Application of a 28-h Method for Monitoring Nutritional Protein Quality Changes during Single-Cell Protein Processing†

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The purpose of this work was to test the suitability of the method described in the accompanying paper which evaluates the nutritional quality of single-cell protein produced from waste carbohydrates through different technological processes. The sources of single-cell protein employed were (1) cellulose-utilizing bacteria grown on sugar cane bagasse and (2) the yeasts *Torula utilis* and *Saccharomycopsis lipolytica* grown on industrial wastes from an alcohol distillery and an olive oil factory, respectively. Processes such as filtration and decantation–spray-dry, normally used in industrial food processing, introduced significative differences in the nutritional quality (biological values from 48.5 to 61) of bacterial protein which could be detected by the rapid method. Furthermore, very low biological values obtained after decantation–spray acid treatment could be improved by addition of methionine or methionine plus lysine, as determined by the rapid method. We conclude that the method proposed can be applied to any technological problem, given that the interest is mainly focused on major differences in nutritional protein quality.

In the accompanying paper (Sammán and Farías, 1993), we reported on the close correlation observed between the traditional method of Miller and Bender (1955) and the 28-h method using a protein concentration in the diet of 10%. The present paper shows that a reasonable agreement was found between both methods when they were applied to the determination of the changes in nutritional quality of protein during single-cell protein processing. Production of microbial protein, also called single-cell protein, derived from yeasts, molds, bacteria, and algae, has been studied with great interest during the past years. Various industrial wastes have been used as substrates for cultivation (Kihlberg, 1972). We believe that this paper will contribute to the assessment of new technological approaches in the area of industrial waste processing and other areas. The main reason is that the evaluation of protein quality by the semiquantitative method is a simple and rapid technique for obtaining reliable information during adjustment of a technological variable.

MATERIALS AND METHODS

Bacterial Protein. Cellulose-degrading aerobic and mesophilic bacteria were isolated (Molina and Callieri, 1978) from sugar cane bagasse, according to the method of Han and Srinivasan (1968). The organisms were identified as a mixture (9:1) of *Cellulomonas* sp. and *Bacillus subtilis* (Molina et al., 1983). Alkali tretment of sugar cane bagasse was applied according to the procedures of Han and Callihan (1974) and Pamment et al. (1978). The cellulolytic process was carried out for 72 h at 34 °C with stirring in a batch fermentor with sugar cane bagasse pith as the hydrocarbon source and $NaNO_3$ as the nitrogen source (O. E. Molina, Ph.D. thesis, Universidad Nacional de Tucumán, Argentina, 1980). After this period, aeration and temperature control were stopped. The suspension was filtered or, alternatively, allowed to settle, and bacteria were recovered from the supernatant. In the last case, the residual pith and the cellulosic colloids were allowed to decant for 2–3 h. The bacterial biomass remaining in the supernatant was collected by syphoning at the top of the vessel and then centrifuged at 10000*g*. This is a crucial step to harvest cells with low crude fiber content (Molina et al., 1983). The nucleic acid content was lowered with either high or low pH treatment according to the method of Zee and Simard (1975). Different procedures to obtain bacterial protein are summarized in Figure 1.

Yeast Protein. Two sources of yeast protein were used: *Torula utilis* var. Major and *Saccharomycopsis lipolytica*, grown on industrial wastes from an alcohol distillery (stillage) (Cabib et al., 1983) and from olive oil manufacture (olive black water) (Ercoli and Ertola, 1983), respectively.

Diets. Ingredients of the basic experimental diet were described previously (Sammán and Farías, 1993). Protein was added to the basal diet by substituting for an equivalent amount of wheat starch/sucrose (1:1) to obtain a protein level of 10%, as recommended (Farina et al., 1979), or 20% for the body weight gain experiment.

Sprague-Dawley rats weighing 60 ± 1 g of either sex from our own breeding laboratory were used. They were housed in individual screen-bottom cages and assigned at random to the experimental groups. The room temperature of the breeding laboratory was maintained at 21 ± 1 °C with lighting from 7:00 a.m. to 7:00 p.m.

The protein efficiency ratio (PER) was determined with weanling rats fed with a 10% protein diet during 28 days according to the AOAC standard method for biological evaluation of protein quality (AOAC, 1980).

