RESEARCH ARTICLE OPEN ACCESS

Utilisation Of Spirulinasp. And Chlorellapyrenoidosa Biomass For The Productionof Enzymatic Protein Hydrolysates

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ABSTRACT
This aim of this study was to assess the hydrolysis reaction of the biomass of *Chlorella pyrenoidosa*and*Spirulina*sp. LEB 18,using commercial proteases that act in different pH ranges, to obtain protein hydrolysates with promising application in food or food supplement, improving functional and nutritional food properties. Threecentral composite study designs were carried out for each microalga *(Chlorella* and *Spirulina).* The 2^3 type central composite design was utilized with three replications at the central point, varying the enzyme concentration (5 to 10 $U.mL^{-1}$), the concentrationof substrate (5 to 10 %) and reaction time (60 to 240 min), for a total of 11 experiments per planning. The highestdegrees of hydrolysis (52.9% and 55.31%) for*Spirulinaand Chlorella*,respectively, were obtained with 4 h of reaction. The results show that it is possible to obtain enzymatic protein hydrolysates with different DH from microalgae biomass.
Keywords- experimental design*;* enzymatic hydrolysis,microalgae, proteases

I. 1.INTRODUCTION

Microalgae have been the subject of biotechnology research due to their nutritional, economic and ecological importance. Many microalgae are used in food production because they provide various useful substances, such as vitamins, minerals, pigments, fatty acids and proteins [1].

*Spirulina*and*Chlorella*are microalgae with biomass that is rich in proteins, with values above 50%,biocompounds with high added value can be extracted from them.These microalgae are notable as theypossess a GRAS(Generally Recognized as Safe)certification from the FDA(Food and Drug Administration),which ensures they can be used as a food and drug [2].

The enzymatic hydrolysis of polymers in foods is an important process, used to improve the physical, chemical and functional properties of foods without damaging their nutritional value. It improves the absorption characteristics of proteins.[3] Protein hydrolysates have been consistently reported as a suitable source of protein for human nutrition because they are absorbed more effectivelyin the gastrointestinal tract when compared with intact protein or free amino acids[4], [5] and [6].

Many scientific studies have been carried out under different experimental conditions to obtain hydrolysates from different protein sources such as: whey [7] and [8], fish [9] and [10]and chicken [5]. Recently, proteins from microalgae have been recommended as an alternative source due to their abundant protein content and amino acid profile [11].The microalgae*Spirulina*sp.LEB 18 and*Chlorella pyreinodosa*have a high protein concentration in their biomass and are promising sources of protein for protein hydrolysates.

The enzymatic hydrolysis of cellular proteins fromgreen algae has been described as a highly promising method of improving the digestibility of protein and obtaining a balanced protein for human consumption [4] and [12].However, there are hardly any data available on the use of proteinfrom *Chlorella*and *Spirulina*asprotein hydrolysates for human nutrition.

The aim of this study was to assess the influence of variables of the process involved in enzymatic reactions to obtain protein hydrolysates, using three different commercial proteases that act in different pH ranges (alkaline, neutral and acid) with amicroalgal biomasssubstrate of *Spirulina and Chlorella.*

II. MATERIALS AND METHODS

2.1 Microalgae

The microalgae used for obtaining protein hydrolysateswere *Chlorella pyrenoidosa*,in powder form, produced by Fuqing King Dnarmsa Co. Ltd., China and *Spirulina*sp.LEB 18, isolated from Mangueira Lagoon[13]and produced in the pilot plant of the Laboratory of Biochemical Engineering,

atMangueira Lagoon (33° 30' 13''S e 53° 08' 59'' W) in Santa Vitória do Palmar, Brazil.The biomass of*Spirulina*sp.LEB 18 was ground in a ball mill (Model Q298, QUIMIS) and sieved.

2.2 Enzyme

The enzymes used wereProtemax 580Lof *Bacillus lichenformis*, ProtemaxN200 of *Bacillus subtilis*(Prozyn, São Paulo, Brazil) and pepsin derived from pig stomach (VetecQuímicaFina LTDA, Rio de Janeiro, Brazil).

Different proteases were used in order to assess the behavior of hydrolysis at different pH (alkaline, acid and neutral) and to enable a comparison between proteases that do not exist in the human gastrointestinal tract (GIT),such as pepsin, to obtain protein hydrolysates.

2.3 Enzymatic activity

The enzymatic activity of commercial proteases was determined according to the method described by Ma and colleagues [14].The tyrosine content of the supernatant was determined by colorimetry at 650nm usingFolin phenol reagent [15].The enzyme was inactivated by the addition of 10% trichloroacetic acid (TCA) and the activity defined as the amount of enzyme that releases 1μg of tyrosine per minute, under the conditions used in the study.

