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New Protein Hydrolysate from *Spirulina platensis* used as Peptone in Microbiological Culture Media

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ABSTRACT

Spirulina platensis algae has been used worldwide as an additive in foods and as nutraceutical for different deficiencies and illness due to its high protein content. The main goal of this study was to obtain a peptone from *S. platensis* appropriate for the growth promotion of bacteria and yeast in culture media. The hydrolysis process of *S. platensis* by papain was optimal at 70 °C, pH 5, with an enzyme to substrate ratio of 20 % (papain with 3000 Umg^{-1}). *S. platensis* papainic peptone (SPPP) showed high amino-acid content (67.09 g/100 g), satisfactory level of N_{am}/N_t ratio (32.5 %), and low loss on drying (3.8 %). Furthermore, we evaluated the microbiological performance of SPPP in general and differential media for eight different microorganism strains. The results indicate that SPPP can be considered an efficient nitrogen source in solid and liquid microbiological culture media for gramnegative, and grampositive bacteria, and yeast. This new peptone is safe for microbial growth and is suitable for microbiological diagnostic, supporting the value of a non-animal protein source in the pharmaceutical industry.

1. Introduction

Protein hydrolysates are key components of a great number of microbiological culture media [1-3]. They contribute to bacterial and fungal growth promotion with significant amounts of amino acids, macro- and micro-elements, vitamins and other organic compounds [1, 4-7]. Protein hydrolysates also improve the medium performance due to their buffering capacity, helping to keep the pH in an optimum range [8]. Until now, most of the protein sources for hydrolysate production are obtained from food industry in which high valuable edible products such as casein are processed [1, 9]. In that way, the protein hydrolysates production competes with human nutrition [10]. Nowadays food by-products, such as blood, liver, brain, and beef hearts [11]; sheep visceral mass, horns [12]; poultry by-products [13]; and fish by-products [14] have being widely used in order to decrease the dependence of culture media production from these valuable protein sources. Nevertheless, these by-products are worldwide incorporated to less expensive, but nutritive foods and feeds.

Given the growing requirements for raw materials of animal origin to be approved and certified for the use in pharmaceutical industry, the development of peptones from non-animal protein sources is becoming more interesting. As an alternative protein source for hydrolysate production, the use of vegetable [1], microbial and algal sources [15] is gaining more interest.

Arthrospira platensis, also named *Spirulina platensis* (SP), is classified as a cyanobacterium [16, 17] and commonly considered as green-blue algae [18]. It has been used worldwide as an additive in foods and as nutraceutical for different deficiencies and illness [19]. SP is rich in proteins, vitamins, *β*-carotene, micro- and macro-elements and polyunsaturated fatty acids [16, 20-23]. *Spirulina* grows in alkaline waters in Africa, Asia, North and South America [24], areas of incident solar irradiation from 600 to 850 KJ/cm^2 per year [25]. Like other microorganisms, *Spirulina* grows fast with low nutritional needs and therefore is superior for biomass production over higher plants [26]. Furthermore, it can been cultivated in a semi continuous system and harvested continuously all year round.

Only few works have used SP as a substrate for protein hydrolysates [15] or in culture media composition [27]. However, the entire SP biomass has been used as starter culture promoter for lactic-acid bacteria in the production of probiotics [28, 29]. In this work, we developed a peptone from *Spirulina platensis* using enzymatic hydrolysis with papain and evaluated their suitability as microbial growth media appropriate for the culture of gramnegative, and grampositive bacteria, and yeast. Our study takes advantage of the excellent nutritional properties of SP protein hydrolysate for microorganism growth promotion in general purpose and differential culture media.

2. Experimental Methods

2.1 Material

SP mass was obtained from Genix S.S. (Cuba). Briefly, the *Spirulina* culture was started from subcultures of single cyanobacterial laboratory isolates in Zarrouk's synthetic medium [30]. Further, the cultures were continued in 5000 m^2 of water in open pounds with adequate deep conditions, stirring, and optimal nutrients concentration. The algae was harvested by filtration under vacuum, washed and the remaining biomass with a 20 % of solid content was dehydrated and pasteurized. A dry product was obtained as 4 cm length and 0.8-1.6 mm thickness flakes of intense green-bluish color and with 5-7 % humidity.

Papain from *Carica papaya* (6000 Umg^{-1} and 3000 Umg^{-1}) was purchased from Merck (Darmstadt, Germany).

2.2 Preparation of Spirulina platensis Hydrolysates

Twelve hydrolysates were prepared according two fully randomized factorial experimental designs. The effects of the enzyme concentration (% of substrate weight, w/w), substrate to water ratio (SWR), and pH were evaluated (Table 1). SP flakes (50 g) were mixed with deionized water at different SWR and hated up to 70 °C in a water bath (Haake-DC10, Germany). The pH of the mixture was adjusted to the values referred in Table 1 by adding HCl (1:1) or NaOH solutions (10 N). Then, papain was added at the desire concentration and the hydrolysis was carried out for 4 h at 70 °C in a water bath under constant stirring with a Heidolph helicoidal mixer (Germany). After passing the reaction time, the enzyme

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was inactivated by heating at 98 °C for 15 min. The pH was adjusted to 6.5 and the hydrolysate mixtures were centrifuged at 4043 *g* (Jouan CR4-12, France). Finally, the supernatants were filtered through a clarifying plate filter under negative pressure and stored at -20 °C for further experiments.