The classical method net protein utilization (NPU) was determined by a modification of the Bender and Miller (1953) method using body water as an index of body nitrogen (Miller and Bender, 1955). Biological values (BV = NPU/digestibility) were calculated according to the method of Miller and Payne

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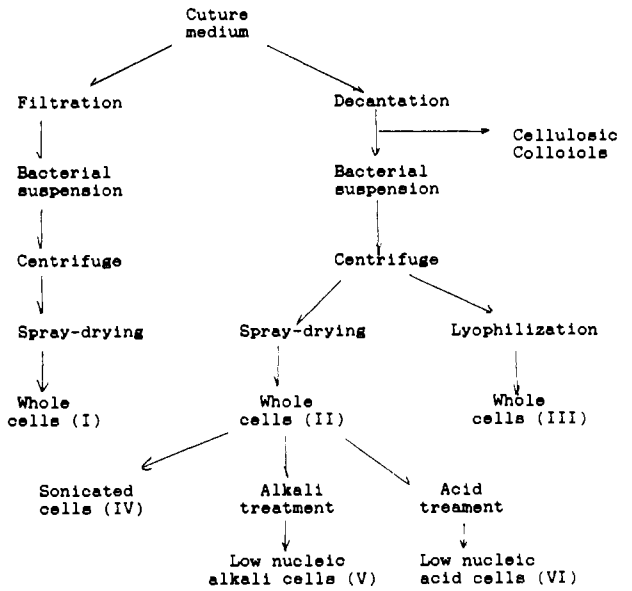

Figure 1. Flow chart of the obtention procedure of single-cell protein from *Cellulomonas* sp. and *B. subtilis* developed in sugar cane bagasse. I, filtration; II, decantation–spray; III, decantation–lyophilization; IV, decantation–spray-drying–sonication; V, decantation–spray-drying–low nucleic acid–alkali treatment; VI, decantation–spray-drying–low nucleic acid–acidic treatment.

(1963). Three groups of four rats each were used for each protein sample during 10 days.

Rapid Method. The method of Farina et al. (1977) was performed as described in Sammán and Farías (1993). Biological values and NPU values were calculated from the following equations: BV = 219.5 − 95.5 log (WL%); NPU = 211.8 − 95.1 log (WL%) (Sammán and Farías, 1993). The protein nutritional quality (NQ) index was calculated as in Sammán and Farías (1993).

Food intake was determined during the 24-h feeding period.

Dietary and fecal nitrogen were determined according to the Kjeldahl procedure (AOAC, 1980), and the crude protein was calculated as N × 6.25.

Amino Acid Analysis. Fifty milligrams of dried material was hydrolyzed for 24 h at 105 °C in vacuo with HCl (6 mol/L) containing 0.13 mol/L mercaptoethanol and 0.1 mol/L phenol to avoid the loss of cysteine and methionine. Amino acid composition was determined by running the hydrolysates through a single-column LKB analyzer, Model 4101, according to the manufacturer's instructions.

Statistics. Means are expressed with their standard deviation. Analysis of variance (ANOVA) was used to test for an effect, and when more than two means were to be compared, it was followed by Tukey's multiple-range test.

RESULTS

Bacterial Protein. Previous studies from the literature used various cellulolytic bacterial species to produce single-cell protein from cellulose wastes (Han and Srinivasan, 1968; Callihan and Dunlap, 1971; Perotti and Molina, 1988). The amino acid profile of *Cellulomonas* sp.–*B. subtilis* mixed culture used in the present work is in agreement with that of the FAO/WHO-73 reference protein (FAO, 1973). Except for a high level of isoleucine and leucine, the essential amino acids of the cellulose-descomposing microorganism mixture show a pattern similar to that reported previously for a *Cellulomonas* pure culture (Han and Callihan, 1974; Hitchner and Leatherwood, 1980). Limiting amino acids were mainly the sulfur-containing ones, a typical feature of single-cell protein products (Kihlberg, 1972). Lysine content was higher than in the reference protein (Table I).