2.4 Percentual Composition

Total protein, ash, moisture and lipids were determined according to methods described by AOAC [16]. To determine the amount of protein, the total nitrogen micro-Kjeldahlmethod was used with a conversion factor of 6.25. Ash was determined by the gravimetric method in a muffle (550-600 °C) and moisture content by the gravimetric method in an oven (105 °C). The lipids were extracted with 2:1 (v/v) chloroform/methanol, purified with 0.9% (w/v) NaCl and 2:1 (v/v) methanol/water mixed according to Folch and Lees [17]and transferred to a rotary evaporator; the solvent was removed at approximately 37°C, the content of lipids was determined gravimetrically. The remainder was considered to be carbohydrates.

2.5 Experimental design

In this study, the factorial planning was used to determine which variables had significant effects on the degree of hydrolysis. Thus, the study assessed the influence of the enzyme concentration, substrate concentration and reaction time. The initial content of the substrate, the concentration of added enzyme and reaction time were the variables studied througha factorial design. Six2^3 type factorial planningswere carried out with three repetitions at the central point; the degree of hydrolysis of proteins was the dependent variable.Analyses were carried out in duplicate and the datawere statistically analyzed.Table 1 presentsthe matrix of the 2^3 central composite design with the levels and values of the independent variables used in the factorial designs.The proposed levels for each variable were based on preliminary experiments (data not shown).

Table 1. Matrix of the2^3 centralcomposite designwithactualandcodedvariables

Assay	CE ($U.mL^{-1}$)	CS (%)	t (min)
1	-1 (5)	-1 (5)	-1 (60)
2	+1 (10)	-1 (5)	-1 (60)
3	-1 (5)	+1 (10)	-1 (60)
4	+1 (10)	+1 (10)	-1 (60)
5	-1 (5)	-1 (5)	+1 (240)
6	+1 (10)	-1 (5)	+1 (240)
7	-1 (5)	+1 (10)	+1 (240)
8	+1(10)	+1 (10)	+1 (240)
9	0 (7.5)	0 (7.5)	0 (150)
10	0 (7.5)	0 (7.5)	0 (150)
11	0 (7.5)	0 (7.5)	0 (150)

[a]CE = enzyme concentration; CS =substrate concentration; t = time

The influences of the enzyme concentration, substrate concentration and reaction time on the degree of hydrolysis response were statistically analyzedin order to assess the effects and verify the empirical models through regression coefficients and analysis of variance (ANOVA) with a significance of 95%.

2.6 Enzymatic Hydrolysis Process

Six experimental designswerecarried out, namely C1, C2 and C3, for hydrolysis using *Chlorella pyrenoidosa* as a protein source,and S1, S2 and S3 for reactions using*Spirulina*sp.LEB 18. Thenumbers represent the use of different enzymes in the study (1: Protemax 580L, 2: Protemax N200, and 3: Pepsin); the biomasses were previously

characterized regarding their percent composition.[16]The assaysC1 and S1 were carried out withProtemax 580L,in bicarbonate-sodium carbonate buffer pH 9.5 at the optimum enzyme activity temperature of 60 °C; C2 and S2 were carried out with Protemax N200, in sodium phosphate buffer 0.2M pH 7.0 at the optimum enzyme activity temperature of 55 °C;and C3 and S3 were carried out the enzyme pepsin, in 0.1 M KCl-HClbuffer pH 2.3 at the optimum temperature of 37°C. All experiments were carried out with agitation of 180 rpm in a "Shaker" (Certomat BS-1), in aqueous solution with a total volume corresponding to 100mL in erlenmeyertype reactors.The amounts of enzyme and substrate added corresponded to the values established during the factorial design. The hydrolysis reactions were accompanied for 4 h.

2.7 Determination of the Degree of Hydrolysis

The analysis of the degree of hydrolysis (DH) was carried out at 0h, 1h, 2.5h and 4h. Aftereach elapsed time, 1 mL aliquots of hydrolysate were inactivated by the addition of 9 mL of trichloroacetic acid (TCA) 6.25% and maintainedat rest for 10 min. They were subsequently centrifuged for 5 min at 5000 rpm to remove the insoluble material precipitated by the TCA. The content of soluble proteins in the filtrate was determined using the Folin-Lowrymethod, expressed as mg of albumin.The degree of hydrolysis was estimated according to the method described by Hoyle and Marrit [18]and expressed as the percentage of soluble proteins in the TCA compared with the total initial protein amount, and calculated according to Equation (1):

$$\%DH = \frac{(PS_{tf} - PS_{t0}) \times 100}{P_t} \quad (1)$$

wherethe blank, PS_{t0}, corresponded to the amount of protein soluble in TCA 6.25% before addition of the enzyme;$PS_{tf}$was the amount of soluble protein in a given time after addition of enzyme and P_t was the total protein in the sample determined by micro-Kjeldahl (N x 6.25).

