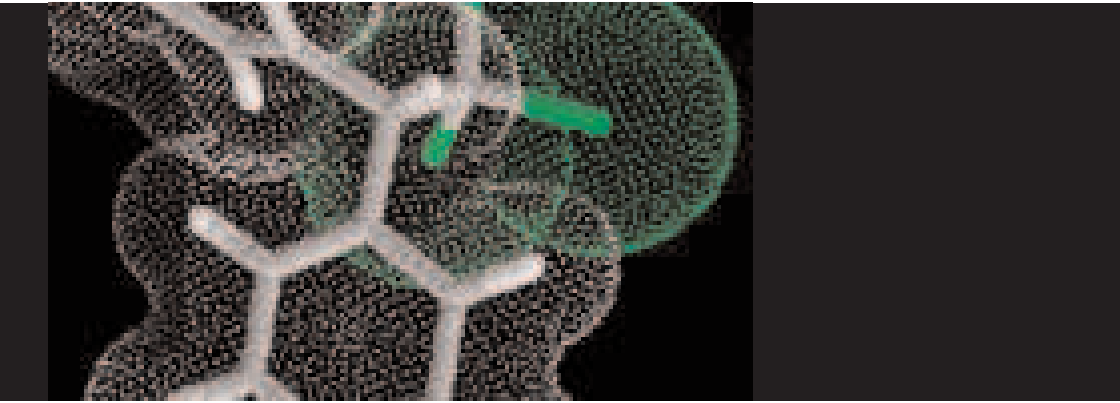JOSÉ A. TEIXEIRA

VALNATURA

A Europe-Latin America post-graduate research network in the VALorization of NATURAl resources

ALICIA CASARIEGO AÑO | GUILLERMO CRISTIAN GUADALUPE MARTÍNEZ ÁVILA | GEORGINA BASSANI
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ACKNOWLEDGEMENTS

PREFACE

The interest on bio-molecules from natural origin for their incorporation in novel and functional foods, for their use in therapeutics and diagnostics and for novel industrial applications has been gaining an increasing importance. The challenges associated are very high and new and high added value products and new processes are expected to be developed, aiming at improving the quality of life. The training of students that are able to successfully integrate life sciences, chemistry and engineering is also of the highest importance once working with bio-molecules covers a wide range of scientific areas, including Biology, Biochemistry, Chemistry and Chemical, Biological and Food Engineering. Taking this in consideration, a network formed by institutions with experts in one or more of those fields was created. This network - VALNATURA - established as main objective to complement the views of post-graduate students with different skills in order to allow them to have an adequate overview on the purification, bio-transformation and utilization of bio-molecules from natural origin for different applications. Simultaneously, the interaction of the different expertises of the institutions involved would be reinforced by the completion of MSc and PhD thesis contributing to the cross-fertilization of the teaching and research know-how of the institutions involved.

The specific objectives of the network included:
- to extend the mobility scope to other environment and realities and to give the students the opportunity to carry on post-graduate research work that is not offered at their home institutions
- to promote the mobility of postgraduate students at MSc, PhD and post-graduate level between institutions working on the purification, bio-transformation and use of bio-molecules
- to allow students of postgraduate courses to undertake their dissertations in other countries and using complementary know how
- to allow the implementation of academic degrees recognized by, at least, two institutions
- to stimulate the scientific cooperation between institutions working on the same subject but having different approaches
- to contribute for the improvement of the research work on the use of bio-molecules from natural origin

The VALNATURA network involved 8 institutions – 3 from the EU: Universidade do Minho (Portugal), Universidad de Vigo (Spain) and University College Cork (Ireland); and 5 from Latin America: Universidade Federal do Ceará (Brazil), Universidade Federal de Pernambuco (Brazil), Universidad Nacional de Rosário (Argentina), Instituto Superior Politecnico "Jose A. Echeverria" (Cuba) and Universidad Autónoma de Coahuila (Mexico). The different scientific backgrounds of the coordinating researchers in these institutions guaranteed the required expertise to fulfill the objectives of the network.