Table I. Amino Acid Content of *Cellulomonas–B. subtilis* Culture Mixture and FAO/WHO-73 Reference Protein

amino acid	g/100 g of protein			
	FAO/ WHO-73	*Cellulomonas–B. subtilis*	*Cellulomonas*	
			a	*b*
isoleucine	4.0	6.3	3.3	4.7
leucine	7.0	13.2	8.1	11.2
lysine	5.5	6.4	6.5	6.8
methionine + cystine	3.5	2.1	1.5	1.8
phenylalanine + tyrosine	6.0	7.0	7.0	6.9
threonine	4.0	4.7	4.7	5.3
valine	5.0	6.9	5.8	10.7
tryptophan	1.0	ND[c]	ND	ND

[a] Hitchner and Leatherwood (1980). [b] Han and Srinivasan (1968). [c] ND, not determined.

The biological values of bacterial proteins obtained were measured by the 28-h method (Sammán and Farías, 1993; Table II). The filtration procedure yielded single-cell protein preparations with a high content of fiber (25% of dried cells). The biological values of this protein preparation could not be determined by the rapid method (Table II, row 1) since the weight loss after the 4-h fast was higher than the weight gain over the 24-h feeding period. The latter was reflected by a very low NQ index.

When decantation was used in place of filtration, the fiber level of the single-cell preparation diminished from 25 to 7%. As expected from the work of Shah et al. (1982), the latter procedure improved the biological value, which was 48.5 (low NQ index) when the preparation was spray-dried and 61.8 (good NQ index) when it was lyophilized (Figure 1 and Table II, rows 2 and 4). When spray-dried cells were disrupted by sonication, the biological values were similar to those of lyophilized whole cells (Table II, rows 4 and 5).

To reduce the nucleic acid content from spray-dried cells, we assayed two different methods: alkali and acid treatments (Figure 1). In both cases, the NQ index decreased from good to very low or low NQ (Table II, rows 5, 6, and 8). In the case of the acid treatment, good and high NQ indices were obtained when the bacterial protein was supplemented with methionine or methionine plus lysine (Table II, rows 9 and 10). This supplementation was ineffective when the cells with low nucleic acid content were obtained by alkali treatment (Table II, rows 6 and 7) since the NQ index increased only from very low to low values.

Yeast Protein. The BV and NQ indices obtained by applying the 28-h method to yeast grown on industrial wastes are shown in Table III. They could not be determined for *T. utilis* when cells were dried in a rotary drum (Table III, row 1) due to the fact that the weight loss of the rats (4 h) was higher than the weight gain (24 h). When *T. utilis* cells were spray-dried, a very low NQ index (WL% > 100) was also found (Table III, row 2). The amino acid composition of the yeast protein revealed that methionine, not lysine, was the limiting amino acid (Kihlberg, 1972). Accordingly, supplementation with methionine (good NQ index), but not lysine (low NQ index), significantly improved the BV indices of *T. utilis* (Table III, rows 3–5). The BV and NQ indices of a *S. lipolytica* cell preparation were increased by methionine supplementation from low to good NQ index (Table III, rows 6 and 7).

Food Intake. The 24-h food intake ranged from 6.0 to 8.0 g, except for the protein shown in Table II, row 6, and

Table II. BV and NQ Indices of Mixture Culture of *Cellulomonas* Species and *B. subtilis*

bacterial protein	biological values[h]	NQ[h]	food intake, g/24 h
filtration (I)[a]	WL% > 100	very low	6.5 ± 0.8
decantation–spray (II)[b]	48.5 ± 6.9[f]	low	6.8 ± 0.7
decantation–spray (II) (50%) plus corn protein (50%)	58.9 ± 7.3[f]	good	7.1 ± 0.8
decantation–lyophilization (III)	61.8 ± 5.4[f]	good	7.4 ± 0.6
decantation–spray–sonication (IV)	61.0 ± 6.7[f]	good	6.8 ± 1.0
decantation–spray alkaline treatment (V)[c]	WL% > 100	very low	5.3 ± 0.2
plus Met, Lys[d]	30.1 ± 8.9[e]	low	6.9 ± 0.7
decantation–spray acid treatment (VI)[c]	32.3 ± 12.1[e]	low	6.4 ± 0.4
plus Met	61.5 ± 4.7[f]	good	6.3 ± 0.4
plus Met, Lys	84.2 ± 4.7[g]	high	8.1 ± 0.3

[a] Fiber 25%, protein 50.7% of dry cells. [b] Fiber 7%, protein 58.5% of dry cells. [c] The amount of nucleic acid was decreased from 18–20 to 4–6% of dried cells. [d] Met and Lys were added to 1.0 and 0.5 g/100 g of dietary protein, respectively. [e–g] The results are expressed as mean ± SD. The values differ significantly when not followed by the same letters ($p < 0.05$). [h] BV and NQ values were determined by the 28-h method (Sammán and Farías, 1993) as indicated under Materials and Methods.