III. RESULTS AND DISCUSSION

The high protein content of some microalgae is the main reason for the selection of these micro-organisms as an unconventional source of proteins, with the aim of producing protein hydrolysates. The mean chemical composition and standard deviation of *Chlorella pyrenoidosa*and*Spirulina*sp.18 LEB used in this study is shown in Table 2.

Table 2. Percent composition of the microalgae*Chlorella pyrenoidosa*and*Spirulina*sp.LEB-18

Determinations (%)	*Spirulina* sp. LEB-18 $\left(\overline{X} \pm D.P\right)*$	*Chlorella pyreinoidosa* $\left(\overline{X} \pm D.P\right)*$
Humidity	11.99 ± 0.07	6.91 ± 0.06
Proteins	51.66 ± 1.87	50.80 ± 0.63
Ash	7.84 ± 0.02	6.66 ± 0.04
Lipids	7.06 ± 0.11	13.53 ± 0.22
Carbohydrates	27.45 ± 0.00	22.10 ± 0.00

* means ($\overline{X}$) and standard deviations (SD), results were expressed as mean and standard deviation of three determinations.

The protein concentration of the microalgalbiomasses was used to calculate the amount of biomass needed to obtain the suspensions of each experiment studied. The values found of protein concentration for both microalgae studied resemble those found in previous studies [13], justifying the choice of appropriate and high biomass potential of these micro-organisms for the production of protein hydrolysates with high value.Table 3 presents the 2^3 central composite design matrix with real and coded variables and the degree of response of hydrolysis corresponding to the maximum values achieved in each experiment.

Table 3. Matrix of the 2^3central composite design and degree of response of hydrolysis using*Chlorellapyreinoidosa*and*Spirulina*sp. LEB 18 for assayswith the enzymes Protemax 580L, Protemax N200 and pepsin.

Exp.	Codified values			DH (%)					
	CE ($U.mL^{-1}$)	CS (%)	t (min)	Protemax 580L		Protemax N200		Pepsin	
				C1	S1	C2	S2	C3	S3
1	-1	-1	-1	34.4	38.2	14.6	24.6	1.4	2.3
2	+1	-1	-1	34.8	42.7	18.7	24.4	2.3	2.8
3	-1	+1	-1	20.8	30.2	7.8	8.5	1.0	2.1
4	+1	+1	-1	25.2	33.9	10.3	12.2	1.6	1.9
5	-1	-1	+1	48.0	52.3	27.5	29.9	16.7	28.3
6	+1	-1	+1	52.9	55.3	29.8	35.3	18.3	31.8
7	-1	+1	+1	30.7	35.7	9.2	17.31	9.7	15.1
8	+1	+1	+1	34.0	42.1	16.3	22.8	10.6	16.7
9	0	0	0	32.5	49.7	13.1	27.3	8.6	8.2
10	0	0	0	33.0	48.8	12.6	26.6	8.5	8.2
11	0	0	0	33.6	49.7	12.7	26.1	8.4	8.1

Exp = experiment, CE = enzyme concentration, CS = substrate concentration, t = reaction time, DH = degree of hydrolysis, C1 = DH with Prozyn 580L in *Chlorella pyreinoidosa*, C2 = DH with Prozyn N200 in *Chlorella pyreinoidosa*, C3 = DH with pepsin in *Chlorella pyreinoidosa*, S1 = DH with Prozyn 580L in *Spirulina* sp. Leb18, S2 = DH in Prozyn N200 in *Spirulina* sp. Leb 18, S3 = DH with pepsin in *Spirulina*sp. Leb 18.

As we can see in Table 3 the degree of hydrolysis (DH) varied from 1% to 55.31% among the experiments studied,this can be justified by the variety of parameters used in preparing of enzymatichydrolysates obtained. The highestDH for both microalgae with the 3 different enzymes was achieved in experiment 6 (the reactional system consisting of 10 $U.mL^{-1}$ of enzyme and 5% substrate) at 4 h reaction.Schmidt e Salas-Mellado studiedthe same enzyme for the DHof chicken breast protein, and obtained a variation of 20.93% to 57.42%.[5]Zavareze and colleaguesstudied the influence of the action of different proteolytic enzymes (Alcalase, Flavourzymeand Novozym) in the DHof goatpulp *(Prionotuspunctatus),*and obtained a variation of 16.91% to 27.96% for different hydrolysis times [19].

