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NH_4OH-Based Pretreatment for Improving the Nutritional Quality of Single-Cell Protein (SCP)

J. C. PARAJÓ,* V. SANTOS,
H. DOMÍNGUEZ, AND M. VÁZQUEZ

Department of Chemical Engineering, University of Vigo, Campus Orense, Faculty of Sciences, Las Lagunas, 32004 Orense, Spain

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ABSTRACT

The potentiality of treatments with NH_4OH solutions for improving the quality of protein concentrates from *Candida utilis* biomass was studied. The effects of NH_4OH concentration, reaction time, and temperature on both biomass recovery and composition of processed samples (including nucleic acid and protein contents) were studied. The results obtained were used to develop empirical models providing a quantitative interpretation of the interrelationships among the variables involved. Additional discussion of the reaction selectivity is provided. Under selected conditions, 96% of nucleic acid removal was achieved with 88% protein recovery. The treated cells were high in vitro digestibility and showed an amino acid profile similar to that of untreated biomass.

Index Entries: Nucleic acids removal; single-cell protein.

INTRODUCTION

The increasing world deficit of protein is becoming a main problem of humankind (*1*). Microbial biomass (single-cell protein, SCP) has been considered an alternative to conventional sources of food or feed.

*Author to whom all correspondence and reprint requests should be addressed.

Large-scale processes for SCP production show interesting features, including:

1. The wide variety of methodologies, raw materials, and microorganisms that can be used for this purpose;
2. High efficiency in substrate conversion;
3. High productivity, derived from the fast growth rate of microorganisms; and
4. Independence of seasonal factors (*2,3*).

From a nutritional viewpoint, the content of SCP in nucleic acids (NA) is one of the main factors hindering its utilization as food. The end product resulting from the human metabolism of NA is uric acid, a compound with reduced solubility in physiological fluids (blood or urine). Excessive intakes of NA lead to uric acid precipitation, causing health disorders, such as gout or kidney stone formation. On the basis of the results reported by Waslien et at. (*4*) and Edozien et al. (*5*), the upper limit of NA consumption was fixed at 2 g/d. This amount corresponds to a diet containing 20–30 g of yeast biomass with roughly 50% true protein. Hence, in order to satisfy a substantial part of the protein intake requirements in human diets from SCP-derived foods, their NA contents must be reduced below 2% (*6*).

Several technologies to reduce the NA content of microbial cells have been reported, including both chemical and enzymatic procedures. Among the chemical methods, the removal of NA has been tried using alkalis, acids, or salts. Several authors (*7–11*), reported the utilization of NaOH solutions to obtain protein concentrates with low-NA contents. Alvarez and Enriquez (*12*) accomplished the reduction of NA from *Kluyveromyces fragilis* cells in media containing NH_4OH. Both types of alkaline processes allowed the reduction of NA below 2% with good protein recoveries. Otero et al. (*13*) proposed HCl solutions for hydrolysis of NA from *Candida utilis*. Under selected conditions, isolates containing 0.55–3% NA were obtained, with protein losses accounting for the 10–24% of the initial amount. Gibert (*14*) developed an effective two-stage treatment for yeast biomass, using acid media in the first step and alkaline solutions in the second one. The end product contained 0.2–0.7% nucleic acids, and reduced protein losses occurred (15–17% of the initial amount). Several methods based on the utilization of salts, such as NaCl, $NaClO_4$, or Na_2SO_3 have been reported (*2,15,16*).

Enzymatic methods for SCP processing involve endogenous or exogenous nucleases. The endogenous nucleases of *C. utilis* can be activated by a heat shock, allowing a substantial reduction of NA (*17*). Bacterial or pancreatic nucleases have been studied for NA removal from yeast cells (*18–23*). Hydrolysis of NA has also been performed in experiments using immobilized enzymes (*24–26*).

Although both chemical and enzymatic methodologies are effective for NA removal, the nutritional quality of substrates may be altered by these treatments. De Groot and Slump (*27*) reported the alterations in both amino acid profile and biological utilization of proteins caused by alkaline processing of foods. Shetty and Kinsella (*28*) also found undesired effects associated with protein treatments by alkaline solutions, such as destruction of essential amino acids and chemical reaction between lysine and alanine to give lysinoalanine, a potentially toxic compound. In the same way, during the enzymatic hydrolysis of NA, the endogenous proteases give protein degradation reactions (*29*).