Table III. BV and NQ Indices of Yeast Grown on Industrial Wastes

yeast protein	biological value[g]	NQ[g]	food intake, g/24 h
T. utilis[a] plus Met 1%	WL% > 100	very low	7.0 ± 1.0
T. utilis[b]	WL% > 100	very low	10.5 ± 2.0
plus Lys 1%	28.3 ± 6.5[d]	low	7.9 ± 1.8
plus Met 1%	61.9 ± 5.8[e]	good	7.2 ± 0.6
plus Lys 0.3% Met 0.7%	70.6 ± 4.0[f]	good	8.0 ± 0.6
S. lipolytica[c]	35.4 ± 3.2[d]	low	6.4 ± 0.9
plus Met 1%	65.2 ± 6.6[e,f]	good	5.7 ± 0.9

[a] Rotary drum dry. Protein content: 45.6% of dry weight (Cabib et al., 1983). [b] Spray-dry. Protein content: 47.90% of dry weight (Cabib et al., 1983). [c] The sample was recovered at the end of batch process conducted in fermentor scale by centrigutation and used unwashed after drying at 50–55 °C. Protein content: 45.2% of dry weight (Ercoli and Ertola, 1983). [d–f] The results are mean ± SD. The values differ significantly when they are not followed by the same letters ($p < 0.05$) (Tukey's test). [g] BV and NQ indices were determined by 28-h method (Sammán and Farías, 1993) as indicated under Materials and Methods.

Table III, row 2. The difference in BV and NQ indices shown in Tables II and III is clearly not related to food intake.

Comparison of the Rapid and Classical Methods. Table IV lists the nutritional quality values obtained by the 28-h and Miller and Bender (1955) methods for six single-cell protein preparations.

Complementation between Bacterial and Vegetable Proteins. A classic example of complementation between corn and soybean proteins evaluated by both methods is included at the bottom of Table IV, rows 7–9. These results confirm the validity of our single-cell protein complementation studies with corn protein (Table II, row 3).

Body Weight. The average body weight was studied for 10 rats/group fed with diets containing 10% casein and 10 or 20% bacterial protein (VI). Cells were obtained by decantation, spray-dried, treated with acid, and supplemented with lysine and methionine (BV = 78; Table IV, row 2). With 10% dietary protein level, a similar body weight gain was obtained in bacterial protein- and casein-fed rats. When bacterial protein was increased to 20%, an improvement in the body weight gain was observed. The latter indicated that, in addition to the high NQ index, this bacterial protein preparation was palatable and suggested no adverse effects on growing rats during the 30-day experimental period.

DISCUSSION

Our results indicate that changes in the preparation process of single-cell protein are detected with high reliability by the 28-h method (Sammán and Farías, 1993). Statistically similar biological values were found with both methods in four of the six single-cell protein preparations analyzed (Table IV, rows 2 and 4–6). As indicated in the text, in the case of bacteria obtained by filtration, the 28-h method failed to estimate the biological values (Table II, row 1) as well as the classical method of 10 days (Miller and Bender, 1955) (Table IV, row 1). The rats lose weight during the former 5 days of the biological assay. Accordingly, Shah et al. (1982) reported that fiber negatively influenced the biological values. Then, from the six samples shown in Table IV, only the values for *T. utilis* dried by rotary drum showed an apparent difference. In fact, a biological value of 38.6 was found using the Miller and Bender (1955) method, suggesting a low NQ index (Table IV, row 3), biological values could not be calculated