Of the three enzymes studied,*Spirulina*sp. LEB 18 presented the best results in the process. There are hardly any comparative studies using commercial exogenous enzymes (Protemax 580L and Protemax N200) andendogenous enzyme (pepsin), since these enzymes have different optimum conditions (pH, temperature and time ofperformance) and different specific activities.

Enzymes have been compared by the use of optimal conditions in the process in the study using data provided by the manufacturers, without taking into account that the optimal conditions should also depend on the substrate used for the hydrolysis [20]. In this study, themicroalgae grew at different pH ranges,*Spirulina* LEB - 18 grows in culture media at alkaline pH in the range above 9.0 to 10.5 [21], [22] and [23].The*Chlorellapyrenoidosa*is grown in pH around 6.5-9.0 [24] and [25].Low degree of hydrolysis results were observed with the use of pepsin, which mayhave been caused by the low pH of the reaction mediumaltering the protein structure, thus hindering the action of the enzyme.Another possible factor that influenced the outcome of the degree of hydrolysis of proteins may be the differences between the optimum pHfor pepsin activity and the pHof media of both microalgae.Therefore, when the hydrolysis reaction was carried out using the enzymeProtemax 580L, which operates at alkaline pH, there were higher degree of hydrolysis values for both microalgae.

All the experiments with*Chlorella pyrenoidosa*as a source of proteins, presentedhydrolysis degree values lower than the experiments with *Spirulina*sp.LEB 18. This behavior of the degree of hydrolysis compared with raw materials may be due to the higher levels of lipids in*Chlorella*(91.64 % higher than the level found in *Spirulina*).The amount of lipids in raw material influences the hydrolysis process, because a relatively high amount could form protein/lipid complexes, which may be more resistant to enzymatic breakdown [26].

The enzymatic hydrolysis of algae cell proteins depends on the initial enzyme concentration. Independently of CE the enzymatic hydrolysis presented two major stages [27]. During the first two hours the hydrolysis could account for the soluble algae proteins. Afterwards, the insoluble proteins inside the cells presumably come into contact with the enzyme molecules. The globular structure of the major proteins of Chlorella biomass could be per se

an important limitation on the action of proteolytic enzymes.

Analysis of variance (ANOVA) was used to verify the mathematical model, shown in Table 4 for the DH of hydrolysed proteinsof *Chlorella pyrenoidosa*and*Spirulina*sp.LEB 18, obtained with the threestudied enzymes.

The data obtained in the experimental design were treated by statistical methods. This enabled us to obtain six encoded linear mathematical models of first order according to the equations shown in Table 5, which shows the response, as a function of the significant independent variables ($p < 0.05$); the results received an SSresidual treatment.

Table 4. Analysis of variance of the values of the degree of hydrolysis for the hydrolysates of*Chlorella pyrenoidosa*and*Spirulina*sp. LEB-18 obtained with Prozyn 580L, Prozyn N200 and pepsin, at a confidence level of 95%.

Outcome DH (%)	SourceofVariation	Sum ofSquares	DegreesofFreedom	MeanofSquares	F Calculated	Tabular F
C1	Regression	761.55	2	380.77	50.63	4.46
	Residues	60.21	8	7.52		
	Total	821.76	10			
C2	Regression	400.07	2	200.03	14.80	4.46
	Residues	108.93	8	13.50		
	Total	509.00	10			
C3	Regression	354.45	3	118.15	227.21	4.35
	Residues	3.69	7	0.52		
	Total	358.14	10			
S1	Regression	273.06	1	273.06	6.49	5.12
	Residues	378.5	9	42.05		
	Total	651.56	10			
S2	Regression	515.89	2	257.94	23.43	4.46
	Residues	88.13	8	11.01		
	Total	604.02	10			
S3	Regression	1057.5	3	352.5	48.48	4.35
	Residues	50.92	7	7.27		
	Total	1108.42	10			

Table 5. Equations obtained for the mathematical models from the values of degree of hydrolysis of *Chlorella pyrenoidoda* and *Spirulina*sp.

Outcome	PreditiveModelEquation	R^2
C1 (%)	= **34.51** + 1.66CE – **7.49CS** + **6.25t**+0.36CE*CS + 0.48CE*t – 1.67 CS*t	0.9847
C2 (%)	= **15.72** + 1.99CE – **5.88CS** + **3.92t**+0.40CE*CS + 0.35CE*t – 2.09 CS*t	0.9202
C3 (%)	= **7.91** + 0.49CE – **1.96CS** + **6.13t** - 0.12CE*CS + 0.12CE*t– **1.70 CS*t**	0.9960
S1 (%)	= **43.20** + 2.38CE – **5.73CS** + **4.95t**+0.16CE*CS + 0.31CE*t – 1.54 CS*t	0.8155
S2 (%)	= **23.18** + 1.81CE – **6.67CS** + **4.47t**+0.49CE*CS +0.92CE*t – 0.39 CS*t	0.9143
S3 (%)	= **11.41** + 0.67CE – **3.67CS** + **10.35t**+0.32CE*CS +0.60CE*t – **3.40CS*t**	0.9608

Values in bold are significant at 95% confidence.