During the period 2005-2008, the project enabled the mobility of 17 students - 5 from EU to LA and 12 from LA to EU. Most of the students engaged in this network - 14 - were PhD students, the remaining 3 being MSc students. The PhD student mobility was supported by a 1-year grant that was reduced to 6 months for MSc mobility.

The activities at the receiving institution were of practical/applied nature and the work to be developed resulted from a mutual agreement between institutions/supervisors involved. This approach proved to be very successful as confirmed by the execution of the planned student mobility and by the definition/development of research projects that fitted within the thematic of the network. This model is, undoubtedly, a good way of promoting mobility at PhD and MSc level. VALNATURA network has reached its main goal and its consolidation for the future is a compromise of each individual participant, including researchers and students.

This publication contains a collection of papers that were prepared by the students and their supervisors within the VALNATURA project. The work within each paper describes the results of the thesis work that was developed during the mobility period abroad. Some of these results have already been published in peer reviewed papers and presented in international conferences, including two conferences that had a session devoted to the VALNATURA network. Most of the papers are focused on the production, purification and application of bio-molecules (lectins and enzymes) for environmental, food and therapeutic application and on the development of edible coatings and films for food preservation.

Finally, I would like to point out that the implementation of this VALNATURA network was a stimulating activity for everyone involved and that it played an important role in the increment of post-graduate student training and research cooperation between EU and LA.

Braga, 12th November 2008

José António Teixeira
(VALNATURA coordinator)

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ALICIA CASARIEGO AÑO

Chitosan coating and films: evaluation of surface, permeation, mechanical and thermal properties

ABSTRACT

The potentialities of chitosan (from lobster of the cuban coasts) coating to extend the shelf life of vegetables were evaluated. To do so, the surface properties of tomato and carrot were characterized and the wettability properties of chitosan coatings were studied. In such coatings, chitosan concentration and effects of type and concentration of plasticizer or surfactant on wettability of chitosan coatings were evaluated, as well as the respective barrier and mechanical properties. Additionally, a blend of chitosan and clay microparticles was performed and the films obtained were characterized in terms of barrier, mechanical and thermal properties.

The values of the polar and dispersive components of the superficial tension for the tomato and the carrot were determined, being the superficial tensions of the tomato and carrot 28.55 and 26.40 mN/m, respectively. The results of wettability determinations allowed the construction of one factorial model. The best values of wettability correspond to the following coating composition: 1.5 % (w/v) of chitosan and 0.1 % (w/w) of Tween 80.

A correlation has been found between the gas permeability coefficients and chitosan concentration (while keeping Tween concentrations constant).

The water vapour barrier property of the chitosan films was significantly improved by incorporation of clay, the tensile strength increased significantly with increasing chitosan and clay concentrations, while the values of elongation decreased slightly for high values of chitosan concentration. The obtained mo-dels are meant to predict the properties of chitosan/clay films to be prepared.

TESTIMONIAL

"The stay was profitable in every sense."
"It allowed us to know more about Portugal, its persons, culture, but also persons of other countries that they turned out to be friends later."

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Chitosan coating and films: evaluation of surface, permeation, mechanical and thermal propertiess

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Abstract

The potentialities of chitosan (from lobster of the cuban coasts) coating to extend the shelf life of vegetables were evaluated. To do so, the surface properties of tomato and carrot were characterized and the wettability properties of chitosan coatings were studied. In such coatings, chitosan concentration and effects of type and concentration of plasticizer or surfactant on wettability of chitosan coatings were evaluated, as well as the respective barrier and mechanical properties. Additionally, a blend of chitosan and clay microparticles was performed and the films obtained were characterized in terms of barrier, mechanical and thermal properties.

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A correlation has been found between the gas permeability coefficients and chitosan concentration (while keeping Tween concentrations constant).