Table 1 Hydrolytic conditions used in preparing SP hydrolysates

Hydrolysates	pH	SWR	PC
	First design	Papain (6000 Umg^{-1})	
H1	5.0	1:40	1
H2	7.5	1:40	1
H3	5.0	1:20	1
H4	7.5	1:20	1
H5	5.0	1:40	5
H6	7.5	1:40	5
H7	5.0	1:20	5
H8	7.5	1:20	5
	Second design [a]	Papain (3000 Umg^{-1})	
H9	5.0		30
H10	6.5		30
H11	5.0		40
H12	6.5		40

SWR =subsrate:water ratio (w/w), PC = papain concentration (w/w of substrate weight), [a] In this group of experiments, SWR was set up to 1:20

In order to obtain *S. platensis* papainic peptone (SPPP) at a pilot scale, 5 kg of SP flakes were hydrolyzed as described above, setting the SWR to 1:20 (w/w), adding papain (3000 Umg^{-1}) at 20 % and adjusting the pH to 5. In this case, after the hydrolysis process, the clarified supernatant was concentrated in a rotary vacuum evaporator (IKA-Labortechnik, Germany) up to 40 % dry matter and dried at 180 °C to 90 °C in a spray dryer (Niro Atomizer, Denmark).

2.3 Analytical Methods

The amino nitrogen (N_{am}) was determined using the formol titration method [31] and the total nitrogen (N_t) content was assessed by the Kjeldahl method [32]. The degree of hydrolysis (DH, %) was calculated using the ratio between N_{am} and N_t [(N_{am}/ N_t)*100 %] [33]. Chloride ions (as NaCl) were determined by the Volhard method [34]. Loss on drying (LD, %) was estimated by a standard gravimetric method [35]. The nitrogen content in different hydrolysate fractions (total proteoses, primary and secondary proteoses, peptones and amino-acids) was measured as described by Bridson and Brecker [36]. The amino-acid analysis was carried out after hydrolysis of the SPPP [37] in an Alpha Plus automatic amino-acid analyzer (Alpha Plus, Pharmacia, Sweden). The free tryptophan content was measured with a spectrophotometer by a colorimetric method [34] (PU 8620, PHILIPS, United Kingdom).

2.4 Bacterial and Yeast Strains

Different bacterial and yeast strains from the American Type Culture Collection (ATCC) were tested in this study: *Escherichia coli* 25922, *Staphylococcus aureus* 25923, *Salmonella* Typhimurium 14028, *Enterobacter aerogenes* 13048, *Shigella flexneri* 12022, *Candida albicans* 26790, *Saccharomyces cerevisiae* 9763, and *Saccharomyces uvarum* 9080. All the strains were maintained between transfers on tryptic soy agar slants at 2-8 °C.

2.5 Microbiological Analysis

The capacity of SPPP to serve as a source of carbon and nitrogen for growth of microorganisms was tested in different culture media. The culture media used were nutrient agar, Saboraud dextrose agar, and Kligler iron agar prepared according to Oxoid Ltd. (United Kingdom) [38] with either SPPP or bacteriological peptone (BP, Oxoid) [38], as reference peptone.

In order to prepare an initial suspension, colonies from fresh (18-24 h) reference culture in tryptic soy agar were suspended in sterile saline solution (0.85 % w/v) and homogenized by vortex (VF2, IKA-Labortechnik, Germany). The microbial cell concentration was adjusted to OD_{580} of 0.25 (approximately $3.0x10^8$ $CFUmL^{-1}$) using a UV-visible spectrophotometer (PU 8620, PHILIPS, Pye Unicam Ltd., United Kingdom). SPPP biological reactivity was assessed by three different tests (indol production, Voges-Proskauer reaction, and H_2S production) according USP [35] after the inoculation of 0.2 µL of the initial suspension. The performance characteristics of SPPP in Kligler iron agar were tested with of 0.2 µL of the initial suspension by streaking the slant and stabbing the butt with an inoculating needle. Further, decimal dilutions from the initial suspension were prepared in sterile saline solution to evaluate the performance characteristics of SPPP in nutrient agar and Saboraud dextrose agar. Inoculation of 0.2 mL suspension with 3.0×10^2 $CFUmL^{-1}$ were applied by spread surface in both media. All cultures were incubated at 35 ± 2 °C and read after 18–24 h for growth and reactions. Colony forming units (CFU) enumeration was executed with a colony counter (Gallenkamp, United Kingdom) in triplicates. The productivity ratio (P_R) of SPPP was calculated using the following equation: $P_R=(N_s/ N_o)* 100$; where N_s is the total colony count obtained from the culture medium with SPPP and N_o is the total colony count obtained from the defined reference culture medium tested [39].

2.6 Experimental Design and Statistical Analysis

In the present study we follow two experimental designs. The first one included 3 independent variables, x_1 (pH), x_2 (SWR), andx_3 (PC) at two levels (-1 and 1). The second experimental design involved 2 factors as independent variables where x_1 (PC) and x_2 (pH) at two levels (-1 and 1). The dependent variables (y) evaluated in both designs were the N_{am} and N_t. The following polynomial equations were fitted for each dependent variable in the first (1) and second (2) experimental designs:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{1\,2} x_1 x_2 + b_{2\,3} x_2 x_3 + b_{1\,3} x_1 x_3 + b_{1\,2\,3} x_1 x_2 x_3 \quad (1)$$

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 \quad (2)$$

where, b_0 - constant coefficient, b_i – coefficient for each independent variable.