Table 5 shows the correlation coefficient and the equation of the model for the experiments C1, C2, C3, S1, S2 and S3, where the calculated F values higher than the tabulated values.When calculated F ≥ tabulated F is obtained, we canstatistically validate the model ($p < 0.05$) [28].

The model enabled us to obtain response surfaces to establish the best conditions of substrate concentration, enzyme concentration and reaction time, to provide the highestdegree of hydrolysis of the proteins.The response surfaces are presented in Fig.1 (*Chlorella pyrenoidosa*) and Fig. 2 (*Spirulina* sp. LEB 18).

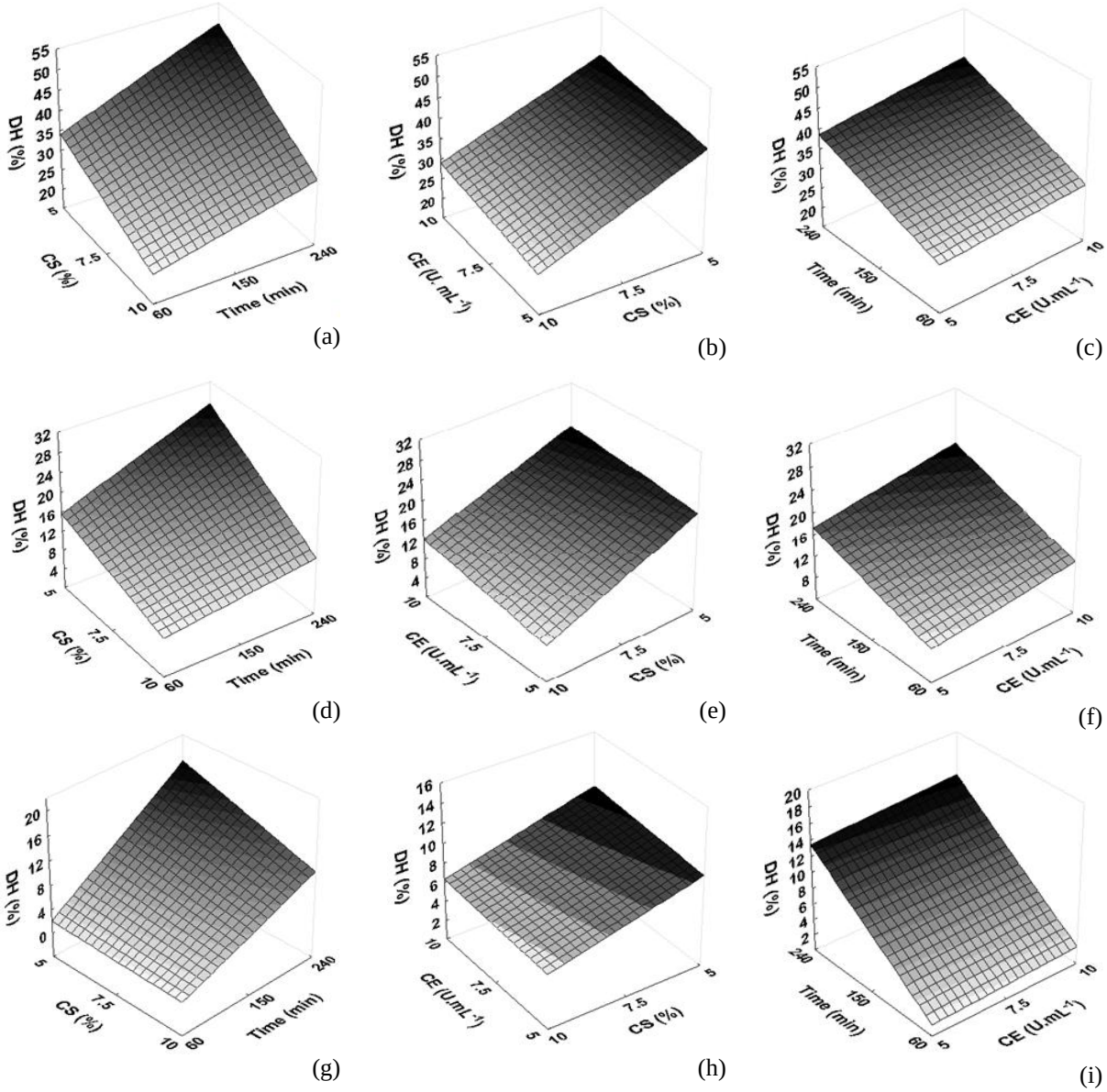
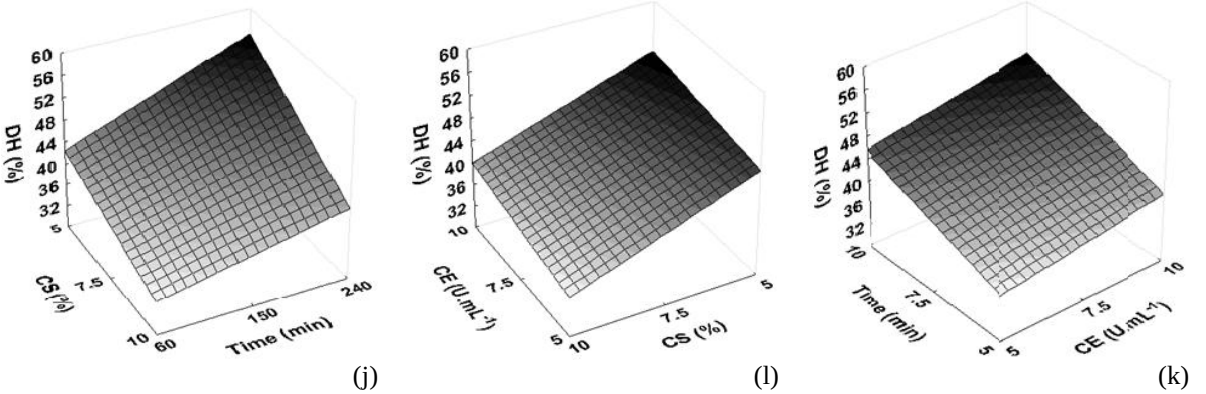