The water vapour barrier property of the chitosan films was significantly improved by incorporation of clay, the tensile strength increased significantly with increasing chitosan and clay concentrations, while the values of elongation decreased slightly for high values of chitosan concentration. The obtained models are meant to predict the properties of chitosan/clay films to be prepared.

Resumen

Las potencialidades del empleo de coberturas de quitosana (obtenida a partir de langosta) para extender la vida de anaquel de los vegetales fueron evaluadas. En este sentido fueron determinadas las propiedades de superficie del tomate y la zanahoria y el efecto de la concentración de polímero, tipo y concentración de plastificante, así como la concentración de agente surfactante sobre la humectabi-lidad de las coberturas de quitosana. Las coberturas que mostraron mejor humectabilidad fueron ca-racterizadas en relación a sus propiedades de barrera y mecánicas. Adicionalmente fueron obtenidas películas de quitosana/arcilla y caracterizadas sus propiedades de barrera, mecánicas y térmicas

Los valores de las componentes polar y dispersiva de la tensión superficial del tomate y la zanahoria fueron determinados, siendo la tensión superficial de los mismos 28.55 y 26.40 mN/m, respectivamente. Los resultados de las determinaciones de humectabilidad se ajustaron a un modelo factorial. Los mejores

valores en términos de humectabilidad correspondieron a la cobertura con una composición de: quitosana 1.5% (m/v) y 0.1 % (m/v) de Tween 80.

Una incidencia de la concentración de quitosana (concentración de Tween constante) sobre las propiedades mecánicas y de barreras de las coberturas fue encontrada.

La permeabilidad al vapor de agua de las películas de quitosana fue significativamente mejorada con la adición de arcilla en su composición. El esfuerzo tensil aumentó significativamente con el aumento de la concentración de quitosana y arcilla, mientras que la elongación disminuyó ligeramente. Los modelos obtenidos permiten predecir las propiedades de las películas de quitosana/arcilla que se quie-ran preparar.

Keywords: Chitosan, edible coating, surface properties, clay, nanocomposite, gases permeability, mechanical properties, thermal properties, modeling.

Introduction

Edible films and coatings have potential in a number of different areas. They can coat food surfaces, separate different components, or act as casings, pouches or wraps. They can preserve product quality by forming oxygen, aroma, oil or moisture barriers; carrying functional ingredients, such as antio-xidants or antimicrobials, and improving appearance, structure and handling.

The characterization of chitosan films obtained from chitosan derived from crab and shrimp has been extensively performed [1-5], however Cuban coasts are very rich in lobsters, and the recycling of their exoskeletons produced by the fishing industry is an important goal, even more important if some value is added to that sub-product. For this reason the objectives of this work were to determine the effects of presence of type and concentration of hydrophilic plasticizer, surfactant and polymer concentration on the wettability of Cuban chitosan based coatings in view of their application on vegetables and to develop a model allowing to optimize coating composition. Besides, the transport, mechanical and thermal properties of chitosan coatings, showing the best wettability, were characterized. The effects of chitosan and clay concentrations on the transport, mechanical, and thermal properties of chitosan/clay films were also evaluated.

Materials and Methods

The materials used to prepare the edible coating solutions or chitosan/clay films were: chitosan (obtained in the Pharmaceutical Laboratories Mario Muñoz, Cuba) with a degree of deacetylation of 90 %, glycerol 87% (Panreac, Spain), sorbitol 97% or polyethylene glycol MW 400 (Acros Organics, Belgium) as plasticizers, Tween 80 (Acros Organics, Belgium) as surfactant, lactic acid (Merck, Germany), clay (courtesy of Instituto Politécnico de Viana do Castelo, Portugal) with a mean particle size distribution of 2 ì and distilled water.