This work deals with the optimization of the operational conditions for improving the quality of protein concentrates from *C. utilis* cells by means of treatments with NH_4OH solutions. The effects of NH_4OH concentration, reaction time, and temperature on both biomass recovery and composition of processed samples (including NA and protein contents) were studied. The results obtained from a design of experiments with incomplete, second-order, factorial structure were used to develop empirical models providing a quantitative interpretation of the interrelationships among the variables involved. Additional discussion of the reaction selectivity is provided. Both the amino acid profile and the in vitro digestibility of protein isolates obtained under selected operational conditions were also determined.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Lyophilized broths of *C. utilis* NRRL Y-900 were proliferated and maintained in agar slants containing glucose and yeast extract. The conditions used for SCP production were:

1. Culture media composition: 2% glucose, 1% yeast extract, and 1% peptone;
2. Temperature = 30°C; and
3. Incubation in Erlenmeyer flasks placed in orbital shakers (agitation speed = 200 min^{-1}).

The cells were collected by centrifugation at the end of the log phase, washed, and used for analytical determinations and chemical processing.

Analytical Determinations

True protein content of yeast cells (before and after treatments) was determined by the biuret method according to Herbert et al. (*30*). The NA content of cells (before and after treatments) was determined by the method

of Schmidt-Thannahauser-Schneider (*30*). The amino acid profile of both untreated and treated samples was determined by the Pico-Tag method (Waters Assoc.). The in vitro protein digestibility of substrates was determined by the HCl-pepsin method.

NH_4OH Treatments of Cells

Yeast cells were treated with NH_4OH solutions at a liquor/solid ratio = 12 g solution/g oven-dried cells. The NH_4OH concentration, temperature, and duration of treatments were considered operational variables (*see* Table 1).

RESULTS AND DISCUSSION

The main factors limiting the nutritional quality of SCP are its NA content and cell-wall digestibility, which can be improved by chemical or enzymatic treatments. In order to allow the access of the hydrolytic agent to the cytoplasmic protein, most of the processes proposed in literature for NA removal include a previous step for cell-wall disruption (such as physical treatment or osmotic shock). However, treatments with NH_4OH under moderate experimental conditions without neither physical nor osmotic pretreatments have been reported as an efficient method for removing NA from *K. fragilis* or *Saccharomyces cerevisiae* cells.

In order to optimize the operational conditions for NA removal from *C. utilis* NRRL Y-900 cells, the experimental scheme depicted in Fig. 1 was followed: Cells from fermentation media were collected, washed, and weighed. The overall cell lot was divided into four aliquots having the same composition, which were used for analytical determinations (moisture, protein, and NA) and for treatments with NH_4OH solutions. The protein isolates were recovered by centrifugation, washed, weighed, and divided in three aliquots, which were used for analytical determinations.

The values determined for NA and protein content of *C. utilis* cells obtained in different batch fermentations varied in a narrow range. The mean values (9.0% NA and 53.2% protein) were used for calculation purposes.

Structure of the Experimental Study Carried Out on the NA Removal

A second-order, incomplete, factorial design of experiments was employed in order to obtain reliable information on the several factors involved in the processing of yeast cells (including extent of NA removal, protein content of concentrates, and reaction selectivity). The goal was the development of empirical models providing accurate information on the interrelationships among the variables considered.