Figure 1. DH response surfaces in relation to substrate concentration, enzyme concentration and reaction time: (a), (b) and (c) surfaces of response for the microalga*Chlorella*with Protemax 580L; (d), (e) and (f) surfaces response for the microalga*Chlorella* with Protemax N200; (g), (h) and (i) surfaces response for the microalga*Chlorella* with pepsin.



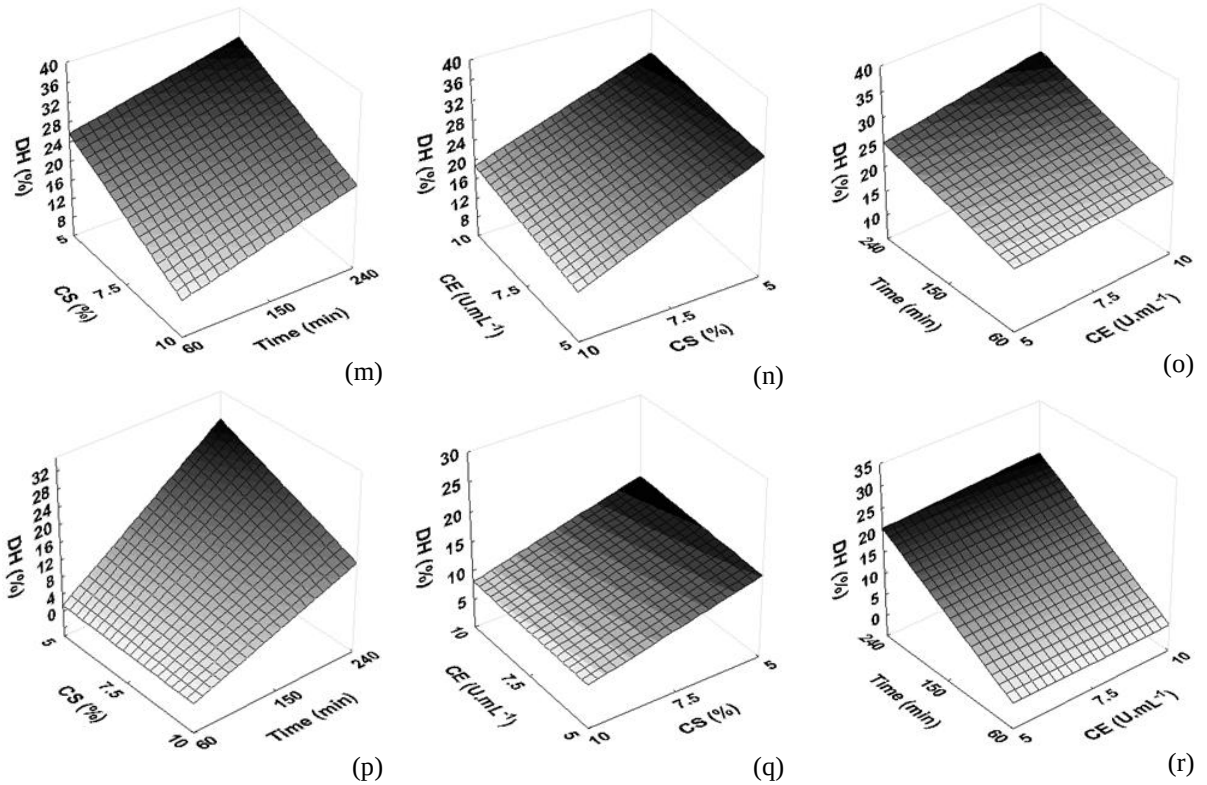


Figure 2. DH response surfaces in relation to substrate concentration, enzyme concentration and reaction time: (j), (l) and (k) surfaces of response for the microalga*Spirulina*with Protemax 580L; (m), (n) and (o) surfaces response for the microalga*Spirulina*with Protemax N200; (p), (q) and (r) surfaces response for the microalga*Spirulina* with pepsin.

The Fig. 1 and 2 show theresponse surfacesobtained for the interactions of the variables in the planningswith the microalga *Chlorella pyrenoidosa*: C1, C2 and C3; and with the microalga *Spirulina*spLEB 18: S1,S2 and S3.

Althoughprotemax 580L, protemax N200 and pepsin refer to distinct enzymesfor actingunder differentoptimal pH and temperature conditions, these studies showed similar patterns of their variables, and they can therefore be analyzed together.

In Fig. 1(a), 1(d), 1(g), 2(j) and 2(p)can be verified that with increasing substrate concentration at different levels of reaction time, there was a decrease in the degree of hydrolysis of the proteins for both microalgae studied.It could be also observed that the influence of the substrate concentration was higher in those studies that the protein source used was *Spirulina* sp. LEB 18, enabling to achieve degree of hydrolysis values of 56% (Fig. 2j). The substrate concentration is one of the factors that can affect the initial rate of enzymatic hydrolysis of protein. High concentrations of substrate can cause inhibition due to the formed product,because theenzymatic activityof the enzymes involved in this study can be inhibited by the product formed during the hydrolysis. Moreover, competition can be expected, due to the active site of the enzyme, between the original substrate and the peptides that are continuously released during the hydrolysis process. Each terminal acyl produced after hydrolysis of a peptide bond is an inhibitor, which can bind to the active site of the enzyme, forming an acyl-enzyme complex.

