various stages of development from preflowering (5 days before anthesis) until time of harvest (89 days after anthesis). Ten whole panicles were harvested for each stage of development (as listed in Figure 1), and after hand threshing, the grains were freeze-dried.

After being dried, the grain was mixed and subsamples were ground for 1 min in a Janke and Kunkel mill (a water-cooled coffee mill). The ground grain was extracted at room temperature with 70% aqueous acetone by percolation on a Buchner funnel using 500 mL of 70% ace-

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