Table IV. Comparison of Nutritional Values Obtained by the 28-h and Classical Methods

		biological values[a]		net protein utilization[b]			
row	protein	rapid	classical	rapid	classical	digestibility, %	PER
	bacteria						
1	filtration (I)	WL% > 100	c	WL% > 100	c	c	c
2	decantation–spray acidic treatment plus Met, Lys (VI)	84.2 ± 4.7*	78.0 ± 2.0*	77.2 ± 4.2*	64.9 ± 1.8**	83.2 ± 2.1	2.41
	T. utilis						
3	rotary drum plus Met	WL% > 100	38.6 ± 5.3	WL% > 100	27.2 ± 6.4	70.4 ± 3.3	ND[d]
4	spray plus Met	61.9 ± 5.6*	62.6 ± 2.8*	55.1 ± 5.2*	47.4 ± 2.0**	75.8 ± 2.8	1.4
5	spray plus Met, Lys	70.6 ± 4.0*	75.7 ± 2.2*	63.7 ± 3.8*	49.0 ± 0.7**	64.9 ± 2.3	ND
	S. lipolytica						
6	plus Met	65.2 ± 6.1*	70.5 ± 3.6*	58.3 ± 5.3*	60.4 ± 4.3*	85.7 ± 4.0	ND
7	corn	WL% > 100	35.4 ± 3.2	WL% > 100	30.4 ± 3.0	85.9 ± 2.4	1.05
8	soybean	67.7 ± 4.0*	65.3 ± 1.6*	60.7 ± 3.5*	57.3 ± 0.1*	87.8 ± 0.1	2.22
9	corn 85%, soybean 15%	70.2 ± 5*	74.0 ± 4.2*	63.3 ± 5.3*	64.6 ± 3.8*	87.3 ± 1.2	ND

[a,b] Mean ± SD values for a protein source in the same row having identical superscript symbols (*) are not significantly different ($p < 0.05$). Comparisons were done within each sample between the rapid and classical methods. [c] The weight loss of the rats is about 5 g/5 day. [d] ND, not determined.

Table V. Agreement between the 28-h and Classical Methods for Protein Classification of the Six Single-Cell Proteins[a]

(A) Biological Values

	28-h method			
classical method	high	good	low	very low
high	1	1		
good		2		
low				
very low				2

(B) Net Protein Utilization

	28-h method			
classical method	high	good	low	very low
high	1			
good		2		
low		1		
very low				2

[a] The data are from Table IV and were classified according to the range of nutritional quality given in Sammán and Farías (1993).

by the equations of the 28-h method since the percent weight loss (WL%) exceeded 100, indicating a very low NQ index. The Miller and Bender methods overestimated the nutritional quality of the proteins when the BV values were lower than 40 because the endogenous essential amino acids became important in the plasmatic pool (Young et al., 1981). Taking into account the latter, the BV of 38.6 could also indicate a very low NQ index. When this observation is included in Table V, the comparison between the 28-h and classical methods for protein classification for six single-cell processed proteins give $\hat{K}$ values of 0.76 for both NPU and BV, indicating a good agreement between the two methods. In three samples the rapid method gave NPU values statistically higher than the classical method (Table IV, rows 2, 4, and 5). According to the data reported in the accompanying paper (Sammán and Farías, 1993), the 28-h method evaluated better BV than NPU values.

The BV and NPU values of the proteins analyzed are all similar to those previously reported for single-cell protein (Table IV) (Kihlberg, 1972).

The small amount of sample required by the 28-h method is an important advantage during the adjustment of technological variables, since the amount of material available for biological evaluation is generally limited. To evaluate protein quality by the 28-h method, the amount of diet consumed is small (36–60 g/6 rats). As the protein level in the assay diet is 10% and its concentration in single-cell protein preparations is usually about 50%, a sample of 8–12 g suffices to carry out the nutritional analysis. During 10 days of feeding and using three groups of four rats each, in the method of Miller and Bender (1955), 20-fold higher amounts of sample are necessary.

ABBREVIATIONS USED

BV, biological values; PER, protein efficiency ratio; NPU, net protein utilization; NQ, nutritional quality.

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LITERATURE CITED

AOAC. *Official Methods of Analysis*, 13th ed.; Horwithz, W., Ed.; AOAC: Washington, DC, 1980.

Bender, A. E.; Miller, D. S. A new brief method of estimating net protein value. *Biochem. J.* **1953**, VII–VIII.