All data collected were processed and analyzed using "Statistica8" package (StatSoft, Inc. USA), based on the response surface methodology (RSM), in which, matrix operation was the main feature to find the coefficients of the regression equation. Significance ($p < 0.05$) was set by Student's test. Model adequateness verification was achieved with the Fisher test. When required, for the determination of significant differences, the Duncan's multiple range test was used ($p < 0.05$). All reactions were conducted in triplicate.

3. Results and Discussion

3.1 Influence of Independent Variables on the Hydrolysis of SP

Response surface methodology was employed to evaluate the enzymatic hydrolysis process on SP by combining three different independent variables (pH, SWR and PC) with N_{am} and N_t as the response. The polynomial equations fitted to the experimental data of the first 2^3 full factorial design for predicting N_{am} and N_t are given in Eqs. 3 and 4, respectively:

$$N_{am} = 1.57 - 0.25x_1 - 0.10x_2 + 0.14x_3 - 0.20x1x_2 \quad (3)$$

$$N_t = 7.03 - 0.33x_1 - 0.97x_1x_2 - 0.31x_1x_3 - 0.41x_2x_3 \quad (4)$$

The influence of the independent variables (pH, SWR and PC) on the N_{am} and N_t can be visualized in the response surface and contour plots of the enzymatic hydrolysis conditions showed in Figs. 1 and 2, respectively. The results indicate that for the Nam content, the pH (x_1) and its combination with PC (x_1x_2) has a significant effect ($p<0.05$), followed by SWR (x_3) and PC alone (x_2).

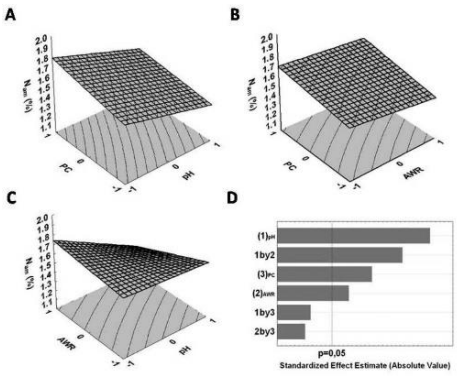

Fig. 1 Surface plots of the influence of different process parameter of the first experimental factorial design on amino nitrogen content. (**A**)relationship between papain concentration (PC) and pH (**B**)relationship between papain concentration (PC) and water ratio (SWR) (**C**)relationship between water ratio (SWR) and pH on the N_{am} content (%) (**D**)Pareto chart of standardized effects of the dependent variable N_{am}. (-1): lower level of the independent variables of the first experimental design; (1): upper level of the independent variables of the first experimental design

Furthermore, the combination of any two of all three independent variables and the pH alone had influence on N_t content; nonetheless, SWR and PC alone were not significant ($p>0.05$). In this first experimental design, the highest N_{am} and N_{am}/N_t values were 1.81 % and 23.78 %, respectively. These values were achieved at pH 5, SWR 1:20, and PC 5 %. Since the highest Nam value (1.81 %) was lower than the value (2.4 %) usually achieved for the bacteriological peptone [38] used as commercial control in our study, we decide to conduct a second experimental design to standardize the enzyme concentration and the pH.

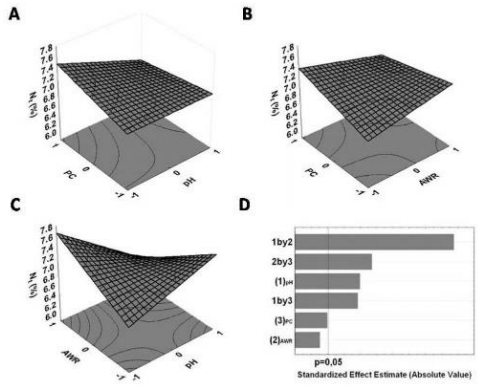

Fig. 2 Surface plots of the influence of different process parameter of the first experimental factorial design on total nitrogen content. (**A**)relationship between papain concentration (PC) and pH (**B**)relationship between papain concentration (PC) and water ratio (SWR) (**C**)relationship between water ratio (SWR) and pH on the N_t content (%) (**D**)Pareto chart of standardized effects of the dependent variable N_t. (-1): lower level of the independent variables of the first experimental design; (1): upper level of the independent variables of the first experimental design

The second 2^2 experimental design showed de following relation between N_{am} and the independent variables pH (x_1) and PC (x_2):

$$N_{am} = 3.35 - 0.13\, x_1 + 0.07\, x_2 \qquad (5)$$

Fig. 3 shows the influence of pH and PC on the N_{am} content during SP hydrolysis. Both variables showed a significant effect on N_{am} content; however, the combination of the two did not influence the hydrolysis process. In this experimental design, the highest N_{am} value (3.42 %) was achieved with a PC concentration of 40 % at a pH value of 5. Nevertheless, at a PC concentration of 30 %, the N_{am} value obtained was 3.41%, higher than the value (2.4 %) usually achieved for the BP [38]. Furthermore, the independent variables did not have any significant impact over the N_t content ($p < 0.05$).