The coating solutions were prepared dissolving chitosan (1.0, 1.5 or 2.0 % w/v) in a 1% (v/v) lactic acid solution; the plasticizers were added in concentrations between 0.25 and 0.50 mL plasticizer/g of chitosan. Tween 80 was added as a surfactant with concentrations between 0.02 and 0.10 % (w/v). The chitosan films were prepared pouring a constant amount (28 mL) of chitosan solution onto an 8 cm diameter glass plate in order to maintain the film thickness constant.

Nanoclay solutions (1 and 3 % w/w chitosan) were prepared according with the methods reported by Xu and others, (2005) [5] by dispersing appropriate amounts of clay into 5 mL of 1% lactic acid solution and vigorously stirring for 24 h. Afterwards, 100 mL of chitosan solution (1.0 to 2.0 % (w/v)) was added slowly into pretreated clay solutions. The mixtures were stirred continuously for 4 h and then cast onto a glass plate.

The films were dried in an oven at 35 ° C. Dried films were peeled from the plate and cut in circles with approximately 80 mm of diameter for property testing. All chitosan films for permeability test were conditioned in desiccators, and maintained at 20 °C and 25 % RH.

Analysis

Both contact angle (è) and surface tension (γ_L) were determined with a face contact anglemeter (OCA 20, Dataphysics, Germany). The (γ_L) of the coating solution was measured by the pendent drop method and Laplace-Young approximation (Song, 1996) [6]. The (è) was measured by the sessile drop method. Ten replicates of contact angle and surface tension measurements were analyzed at 20 (± 1) ºC.

Oxygen permeability (OP) and Carbon dioxide permeability (CO2 P) were determined based on the ASTM (2002) method [7] while water vapor permeability (WVP) of the films was determined gravimetrically based on ASTM E96-92 method [8].

Tensile strength (TS) and elongation-at-break (E) were measured with an Instron Universal Testing Machine (Model 4500, Instron Corporation) following the guidelines of ASTM Standard Method D 882-91[9].

Differential scanning calorimetry and Thermogravimetric analyses (TGA) measurements were performed with a Shimadzu DSC-50 (Shimadzu Corporation, Kyoto, Japan). About 10 mg of the samples were placed in stainless steel DSC pans and were heated from 25 to 350 °C at a heating rate of 10 °C/min in a helium atmosphere, while for the TGA measurement the samples were placed in the balance system and heated from 25 °C to 600 °C at a heating rate of 10 °C/min in a helium atmosphere.

Results and Discussion

BThe Zisman method is based on that a plot of the cosine of the contact angle vs. the superficial tension (liquid-vapor) on a given solid is generally a straight line and is applicable only for systems with a surface tension below 100 nN/m (low energy surfaces) [10,11]. It is therefore necessary to determine the surface energy of tomato and carrot in order to verify the applicability of that method.

The contact angle determinations of at least three pure compounds (water, formamide and bromonaphthalene) on the surface of tomato or carrot combined with their surface tension values [12, 13]· allowed the adjustment of the experimental data to a plot and produces the following equations for tomato (Eq.1) and carrot (Eq.2).

$$\frac{1+\cos\theta}{2} \cdot \frac{\gamma_L}{\sqrt{\gamma_L^d}} = 1.8490 \sqrt{\frac{\gamma_L^p}{\gamma_L^d}} + 5.0231 ; \quad r^2 = 0.9995 \qquad \text{[Equation 1]}$$

$$\frac{1+\cos\theta}{2} \cdot \frac{\gamma_L}{\sqrt{\gamma_L^d}} = 0.6672 \sqrt{\frac{\gamma_L^p}{\gamma_L^d}} + 5.095 ; \quad r^2 = 0,9997 \qquad \text{[Equation 2]}$$

The polar and dispersive components of the surface tension were determined to be 3.32 and 25.24 mN/m. respectively for the tomato and 0.48 and 25.62 mN/m, respectively for the carrot, being the surface tensions of the tomato and carrot the sum of the two components (28.56 and 26.10 mN/m. respectively). These results clearly show that both tomato and carrot are low energy surfaces and that their surface interacts with liquids primarily through dispersion forces [14].The Zisman method can therefore be applied to estimate the critical surface tension. In the present work it has been found that the critical surface tension has values of 17.8 and 24.5 mN/m.