Table 1 lists the fixed, independent, and dependent variables chosen in our study, as well as their nomenclature, definitions, units, and varia-

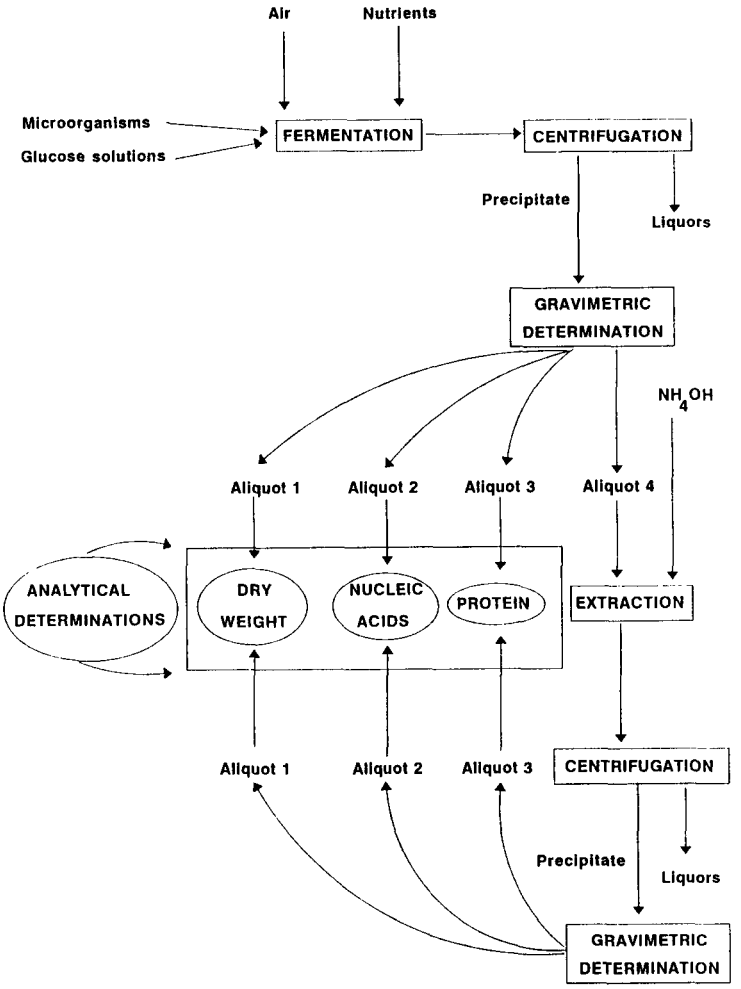

Fig. 1. Scheme of the experimental work developed for processing and analytical characterization of samples.

tion ranges. The same table includes the dimensionless, normalized independent variables (having a variation range [−1,1]) that were defined from the set of independent variables for calculation purposes.

The structure of the experimental design was adapted from a set of experiments proposed in literature. The same structure was previously utilized by the authors for the empirical modeling of chemical and enzymatic processing of lignocellulose (*31*). Table 2 lists the set of experiments performed, as well as the results achieved under the various conditions assayed.

The results shown in Table 2 were used to develop models in which each dependent variable was obtained as the sum of terms giving the contribution of the independent variables through first-order, interaction, and second-order terms, according to the generalized equation:

Table 1
Variables Used in the Study of NH_4OH-Based Pretreatments

Variable	Nomenclature	Units	Variation range	Definition
Fixed variables				
Liquor/solid ratio = 12 g solution/g oven-dried cells				
Independent variables				
NH_4OH concentration	C	g/100 g disolution	1–8	
Temperature	T	°C	55–75	
Reaction time	t	min	15–45	
Dependent variables				
Yield of treatment	$y1$			g biomass recovered/100 g initial biomass, oven-dried basis
NA content of processed biomass	$y2$			g NA/100 g treated biomass, oven-dried basis
Protein content of processed biomass	$y3$			g protein/100 g treated biomass, oven-dried basis
Dimensionless, normalized independent variables:				
Dimensionless NH_4OH concentration	$x1$			(C-4·5)/3·5
Dimensionless temperature	$x2$			(T-65)/10
Dimensionless time	$x3$			(t-30)/15

Table 2
Operational Conditions Assayed and Experimental Results Obtained in the Processing of Yeast Cells with Ammonium Hydroxide Solutions[a]