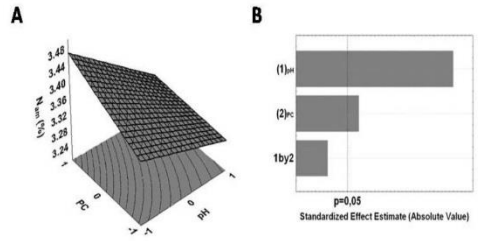

Fig. 3 Surface plots of the influence of different process parameter of the second experimental factorial design on total nitrogen content. (**A**)relationship between papain concentration (PC) and pH on the N_{am} content (%) (**B**)Pareto chart of standardized effects of the dependent variable N_{am}. (-1): lower level of the independent variables of the second experimental design; (1): upper level of the independent variables of the second experimental design

Next, we scale up the hydrolysis process in order to obtain SPPP for further experiments. Taking into account the results from the previous experimental designs, the hydrolysis of SPPP was conducted setting the SWR to 1:20 (w/w), adding papain (3000 Umg^{-1}) at 20 % of substrate weight, and adjusting the pH to 5.

3.2 Characterization of Spirulina platensis Papainic Peptone

Some properties of the SPPP were determined and compared with those of bacteriological peptone used as commercial control (Table 2). SPPP proved to be rich in both organic and inorganic materials essential to microorganism culture media as sources of carbon, nitrogen and minerals. SPPP has lower total nitrogen content than bacteriological peptone. However, SPPP has higher N_{am}/N_t ratio, indicating the quality of the protein hydrolysate. The hydrolysate's total nitrogen content is distributed in fractions of different molecular weight, with the free amino acids (4.24 %) and peptones (2.49 %) fractions having the highest values (Table 2). The loss on drying value was lower than 7 %, common for most commercial peptones and hydrolysates in culture media production and similar to the value observed for bacteriological peptone. The chloride content (measured as NaCl) was 8.42 % and the pH value was close to neutrality (6.97).

Table 2 Composition of *S.platensis papainic* peptone (SPPP) obtained at pilot scale

Component	SPPP (n=3)	BP[a]
Total nitrogen (%)	9.64 ± 0.31	13.6
Amino nitrogen (%)	3.13 ± 0.04	2.4
Amino/Total nitrogen (%)	32.52 ± 0.9	18
Nitrogen of total proteoses (%)	0.68 ± 0.05	
Nitrogen of primary proteoses (%)	0.40 ± 0.02	
Nitrogen of secundary proteoses (%)	0.28 ± 0.04	
Nitrogen of peptones (%)	2.49 ± 0.24	
Nitrogen of amino acids (%)	4.24 ± 0.11	
Loss on drying (%)	3.80 ± 0.01	3.7
Chlorides (%)	8.42 ± 0.04	3.7
pH (2 % w/v solution)	6.97 ± 0.02	7.2

[a]*BP, Oxoid Ltd.*

Table 3 lists the amino-acid composition of the SPPP compared with that of BP. The amino-acid composition of SPPP clearly indicated the presence of several essential amino acids like valine (4.17 %), lysine (3.64 %), leucine (1.81 %), isoleucine (1.57 %), and phenylalanine (1.01 %). The obtained peptone have lower levels of arginine, glycine, proline, and tryptophan than those of the reference peptone. However, an increased content of alanine, aspartic acid, cysteine, glutamic acid, methionine, serine, threonine, valine, and tyrosine was found.

Table 3 Amino-acid composition of *Spirulina platensis* papainic peptone (SPPP) and of commercial bacteriological peptone

Amino-acid	SPPP (g/100 g)	BP[a] (g/100 g)
Alanine	11.75	4.28
Arginine	0.36	4.58
Aspartic acid	6.05	5.86
Cysteine	5.15	0.84
Glycine	7.23	7.75
Glutamic acid	11.23	10.35
Histidine	0.92	NR[b]
Isoleucine	1.57	1.02
Leucine	1.81	3.65
Lysine	3.64	4.04
Methionine	4.52	1.27
Phenylalanine	1.01	2.69
Proline	0.70	6.25
Serine	2.69	1.76
Threonine	2.56	1.47
Tryptophan	0,75	0.89
Tyrosine	0.98	0.33
Valine	4.17	3.85

3.3 Microbiological Test of Spirulina platensis Papainic Peptone

In order to evaluate the microbiological performance of our peptone, we study the behavior of different bacteria strains by performing a group of biochemical tests (Table 4). Indol and acetyl methyl carbinol (AMC) production (Voges-Proskauer reaction) was tested with *E. coli* and *E. aerogenes*. *E. coli* was able to produce indol with either, SPPP or bacteriological peptone, while no production of AMC was detected in any case. In the other hand, *E. aerogenes* did not produce indol but was positive to Voges-Proskauer reaction when culture with SPPP or with bacteriological peptone. Hydrogen sulfide was observed in the culture of *S.* Typhimurium with either SPPP or bacteriological peptone (Table 4).