Cabib, G.; Silva, H. J.; Giuletti, A.; Ertola, R. (1983). The use of sugar cane stillage for single cell protein production. *J. Chem. Technol. Biotechnol.* **1983**, *338*, 21–28.

Callihan, C. D.; Dunlap, C. E. *Construction of a chemical microbial pilot plant for production of single-cell protein from cellulosic wastes*; U.S. EPA Solid Waste Management Series (SW-24C); U.S. Government Printing Office: Washington, DC, 1971.

Ercoli, E.; Ertola, R. SCP Production from olive black water. *Biotechnol. Lett.* **1983**, *5*, 457–462.

FAO. *Amino acid content of food and biological data on protein*; Food Policy & Food Science Service, Nutrition Division; Food and Agriculture Organization: Rome, 1973.

Farina, R.; Río, M. E.; Sanahuja, J. C. A rapid method for the evaluation of protein quality. *Nutr. Rep. Int.* **1977**, *16*, 293–299.

Farina, R.; Río, M. E.; Sanahuja, J. C. A rapid method for the evaluation of protein quality: effect of protein concentration. *Nutr. Rep. Int.* **1979**, *20*, 693–699.

Han, Y. W.; Callihan, C. D. Cellulose fermentation: Effect of substrate pretreatment on microbial growth. *Appl. Microbiol.* **1974**, *27*, 159–165.

Han, Y. W.; Srinivasan, V. R. Isolation and characterization of a cellulose-utilizing bacterium. *Appl. Microbiol.* **1968**, *16*, 1140–1145.

Hitchner, E. V.; Leatherwood, J. M. Use of cellulase-derepressed mutant of *Cellulomonas* in the production of single-cell protein product from cellulose. *Appl. Environ. Microbiol.* **1980**, *39*, 382–386.

Kihlberg, R. The microbe as a source of food. *Annu. Rev. Microbiol.* **1972**, *26*, 427–4656.

Miller, D. S.; Bender, A. E. The determination of the net utilization of protein by a shortened method. *Br. J. Nutr.* **1955**, *9*, 382–388.

Miller, D. S.; Payne, P. R. A theory of protein metabolism. *J. Theor. Biol.* **1963**, *5*, 398–403.

Molina, O. E.; Callieri, D. A. *Rev. Ind. Agric. Tucuman* **1978**, *55*, 13–25.

Molina, O. E.; Pailhé, L. A.; Perotti de Gálvez, N. I.; Popolizio, E. Evaluation of the efficiency of bacterial protein (*Cellulomonas* sp. and *Bacillus subtilis*) in pre-stiarring rations for broiler-chickens. *Rev. Latino-am. Microbiol.* **1983a**, *25*, 253–258.

Molina, O. E.; Perotti de Gálvez, N. I.; Callieri, D. A. Bacterial protein production from sugar cane bagasse pith. *Acta Cient. Venez.* **1983b**, *34*, 59–64.

Pamment, N.; Moo-Young, M.; Hsieh, F. H.; Robinson, C. N. Growth of *Chaetomoium Cellulolyticum* on alkali-treated hardwood sawdust solids and pretreatment liquor. *Appl. Environ. Microbiol.* **1978**, *36*, 284–290.

Perotti, N. I.; Molina, O. E. Corn cob as a bacterial substrate for the production of forage protein. *Biol. Wastes* **1988**, *26*, 125–131.

Sammán, N.; Farías, R. N. A 28-h method for estimating protein nutritional quality. *J. Agric. Food Chem.* **1993**, preceding paper in this issue.

Shah, M.; Atallah, M. T.; Mahoney, R. R.; Peletti, P. Effect of dietary fiber components on fecal nitrogen excretion and protein utilization in growing rats. *J. Nutr.* **1982**, *112*, 658–666.

Young, V. R.; Scrimshaw, N. S.; Bier, D. M. Whole body protein and amino acid metabolism. Relation to protein quality evaluation in human nutrition. *J. Agric. Food Chem.* **1981**, *29*, 443–447.

Zee, J. A.; Simard, R. E. Simple process for the reduction in the nucleic acid content in yeast. *Appl. Microbiol.* **1975**, *29*, 59–62.

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