	Dimensionless indep. var.			Dependent variables		
Experiment	x_1	x_2	x_3	y_1	y_2	y_3
1	0	−1	−1	73.4	2.52	59.8
2	−1	−1	0	77.3	6.06	66.1
3	1	−1	0	71.0	0.63	59.9
4	0	−1	1	71.5	0.81	66.5
5	−1	0	−1	73.6	4.59	63.2
6	1	0	−1	69.4	0.67	49.8
7	0	0	0	72.3	0.59	65.3
8	0	0	0	71.9	0.82	66.3
9	0	0	0	72.0	0.40	63.3
10	−1	0	1	71.6	1.91	66.7
11	1	0	1	68.5	0.53	63.3
12	0	1	−1	71.8	0.40	62.5
13	−1	1	0	71.8	1.10	63.3
14	1	1	0	67.1	0.49	62.7
15	0	1	1	68.7	0.43	63.9
16	0	0	1	72.0	0.56	64.0

[a]Data used for regression and statistical analysis.

$$y_j = b_{oj} + \Sigma_i b_{ij} x_i + \Sigma_i \Sigma_k b_{ikj} x_i x_k \quad (1)$$

where y_j (j: 1–3) is the dependent variable, x_i and x_k (i or k: 1–3) are the dimensionless, normalized independent variable, and b_{oj}, b_{ij}, and b_{ikj} are the coefficients obtained by multiple regression of experimental data.

Table 3 shows the values calculated for the coefficients, as well as their statistical significance (based on the values determined for the *t*-test). The R^2 and *F* parameters (which measure the correlation and statistical significance of models) are also included.

Interrelationship Between Dependent and Independent Variables

The values determined for the treatment yield (variable y_1) varied in a limited range (67.1–77.6%). The yield is significantly affected by x_1 (dimensionless NH_4OH concentration) and x_2 (dimensionless temperature). Increased x_1 or x_2 values resulted in decreased yields, as can be deduced from the respective coefficients. Both terms involving the dimensionless time x_3 (significant at the 90% confidence level) and the interaction or

Table 3
Regression Coefficients and Statistical Parameters

	Dependent variable, y_j			Statistical significance of coefficients (t-test)	Statistical parameters obtained for the models			
Coefficients	y_1	y_2	y_3		Variable	R^2	Corrected R^2	F_{exp}[a]	Prob, $F_{exp} > F_{st}$[b]
				Significant coefficients at the 95% confidence					
b_{0j}	72.28	0.648	64.72	level	y_1	0.9075	0.7678	6.54	<0.02
b_{1j}	−2.29	−1.418	−2.95	Variable y_1: b_{11}, b_{21}	y_2	0.9693	0.9233	21.06	<0.01
b_{2j}	−1.73	−0.950	−0.05	Variable y_2: b_{12}, b_{22}, b_{32}, b_{122}, b_{132}, b_{112}	y_3	0.8830	0.7074	5.03	<0.04
b_{3j}	−0.83	−0.529	3.01	Variable y_3: b_{13}, b_{33}					
b_{12j}	0.40	1.205	1.40	Significant coefficients at the 90% confidence					
b_{13j}	0.28	0.635	2.50	Variable y_1: b_{31}					
b_{23j}	−0.30	0.435	−1.20	Variable y_2: b_{232}					
b_{11j}	−0.61	1.137	−1.92	Variable y_3: b_{133}, b_{333}					
b_{22j}	−0.03	0.252	0.38						
b_{33j}	−0.74	0.177	−2.24						

[a] F_{exp} defined as the ratio between the mean squares of model and error.
[b] F_{st} defined as the statistical value of F for the degrees of freedom of model and error.