Table 4 Biological reactivity of the *Spirulina platensis* papainic peptone (SPPP) with reference collection bacterial strains

Microorganism	Variant	H_2S	Indol	AMC
Escherichia coli	SPPP		+	–
ATCC 25922	Control		+	–
Enterobacter aerogenes	SPPP		–	+
ATCC 13048	Control		–	+
Salmonella Typhimurium	SPPP	+		
ATCC 14028	Control	+		

Next, we study the performance characteristics of SPPP in Kligler iron agar with different bacteria strains (Table 5). Both *S.* Typhimurium and *S.*

flexneri initially produced a yellow slant due to the acid created in the dextrose fermentation process. Once the dextrose supply was exhausted in the aerobic environment of the slant, the reaction was reverted to alkaline (red slant) due to oxidation of the acids. In the butt, the color remained yellow (acid), due to the absence of enough oxygen to revert the fermentative reaction. In the other hand, *E. coli* produced a yellow slant and butt due to the lactose fermentation, which originates enough acid to maintain an acidic media under aerobic conditions. This bacterium also produced gas in the butt, while no gas production was observed for *S.* Typhimurium and *S. flexneri*. Hydrogen sulfide production was detected for *S.* Typhimurium, and not for the other two strains, as evidence by the black color throughout the butt of the tubes.

Finally, we evaluated the P_R of two media containing SPPP: nutrient agar for bacteria and dextrose Sabouraud agar for yeast. A P_R of 100 % was achieved for *E. coli*, and of 85.4 % ± 9.5 for *S. aureus*, based on triplicate counts of plates containing nutrient agar formulated with SPPP against the medium containing bacteriological peptone (Table 6). Dextrose Sabouraud agar showed a P_R of 89.5 % ±19.3 for *C. albicans*, 102.6 % ± 23.5 for *S. cerevisiae,* and 113.9 % ± 21.7 for *S. uvarum*.

Table 5 Performance of the *Spirulina platensis* papainic peptone (SPPP) and bacteriological peptone from Oxoid Ltd. (Control) in Kligler iron agar

Microorganism	Variant	Slant	Butt	H_2S	Gas
Escherichia coli	SPPP	Acid	Acid	-	+
ATCC 25922	Control	Acid	Acid	-	+
Shigella flexneri	SPPP	Alcaline	Acid	-	-
ATCC 12022	Control	Alcaline	Acid	-	-
Salmonella Typhimurium	SPPP	Alcaline	Acid	+	-
ATCC 14028	Control	Alcaline	Acid	+	-

H_2S– hydrogen sulfide

Table 6 Productivity ratio of different bacteria and yeast collection strains in two culture media formulated with the *Spirulina platensis* papainic peptone and with common nutritive bases as control media

Microorganism	Productivity ratio (%) in culture medium			
	Nutrient agar		Dextrose Sabouraud agar	
	Media	Sd	Media	Sd
E. coli ATCC 25922	100.0	18.0	-	-
S. aureus ATCC 25923	85.4	9.5	-	-
C. albicans ATCC 26790	-	-	89.5	19.3
S. cerevisiae ATCC 9763	-	-	102.6	23.5
S. uvarum ATCC 9080	-	-	113.9	21.7

Sd – standard deviation

The main characteristics of protein hydrolysates from food and food by-products are their high amino/total nitrogen content ratio, low humidity and broad essential amino-acid spectrum. Nitrogen from proteins is considered the most relevant source of this element for the metabolic process of bacteria and yeast [1, 10, 40, 41]. In order to evaluate the ability of a non-animal protein source as a peptone suitable for microbiological diagnostic, in the present work we developed and characterized a protein hydrolysate from SP. This cyanobacterium is widely distributed in nature and has been used for many years as additive since it has a high protein content and nutritional value. For example, SP contains high amounts of proteins, typically in the order of 50-60 mass-% of the dry weight [18, 21, 42] and a good array of amino-acids, minerals (K, Se, Zn, Ca, Fe, P, Mg, Cr, Cu, Mn), vitamins (vit C, E, folic acid, B12), and carbohydrates [16, 23].

The data obtained in the present study depicted that the hydrolysis process of SP was dependent on the papain concentration, pH and substrate to water ratio. In the initial experiments, as optimal conditions for SP hydrolysis we used a SWR of 1:20 (w/w), papain (3000 Umg^{-1}) at 30 % of substrate weight, and adjusted the pH to 5. These results were consistent with previous findings in which pH 5 was confirmed to be optimum for different substrates such as bovine meat, beef heart, and soy [11, 41].

For a pilot scale production of SPPP, PC value was diminished to 20 % (w/w of substrate weight) in order to try to reduce the cost of any future industrialized technological process. At this scale, it was possible to achieve N_{am} levels similar to those obtained at laboratory scale for SP hydrolysates. The obtained SPPP showed a loss on drying less than 4 %, value that guarantees a higher stability of the product during the storage due to prevention of chemical reactions [43]. This value agrees with those reported for heart dehydrated enzymatic digests (2.8-6 %) and bacteriological peptones (3-6 %) [8], as well as with the requirements (less than 8 %) stablished by Mourey et al. [44] and USP 35 [35]. Protein hydrolysates contain different levels of N_{am}, with values ranging from 1 to 7.6 % for gelatin and meat peptone, respectively. The N_{am} content in SPPP was equivalent with the levels in heart peptones (2.8-5.6 %) and was very close to the 2.0-4.0 % observed for a heart extract obtained by papainic hydrolysis [8]. The N_t value of the closer peptone prototypes fluctuates between 12.5 and 14 % [8]. Furthermore, SPPP degree of hydrolysis was of 32.52 %, value that fits in the range described for other peptones. Neklyudov et al. [2] reported a 35-40 % degree of hydrolysis for a protein mixture from muscle and bone tissues obtained with an enzymatic system derived from a porcine pancreatic cell suspension. Morris et al. [40] obtained a hydrolysis degree (N_{am}/N_t) of 20-22 % for a *Chlorela vulgaris* hydrolysate while Hongfei et al. [1] described a 20.15 % for soy hydrolysate's.