Table 1: Model equations adjusting to W_a, W_s and W_c as functions of chitosan (q) and Tween 80 (t) concentrations.

Vegetable	Model Equations	R^2	Optimal Experimental	Optimal Model	*R.E (%)
Tomato	$Wa = 29.8998 + 1.7635\,q - 0.2728\,t$ $+ 2.6633\,q^2 - 0.3226\,q\,t + 0.5885\,t^2$	66.5027	37.00	37.87	2.35
Carrot	$Wa = 22.2664 + 2.1876\,q + 0.8803\,t$ $+ 2.0082\,q^2 + 0.4620\,q\,t + 0.3300\,t^2$	55.1187	30.82	30.47	1.13
Tomato	$Ws = -25.9038 - 3.6992\,q + 1.0132\,t$ $- 8.0450\,q^2 - 1.2957\,q\,t$	90.3553	-22.67	-22.17	2.20
Carrot	$Ws = -33.5581 - 3.2493\,q + 2.1710\,t$ $- 8.6743\,q^2 - 0.5041\,q\,t$	85.0101	-30.20	-29.27	3.07
Tomato	$Wc = 55.8036 + 5.4627\,q - 1.2861\,t$ $+ 10.7084\,q^2 + 0.9731\,q\,t + 0.4686\,t^2$	95.9525	74.04	74.01	0.04
Carrot	$Wc = 55.8246 + 5.4370\,q - 1.2906\,t$ $+ 10.6826\,q^2 + 0.9662\,q\,t + 0.4768\,t^2$	95.976	74.07	74.50	0.58

*Relative error defined as $RE = \left| \left(\dfrac{OE - OM}{OE} \right) \right| \times 100$, where OE is the Optimal Experimental and OM is the Optimal Model.

The spreading coefficient (W_s) decreased as the chitosan concentration increased for the vegetables studied, independently of plasticizer concentration, and a statistically significant difference has been found ($p < 0.05$) between the different chitosan coating ($p < 0.05$). Statistically significant differences ($p < 0.05$) were found between the values of adhesion coefficient (W_a) and W_s for tomato and carrot. The influence of plasticizers in the surface properties of the chitosan coating was studied and a tendency can be observed that W_a and W_s decreased and cohesion coefficient (W_c) increased as the plasticizer concentration increased, such differences were statistically significant. The best results, in term of wettability, were obtained with glycerol, polyethyleneglycol and sorbitol in this order Also in this case remarkable differences were found between the behavior of tomato and that of the carrot ($p<0.05$).

The influence of chitosan and Tween 80 concentrations on the W_a, W_c and W_s were described by a polynomial model (Table 1) for both vegetables. The models suggested that chitosan concentration is the variable of higher influence in the values of W_a, W_c and W_s, reaching its higher effect when the concentration is 1.5% (w/v); term Tween 80 (in the concentration studied) shows the lowest influence. The wettability of the solution was therefore optimized by minimizing/maximizing. The optimal composition found (in terms of the wettability) was obtained for a concentration of chitosan of 1.5% (w/v) and 0.1% (w/w) of Tween 80.

The transport properties of chitosan coatings, showing the best wettability, were characterized. The values of OP oscillated in a range of 2.87 to 15.03 x 10^{-3} cm^3 O_2 m^{-1} day^{-1} atm^{-1} similar to those results reported by others authors [1], while that the OP and CO_2P values increase with increasing chitosan concentration. Similar results were obtained with respect to WVP. The chitosan films exhibited WVP of 3.02 to 3.31 10^{-1} g m^{-1} day^{-1} atm^{-1} values lowest that 9.42 x 10^{-1} g m^{-1} day^{-1} atm^{-1} reported [1]. This could be due to the presence of Tween 80 as surfactants which might improve the barrier to water vapor due to their polar side

which can be bonded to the polar part of the chitosan molecule while the non polar groups can place away from the chitosan molecule thus creating an extra barrier to water vapor [5].