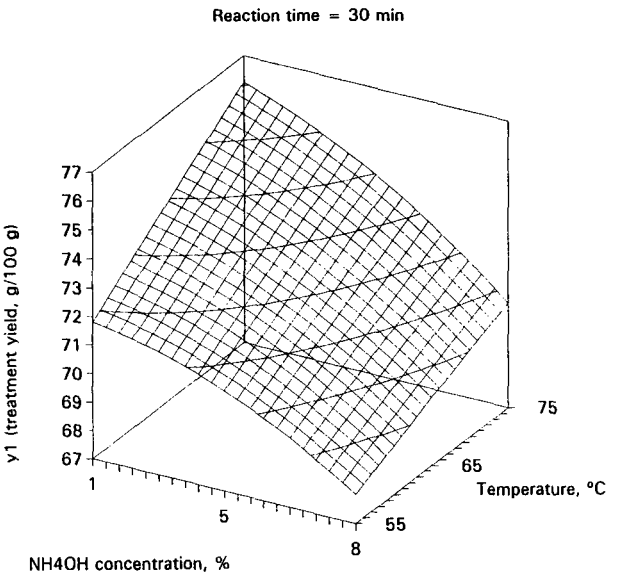

Fig. 2. Dependence of the treatment yield y_1 (g biomass recovered after extraction/100 g biomass subjected to treatment, oven-dried basis) on the ammonium hydroxide concentration and temperature for treatments lasting 30 min.

second-order terms influenced only slightly the treatment yield. This behavior can be confirmed, for example, by comparing the yields obtained in experiments 4 and 15 of Table 2: for the same NH_4OH concentration, a decrease of yield was found when temperature was increased. Similarly, it can be observed that in experiments 2 and 3 (performed at the same temperature), a decrease in yield was determined when the ammonium hydroxide concentration was raised. Figure 2 shows the predictions of the empirical model for the dependence of the treatment yield on the NH_4OH concentration and temperature in experiments lasting 30 min.

The NA percent in treated biomass (y_2) was strongly dependent on the operational conditions assayed. The interrelationship of y_2 with the independent variables was characterized by a broad variation range and a complex influence of the effects considered, being six terms of the model significant at the 95% confidence level and one additional term significant at 90% confidence. The NA removal was enhanced by increased values of the independent variables x_1, x_2, and x_3. The most important effects were associated to the NH_4OH concentration: in experiments carried out using the maximum concentration of ammonium hydroxide, isolates having $<1\%$ NA were obtained independently of the treatment time and temperature considered. The NA concentrations reached in these experiments was far below the maximum content recommended for protein isolates to be used as food. The NA concentration was mainly influenced by

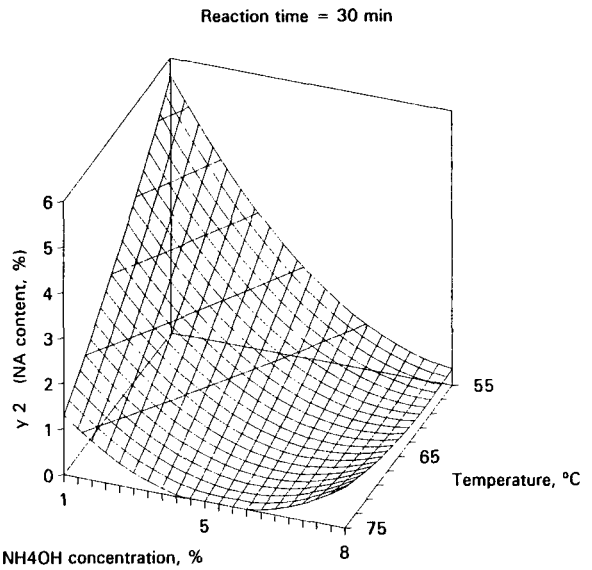

Fig. 3. Dependence of the NA content of processed biomass y_2 (g NA/100 g processed biomass, oven-dried basis) on the ammonium hydroxide concentration and temperature for treatments lasting 30 min.

the dimensionless NH_4OH concentration x_1 (including linear, interaction, and second-order terms) and the dimensionless temperature x_2. The combined effect of x_1 and x_2 on the NA content y_2 can be evaluated by comparison of the results achieved in experiments 2 and 14: in experiment 2, with both x_1 and x_2 fixed at their minimum values, the NA concentration was 6.06%, but in experiment 14, with x_1 and x_2 fixed in their maximum values, the NA content decreased to 0.49%. The dimensionless treatment time (x_3) showed a comparatively limited influence on y_2. Figure 3 shows the predictions of the empirical model for the dependence of the NA content y_2 on the NH_4OH concentration and temperature, relative to experiments lasting 30 min. It can be noticed that under certain experimental conditions, the model predicted a quantitative removal of NA.