The molecular weight distribution of SPPP nitrogen fractions corroborated the high hydrolysis degree of the product, being the nitrogen of the free amino-acids and peptones the most significant fractions. These data agree with experiments carried out by Neklyudov et al. [2], who showed that the content of free amino-acids in a dry hydrolysate reached 25-30 %, and the nitrogen of amino groups in amino-acids and lower peptides was 4-6 % [2]. In another study carried out by Silvestre et al. [45], the best peptide profile was achieved for the hydrolysates obtained with papain in comparison with pancreatin supporting the use of papain in our study. The chloride content (as NaCl) and the pH value were, as expected, similar to the values observed for peptones available in the market [8].

The amino-acid composition of the SPPP clearly indicated the presence of several essential amino acids like isoleucine (1.57 %), leucine (1.81 %), lysine (3.64 %), phenylalanine (1.01 %), and valine (4.17 %), thus, making it nutritionally beneficial. However, amino-acids such as arginine, histidine, leucine, lysine, phenylalanine, and proline were found at lower levels when compared to bacteriological peptone. These differences could be due to the protein source and the method employed to obtain the peptone. The "soft" parameters employed during the hydrolysis process (temperature below 100 °C, no extreme pH, and short time) could account for the high overall amino-acid content (67.09 g/100 g) obtained from the native proteins, enough to supply the nutritive requirements to promote bacterial growth.

Regarding the fact that peptones in microbial media provides the main sources of nitrogen, carbon and other nutrients, a successful peptone source is better evaluated by the growth and performance of the microorganism of interest [46]. In the present work, the P_R values were much higher than 70 % established by ISO/TS 11133-2 (2003E) [39] as a quality control requirement for culture media. Akhapkina et al. [47] developed an enzymatic hydrolysate from *C. vulgaris* able to support the growth of the gramnegative bacterium, *Pseudomonas aeruginosa*. In the other hand, SP powder proved to improve the growth and viability of different lactic bacteria [29]. It has also been proven that green-blue SP and the rest of the metabolites added to the culture medium acted as microorganism growth promoters [48].

Biological reactivity is another key peptone´s attribute designed to be used in culture media for microorganism's identification and differentiation [8]. In general, characteristic positive and negative reactions were observed for both, SPPP (experimental) and bacteriological peptones (control) in Kligler iron agar, a very frequently used solid medium for *Enterobacteriaceae* differentiation by dextrose and lactose fermentation with acid and gas production, as well as hydrogen sulfide (H_2S) production from sulfur containing amino acids. Both media showed same reactions and it was possible to finally demonstrate that the experimental peptone does not contain fermentable carbohydrates in significant amounts that could limit its use as a component in media for bacterial identification or differentiation.

4. Conclusion

Spirulina platensis cyanobacterium, especially in its dried form, is suitable for the highly nutritive hydrolysate protein preparation, rich in amino acids with a high hydrolysis degree and low humidity content. Papain is an appropriate enzyme for such purpose, and the process could be driven upon mild pH and temperature with an appropriate water and enzyme to algal mass ratios, followed by a clarification and spray drying processes yielding a characteristic peptones product. The hydrolysate is useful as a peptone in solid and liquid culture media due to its characteristic biological reactivity and productivity ratio for gramnegative and grampositive bacteria, and yeast. In conclusion, SPPP has a high nutrient content, is safe for microbial growth and is suitable for microbiological diagnostic, supporting the value of a non-animal protein source in the pharmaceutical industry.

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References

[1] Z. Hongfei, B. Fengling, Z. Fang, P. Walczak, J. Xiangning, et al., Characterization of soybean protein hydrolysates able to promote the proliferation of *Streptococcus thermophilus*, J. Food Sci. 78(4) (2013) 575-581.

[2] A.D. Neklyudov, A.V. Berdutina, A.N. Ivankin, B.S. Karpo, Kinetic characterization of enzymatic hydrolysis of complex protein substrates for producing nutrient media, Prikl. Biokhim. Mikrobiol. 38(4) (2002) 381-388 [Article in Russian].

[3] R. Safari, A. Motamedzadegan, M. Ovissipour, J.M. Regenstein, A. Gildberg, et al., Use of hydrolysates from yellowf in tuna (*Thunnus albacares*) heads as a complex nitrogen source for lactic acid bacteria, Food Bioprocess Technol. 5(1) (2012) 73-79.

[4] L. Beaulieu, M. Desbiens, S. Thibault, J. Thibodeau, Pelagic fish hydrolyzates as peptones for bacterial culture media, Can. J. Microbiol. 55(11) (2009) 1240-1249.

[5] M. Hasan-Beikdashti, H. Forootanfar, M.S. Safiarian, A. Ameri, M.H. Ghahremani, et al., Optimization of culture conditions for production of lipase by a newly isolated bacterium *Stenotrophomonas maltophilia*, J. Taiwan Inst. Chem. Engg. 43(5) (2012) 670-677.