Santos and colleagues [29]studies the degree of hydrolysis of proteins in bluewings earobin *(Prionotuspunctatus)* and croaker *(Micropogoniasfurnieri)* and found a negative effect for the substrate concentration. Similar results were found by Schmidt and Salas-Mellado[5] who studied the degree of hydrolysis of chicken meat proteins.

Increasing the concentration of substrate, for Fig. 1(b) and 2(l), decreased the DH for the entire concentration range of the enzyme, reaching approximately 50 % and 56% when 5 % substrate concentration was used, respectively. As shown in Fig. 1(e), 1(h), 2(n) e 2(q) increasing the enzyme concentration from 5 $U.mL^{-1}$to 10 $U.mL^{-1}$and reducing the substrate concentration from 10 % to 5 %, increased the DH, with values higher than 30 % (Fig. 1c) and 36% (Fig. 2l). The enzyme concentration did not significantly influence the outcome, and when this variable is combined with other variables, such as substrate concentration, this interaction leads to an increase in the DH.

In Fig. 1(c), 1(f), 1(i), 2(k), 2(o) and 2(r) show that increasing the concentration of enzyme had a positive effect, but this was not significant in the plannings studied; a positive relationship of the concentration of enzyme with the outcome was expected, however, this variable was not significant in the concentration range studied (5 $U.mL^{-1}$ to 10 $U.mL^{-1}$), since the interaction between this variable with the reaction time (60 min to 240 min) caused up to 54% (Fig. 1c) for *Chlorella*hydrolysates and 56% (Fig. 2k) for *Spirulina*hydrolysates. The increase in enzyme concentration results in increased scission of the protein chains, increasing the amount of lower molecular sized protein and also the DH of the product, which increasedfurther with the increase in enzyme concentration, but not significantly. However, if there is no inhibitory process by the product, the increase of the enzyme concentration only accelerates the reaction, but will achieve the sameDH with a lower concentration of enzyme.