The protein content of processed biomass (y_3) depended on the operational conditions by means of two terms significant at the 95% confidence limit (NH_4OH concentration and temperature) and two terms significant at the 90% confidence level (the interaction between concentration and time and the second-order on the dimensionless time). The negative value of the coefficients b_{13} and b_{113} (which measure the importance of the linear and second-order effects of x_1) predicted decreased protein concentrations for isolates obtained in reaction media containing increased NH_4OH concentrations. This fact can be confirmed comparing the protein content found in experiment 6 (49.8%, obtained with $x_1 = 1$) with the protein contents higher than 63% found in experiments 2, 5, 10, and 13 (performed with $x_1 = 1$). Figure 4 shows the predicted dependence of the protein content y_3 on the NH_4OH concentration and temperature for experiments

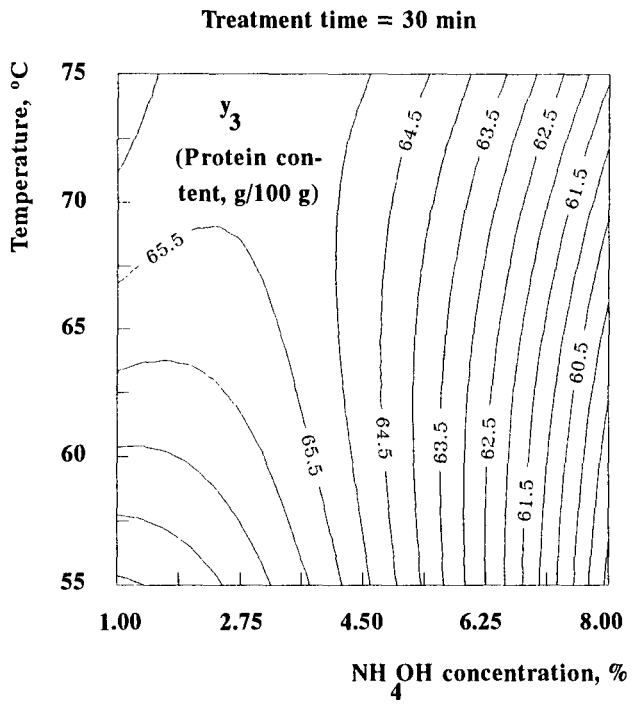

Fig. 4. Dependence of the protein content of processed biomass y_3 (g protein/100 g processed biomass, oven-dried basis) on the ammonium hydroxide concentration and temperature for treatments lasting 30 min.

lasting 30 min. It can be observed from this figure that the effect of temperature is moderate and that the NH_4OH concentration affected the protein content of isolates in the range 4.5–8%.

Further Validation of Empirical Models

The ability of the empirical models to provide predictions in close agreement with the data used in the regression analysis is directly given by their statistical significance, measured by the *F* parameter (*see* Table 3). In order to check the ability of the equations to predict values of $y_1 \ldots y_3$ under experimental conditions different from those used in the development of models, new treatments were performed. The operational conditions considered, the experimental values achieved and the results calculated from the empirical models are shown in Table 4. The means of differences between predicted and experimental results in these experiments were higher than the ones resulting from the data listed in Table 2, but the ability of models to predict was satisfactory in all the cases considered.

Study of Additional Variables

In order to provide a better understanding of the separation selectivity, two variables can be defined to measure the combined effect of yield and

Table 4
Operational Conditions Utilized and Results Obtained in the Additional Experiments Performed to Check the Reliability of the Models

				Dependent variables					
	Independent variables			Experimental values			Calculated values		
Experiment	x_1	x_2	x_3	y_1	y_2	y_3	y_1	y_2	y_3
1	0	1	0	70.5	0.46	63.5	70.5	0.30	64.7
2	1	−1	0	69.9	0.58	60.6	71.7	0.12	61.8
3	−1	1	1	71.8	0.65	65.3	72.9	0.12	65.9
4	−1	−1	−1	77.1	8.58	65.3	76.3	6.52	64.9
5	1	1	−1	66.5	0.53	58.8	68.3	0.52	54.0
6	0	−1	0	74.2	1.51	63.1	74.0	1.60	64.7
7	1	0	0	71.0	0.64	61.7	70.0	0.37	61.8
8	−1	0	0	73.5	3.26	68.4	74.6	3.20	67.7

composition: the "Percent of Nucleic Acids Removal" (PNAR) and the "Percent of Protein Recovery" (PPR). These variables can be defined as:

$$\text{PNAR} = \text{g of NA removed during treatment/ 100 g NA contained in untreated samples} \quad (2)$$

$$\text{PPR} = \text{g of protein recovered after treatment/ 100 g protein contained in untreated samples} \quad (3)$$