[6] A. Salihu, Z. Alam, I. Abdul Karim, H.M. Salleh, Evaluation of nutritional components by Plackett-Burman design for *Penicillium citrinum* lipase production using palm oil mill effluent, Afr. J. Biotechnol. 10(81) (2011) 18704-18708.

[7] S. Turki, I. Ben Kraeima, F. Weeckers, P. Thonart, H. Kallel, Isolation of bioactive peptides from tryptone that modulate lipase production in *Yarrowia lipolytica*, Bioresour. Technol. 100(10) (2009) 2724-2731.

[8] R. Zhurbenko, Metodología para el aprovechamiento de los subproductos de la industria alimenticia y otras proteínas en la evaluación de la calidad sanitaria de los alimentos, Dissertation, University of Havana, Cuba, 2005.

[9] Q. Zhang, J. Ren, H. Zhao, J.M. Regenstein, Y. Li, et al., Isolation and characterization of three noel peptides from casein hydrolyzates that stimulate the growth of mixed cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. Bulgaricus, J. Agric. Food Chem. 59(13) (2011) 7045-7053.

[10] G. Schaafsma, Safety of protein hydrolyzates, fractions thereof and bioactive peptides in human nutrition, Eur. J. Clin. Nutr. 63 (2009) 1161-1168.

[11] R. Zhurbenko, C. Rodríguez Martínez, T. Lobaina Rodríguez, O.D. López Hernández, D.R. Viera Oramas, Peptona papaínica de corazón de vaca como fuente de nutrientes para los microorganismos, SIIC. http://www.siicsalud.com. (Accessed on: 24 February 2012).

[12] E.B. Kurbanoglu, N.I. Kurbanoglu, A new process for the utilization as peptone of ram horn waste, J. Biosci. Bioengg. 94(3) (2002) 202-206.

[13] F. Meli, C. Lazzi, E. Neviani, M. Gatti, Effect of hydrolysates on growth kinetics and aminopeptidase activities of *Lactobacillus*, Curr. Microbiol. 68(1) (2014) 82-87.

[14] M. Fallah, S. Bahram, R.J. Seyed, Fish peptone development using enzymatic hydrolysis of silver carp by-products as a nitrogen source in *Staphylococcus aureus* media, Food Sci. Nutr. 3(2) (2015) 153-157.

[15] H.J. Morris, A. Almarales, O. Carrillo, R.C. Bermúdez, Utilization of Chlorella vulgaris cell biomass for the production of enzymatic protein hydrolyzates. Bioresour. Technol. 99(16) (2008) 7723-7729.

[16] H.H. Abd El Baky, G.S. El Baroty, E.A. Ibrahem, Functional characters evaluation of biscuits sublimated with pure phycocyanin isolated from Spirulina and Spirulina biomass, Nutr. Hosp. 32(1) (2015) 231-241.

[17] X. Xu, S. Yang, J. Xie, J. Zhao, Kinetics and dinamics for light state transition in cyanobacterium *Spirulina platensis* cells, Biochem. Biophys. Res. Commun. 422(2) (2012) 233-237.

[18] I. Ak, Effect of an organic fertilizer on growth of blue-green alga *Spirulina platensis*, Aquacult. Int. 20(3) (2012) 413-422.

[19] J. Teas, M.R. Irhimeh, Dietary algae and HIV/AIDS: proof of concept clinical data, J. Appl. Phycol. 24(3) (2012) 575-582.

[20] G. Markou, Alteration of the biomass composition of *Arthrospira (Spirulina) platensis* under various amounts of limited phosphorus, Bioresour. Technol. 116 (2012) 533-535.

[21] G. Markou, O. Depraetere, D. Vandamme, K. Muylaert, Cultivation *of* Chlorella vulgaris and *Arthrospira platensis* with Recovered phosphorus from wastewater by means of zeolite sorption, Int. J. Mol. Sci. 16(2) (2015) 4250-4264.

[22] J. Sharon Mano Puppu, K. Vojayakumar, V. Ramamurthy, Artificial neural network model for predicting production of *Spirulina platensis* in outdoor culture, Bioresour. Technol. 130 (2013) 224-230.

[23] A. Zafilaza, A. Andriantsimahavandy, D.J. Ramamonjisoa, M. Andrianainarivelo, An evaluation study of the cultivation of *spirulina* in the area of Sambirano, Madagascar Int. J. Food Sci. Nutr. Engg. 5(3) (2015) 101-104.

[24] F. Busson, *Spirulina plantensis* (Gni) Ceîtter et Spirulinageîtteri J de Toni, Cyanophycéesalimentaires, Marseille: Arméefrançais, service de santé, ParcPharo, 1971.

[25] H.E. Landsberg, Global distribution of solar and sky radiation in world maps of climatology, 3rd Ed., Springer, Berlin, 1966.

[26] J.A.V. Costa, M.G. de Morais, The role of biochemical engineering in the production of biofuels from microalgae, Bioresour. Technol. 102 (2011) 2-9.

[27] J.L. Parada, G. Zulpa de Caire, M.C. Zaccaro de Mulé, M.M. Storni de Cano, Lactic acid bacteria growth promoters from *Spirulina platensis*, Int. J. Food Microbiol. 45(3) (1998) 225-228.