Proteins from microalgae can be used for human and animal nutrition, but the enzymatic hydrolysate is usually recommended to improve the digestibility of cell proteins due to the fact that most microalgae are indigestible to monogastric animals and humans [30]. Different proteolytic enzymes were used for production of microalgae cell proteins hydrolysates, and their properties were also studied, especially, including Chlorella. For example, Morris et al. [30] investigated the enzymatic of cell protein in green microalgae *Chlorella vulgaris*.

Morris et al.[4] examined the effects of oral administration of the enzymatic protein hydrolysate from green microalga Chlorella vulgaris. They found increase in hemoglobin concentration, protein content, oligosaccharidase activities in mice, suggesting that Chlorella protein hydrolysate can be used to develop specific formulations suitable for pharmacologic nutrition.

The effect of the type of enzyme was evaluated with the aim of obtaining the hydrolyzate with the highest percentage of degree of hydrolysis for use in foods compared with microbial enzymes Pepsin (animal), enzyme found in the human gastrointestinal tract. The relation of enzyme and substrate (E:S) was analyzed taking in account the process cost reduction for adaptation in large scale. Thus, the use of a lower ratio E:S is associated with the use of a lower amount of enzyme necessary for the hydrolysis and also the use of a lower concentration of the raw material.

The substrate concentration was the most important variable in the overall process.The negative value of its coefficient meant that the degree of hydrolysis of the microalgalproteins washighestfor 5% substrate.Other importantvariables were the reaction time and the interaction between the substrate concentrationand reaction time. The highest reaction time and the lowest substrate concentration resulted in the highesthydrolysis degree.

When the substrate concentration was increased,the hydrolysis of proteins was inhibited in all experiments. This behavior is caused by competition between substrate molecules, because a second substrate molecule binds to a site near the active site of the enzyme, thus reducing the initial speed of reaction, preventing the first molecule from binding properly to the active site or beingtransformed into product [31].

Many enzymes act preferentially on a single or various substrates, but in this case, with reduced efficiency. According to Tauber [32], pepsin hydrolyzesintact proteins, apart from some proteins such as keratin and mucins and slowly hydrolyzes albumin. In the experimental design of this study with pepsin, the variable that most influenced the process was the reaction time, which was not observed in other experiments. Therefore, the fact that longer reaction times resulted in a higher degree of hydrolysis can be explained by the slow hydrolysis of proteins of both microalgal biomasses.

The functional properties of the protein hydrolysates depends on the type of enzymes used in their preparation. This is mainly due to size differences and other physicochemical properties of the polypeptides produced during the hydrolysis [33].

The enzymatic hydrolysis of proteins results in a mixture of free amino acids, di-, tri-and oligopeptides, increases the number of polar groups and the solubility of the hydrolysate and thus modifies the functional characteristics of proteins, which usuallyimproves the functional quality [5].Because pepsin,Protemax N200 and Protemax 580L have different specificities, their hydrolysis process will result in protein hydrolysates with different peptides. The hydrolysates obtained with these enzymes, despite having a similar DH (between enzymes Protemax N200 and 580L), may have a very different peptide profile.

According toVioque et al. [34]hydrolysates with a degree of hydrolysis higher than 10 % can be used in medical and functional diets. The microalgae *Chlorella pyrenoidosa* and *Spirulin*a sp. have been studied due to its high protein content, therefore, can be used as a protein source, and these proteins include all the amino acids essential for growth and human health.Enzymatic hydrolysis of protein is important in obtaining of protein products that can be used in food supplements, increasing the nutritional and functional characteristics of foods. These protein hydrolysates are more easily digested and absorbed by the human body than intact proteins. The intake of hydrolysates rich in small peptides may be important in order to provide a better protein utilization,

particularly in individuals with allergies to certain proteins or in food intolerance, in the case of enzyme deficiency.

IV. CONCLUSION

*Spirulina*had the best results during the process for the three studied enzymes. Protemax 580L was most efficient in the hydrolysis of proteins of both microalgae.Statistical analysis of the results showed a negative effect of the substrate concentration and a positive effect of the reaction timeon the DH.Protein hydrolysates with a wide range of DH,likethose of this study, can be used as food ingredients intended for individuals with allergies to certain proteins or with food intolerance. The results show that it is possible to obtain enzymatic protein hydrolysates with different DH from microalgae biomass.

V. ACKNOWLEDGEMENTS

Coordination of Improvement of Higher Level/Nanofotobiotec Network – Integrated Network of Nanotechnology and Microalgal Biotechnology for Scientific/Technological and Human Resources Training and National Council of Scientific and Technological Development - Nanofotobiotec Network III, for financial support.

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