From material balances, it can be inferred that these variables depend on $y_1 \ldots y_3$ according to the following equations:

$$\text{PNAR} = 100 \cdot [1 - y_1 \cdot y_2/(100 \cdot y_{20})] \quad (4)$$

$$\text{PPR} = y_1 \cdot y_3/y_{30} \quad (5)$$

where y_{20} and y_{30} are the mean weight percents of NA and protein in untreated biomass (9.0 and 53.2, respectively). The dependence of PNAR and PPR on the operational conditions can be predicted from the empirical models developed and the above equations.

Figure 5 shows the predicted dependence of PNAR on the NH_4OH concentration and temperature for experiments lasting 30 min. It can be observed that the predictions show a wide variety of experimental conditions under which more than 96% of NA can be removed. Both increased NH_4OH concentrations and temperatures improved the process. When temperature was fixed in its lowest value, high NH_4OH concentrations were necessary to remove more than 96% of the initial NA. The same level of NA removal can be obtained with notably lower ammonium hydroxide concentrations if the operation is carried out at higher temperatures. According to the behavior found for the NA concentrations, a set of experimental conditions under which a total degradation of NA was predicted can be noticed.

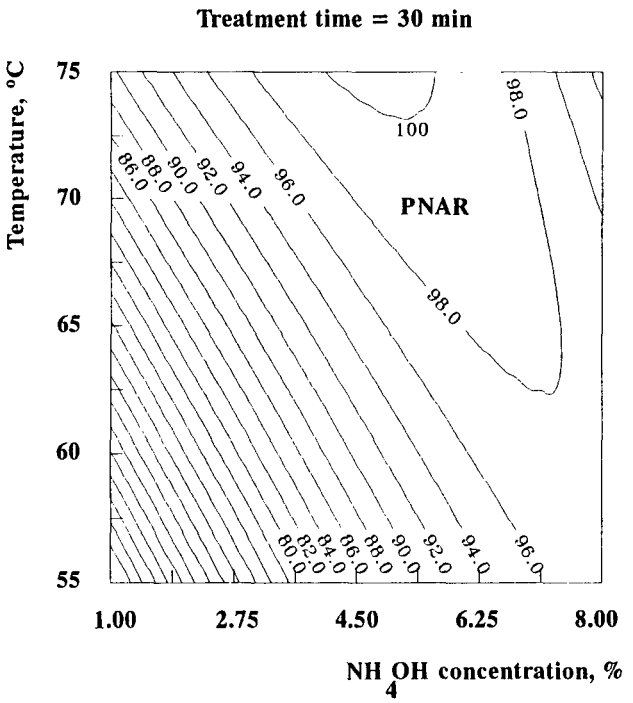

Fig. 5. Dependence of the PNAR (g NA removed during treatment/100 g NA contained in untreated samples) on the ammonium hydroxide concentration and temperature for treatments lasting 30 min.

Figure 6 shows the calculated interrelationship among PPR, base concentration, and temperature for experiments lasting 30 min. The most remarkable features are the little influence predicted for temperature in experiments performed with high NH_4OH concentration and the almost quantitative protein recovery calculated when using low base concentrations.

Selection of Operational Conditions

As a general trend, the variations of PNAR and PPR show opposite dependencies on the independent variables: Figs. 5 and 6 show that mild operational conditions resulted in improved protein recoveries, but in bad extents of NA removal. In order to allow both a significant removal of NA and a good protein recovery, the set of operational conditions shown in Table 5 was chosen for further experimentation. The same table includes the experimental data achieved under the selected conditions, as well as the values predicted by the models.