[28] V. Fadaei, F. Mohamadi-Alasti, K. Khosravi-Darani, Influence of *Spirulina platensis* powder on the starter cultura viability in probiotic yogurt containing spinach during cold storage, Eur. J. Exp. Biol. 3(3) (2013) 389-393.

[29] D. Mocanu, E. Botez, O.V. Nistor, D.G. Andronoiu, G. Vlăsceanu, Influence of *Spirulina platensis* biomass over some starter cultura of lactic bacteria, J. Agroalimentary Processes Technol. 19(4) (2013) 474-479.

[30] C. Zarrouk, Contribution a letuded'unecyanophycee Influence de diversfactours physiques etchimiquessur la croissance et la photosynthese de Spirulina maxima, Dissertation, University of Paris, France, 1966.

[31] S.P.L. Sorensen, Enzymestudien, Uber die quantitative Messung Proteolytischer Spaltungen. Die Formol Titrierung, Z. Biochem. 7 (1908) 45-101.

[32] R.B. Bradstreet, Kjeldahl method for organic nitrogen, Anal. Chem. 26(1) (1954) 185-187.

[33] M.P.C. Silvestre, H.A. Morais, V.D.M. Silva, M.R. Silva, Degree of hydrolysis and peptide profile of whey proteins using pancreatin hydrolysis of whey protein, Nutrire: Rev. Soc. Bras. Alim. Nutr. J. Brazilian Soc. Food Nutr. 38(3) (2013) 278-290.

[34] V.F. Runova, L.G. Bendas, G.A. Maksimova, E.I. Preobrazhenskaya, B.M. Raskin, et al., Mietodicheskie ukazania po primienieniyu fiziko-khimicheskikh mietodov kontrolia pitatielnikh sried, Moscú, 1977. [Published in Russian].

[35] USP, US Pharmacopeia National Formulary, USP 35, United States Pharmacopeial Convention Inc., Rockville, MD, 2012.

[36] E.Y. Bridson, A. Brecker, Design and formulation of microbial culture media. In: J.R. Norris, D.W. Ribbons, Methods in Microbiology, Academic Press, London, New York, 1970, pp. 237-249.

[37] M.G. Davies, A.J. Thomas, An investigation of hydrolytic techniques for the amino acid analysis of foodstuffs, J. Sci. Food Agric. 24(12) (1973) 1525-1540.

[38] Oxoid, Manual. England: Unipath Limited, Basingstoke, Hampshire, 1995, pp.143, 196, 220.

[39] ISO, International Organization for Standarization ISO/TS 11133-2 Microbiology of food and animal feeding stuffs - Guidelines on preparation and production of culture media - Part 2: Practical guidelines on performance testing of culture, International Organization for Standarization, Geneva, Switzerland, 2003.

[40] H.J. Morris, O.V. Carrillo, A. Almarales, R.C. Bermúdez, M.E. Alonso, et al., Protein hydrolyzates from the alga Chlorella vulgaris 87/1 with potentialilities in immunonutrition, Biotechnol. Appl. 26(2) (2009) 162-165.

[41] R. Zhurbenko, C. Rodríguez Martínez, Bases nutritivas para el cultivo de los microorganismos: Parte 1 – Procesos tecnológicos, Salud(i)Ciencia 16 (4) (2008) 420-425.

[42] M.A.B. Habib, M. Parvin, T.C. Huntington, M.R. Hasan, A review on culture, production and use of *spirulina* as food for humans and feeds for domestic animals and fish, Fisheries and aquaculture circular, Food and Agriculture Organization of the United Nations, Rome, 2008.

[43] E.G. Oliveira, G.S. Rosa, M.A. Moraes, L.A.A. Pinto, Characterization of thin layer drying of *Spirulina platensis* utilizing perpendicular air flow, Bioresour. Technol. 100(3) (2009) 1297-1303.

[44] L. Mourey Valdés, M. Gurria Rafols, E. Vidrio Sande, J. Bravo Brash, M.D. Bañales, Manual de normas técnicas sanitarias de agentes de diagnóstico, Secretaria de Salud, México, 1990.

[45] M.P.C. Silvestre, M.R. Silva, V.D.M Silva, M.V.S. Souza, J.R. Lopes, et al, Analysis of protein hydrolysates: peptide profile and ace inhibitory activity, Braz. J. Pharm. Sci. 48(4) (2012) 747-757.

[46] C.A. Mazzucotelli, M.V. Agüero, M.D. Moreira, M.R. Ansorena, Optimization of medium components and physicochemical parameters to simultaneously enhance microbial growth and production of lypolitic enzymes by *Stenotrophomonas sp,* Biotechnol. Appl. Biochem. 63(3) (2016) 407-419.

[47] I.G. Akhapkina, L.P. Blinkova, L.G. Butova, Cultivation of *Pseudomonas aeruginosa* in media with chlorella fermentative hydrolysate as nutrient base. Zh. Mikrobiol, Epidemiol. Immunobiol. 5 (2002) 67-68 [Article in Russian].

[48] O.B. Gorobets, L.P. Blinkova, A.P. Baturo, Stimulating and inhibiting effect of Spirulina platensis on microorganisms, Zh. Mikrobiol. Epidemiol. Immunobiol. 6 (2001) 18-21 [Article in Russian].