Digestibility and Amino Acid Profile of Processed Samples

The digestibility of cell walls has been considered one of the main problems for utilization of SCP as food, although this fact is more restric-

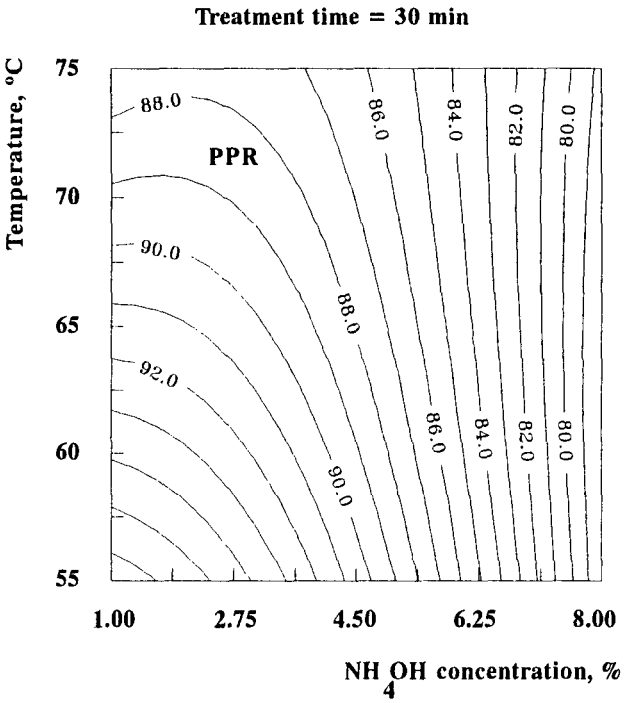

Fig. 6. Dependence of the PPR (g protein recovered after treatment/100 g protein contained in untreated samples) on the ammonium hydroxide concentration and temperature for treatments lasting 30 min.

tive in SCP obtained from algae than in SCP produced from yeast. In order to examine the influence of treatments on the digestibility, three protein isolates obtained under the optimal conditions listed in Table 5 were submitted to in vitro digestibility tests. The results achieved in the assays performed (93.1, 89.4, and 88.5% digestibility) demonstrated that the concentrates were excellent protein sources. Improvements of digestibility after alkaline treatments of protein substrates have been reported in the literature (27).

The biological value of protein concentrates is also dependent on their amino acid profiles. Table 6 shows the distribution of amino acids determined for untreated cells and for protein isolates obtained under the selected conditions. It can be observed that the amino acid pattern was very similar in both cases. The most important differences observed were the concentrations of aspartic and glutamic acid, which can be partly justified by errors in the integration of chromatograms owing to a poor resolution between both peaks. The results for protein content calculated from amino acid analysis were in fairly good agreement with those achieved with the biuret method.

Table 5
Operational Conditions Selected and Results Obtained

Operational conditions selected	Experimental and predicted results obtained under the selected conditions		
	Variable	Experimental value[a]	Predicted value
NH_4OH concentration = 3.6% ($x_1 = -0.25$)	Yield of treatment (y_1)	69%	71%
Temperature = 75°C ($x_2 = 1$)	NA content of processed biomass (y_2)	0.5%	0.5%
Reaction time = 30 min ($x_3 = 0$)	Protein content of processed biomass (y_3)	63%	65%
(Fixed variable: liquor/solid ratio = 12 g/g)	PNAR	95%	96%
	PPR	85%	88%

[a] The reported results are the mean of three experiments.

Table 6
Amino Acid Profile of Untreated Cells and Protein Isolates

Amino acid	Percent of the total amino acids	
	Untreated cells[a]	Protein isolates[a]
Asp	12.7	10.4
Glu	12.5	15.6
Ser	5.8	5.7
Gly	4.6	5.3
His	2.3	2.4
Arg	5.9	5.9
Thr	6.2	6.0
Ala	6.4	7.0
Pro	4.7	4.6
Tyr	3.7	3.6
Val	7.0	6.5
Ile	5.9	5.4
Leu	8.9	9.0
Phe	5.0	4.6
Lys	8.5	9.0

[a]The results are the mean of three experiments.

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