Results also show that increases in chitosan concentration while keeping Tween 80 concentration constants increased the values of TS while E decreased significantly ($p < 0.05$). This behaviour may be due to the fact that chitosan forms hydrogen bonds between hydroxyl groups and amino groups in chitosan film, during the film formation and hydrogen bonding in the chitosan films increased with the increasing amount of amino and hydroxyl groups, due to the increased in concentration of chitosan [2].

The chitosan/clay films showed an improvement of the water vapour barrier property by incorporation of clay in the film matrix ($p = 0.05$) (Table 2). The greatest values of WVP were obtained for those films with the lowest concentration of chitosan and significant influence of both chitosan and clay concentration ($p = 0.05$) was found. The WVP value of the chitosan films were between 2.38×10^{-12} kg m/m^2 s Pa and 2.49×10^{-12} kg m/m^2 s Pa, higher values than to those reported [4], for chitosan films 2% (w/v) in solution of acetic acid 1% (w/v) (1.31×10^{-12} kg/m^{2}sPa), this difference could be due by the acid used to dissolve the chitosan and because we used chitosan obtained from lobster instead of chitosan from another source. The WVP of the nanocomposite films decreased significantly ($p = 0.05$) by 9-32% depending on the chitosan and clay concentration. The decrease in WVP of nanocomposite films is believed to be due to the presence of ordered dispersed nanoparticle layers with large aspect ratios in the polymer matrix [15]. This forces water vapor traveling through the film to follow a tortuous path through the polymer matrix surrounding the particles, thereby increasing the effective path length for diffusion [4]

The influence of chitosan and clay concentration (q and c) on the WVP was described by a polynomial model that suggested that chitosan concentration is the variable of higher influence.

$$WVP = 3.05 \times 10^{-12}\ q - 8.32 \times 10^{-13}\ c + 2.46 \times 10^{-13}\ c^2; \quad p = 0.05 \qquad R^2 = 0.75 \qquad \text{[Equation 3]}$$

Table 2: Chitosan and clay concentration: effects on barrier and mechanical properties of chitosan/clay films. (Standard Deviations given in parentheses)[a]

| Film | | WVP x 10^{-12} | OP | CO$_2$P | TS | E |
Chitosan (%w/v)	Clay (% w/w chitosan)	(Kg m^{-1} s^{-1} Pa^{-1})	(cm^{3}m^{-1}day^{-1} atm^{-1})	(cm^{3}m^{-1}dia^{-1} atm^{-1})	(MPa)	(%)
1.0	0	2.41 (0.19) b	0.012 (0.0007) c	23.15 (0.98) a	11,69 (1.98) a	14,33 (3.36) c
1.0	1	1.75 (0.19) a	0,013 (0.0001) c	23.46 (1.54) a	17,05 (3.68) a	6,79 (1.18) b
1.0	3	1.96 (0.12) a	0.013 (0.0006) c	24.89 (2.95 a	21,54 (3.52) a	6,14 (1.37) ab
1.5	0	2.48 (0.10) b	0.012 (0.0003) bc	23.24 (1.70) a	47,46 (7.6) bc	4,77 (1.26) ab
1.5	1	2.49 (0.02) b	0.008 (0.0005) ab	26.40 (1.86) a	44,97 (0.04) b	6,86 (1.18) b
1.5	3	2.36 (0.13) b	0.010 (0.0008) a	22.34 (1.46) a	45,21 (4.99) b	6,41 (1.12) ab
2.0	0	2.94 (0.08) c	0.012 (0.0008) ab	23.19 (0.49) a	58,85 (7.25) cd	3,56 (1.25) ab
2.0	1	1,97 (0.07) a	0.007 (0.0009) a	22.84 (0.68) a	61,26 (5.44) d	3,32 (0.32) a
2.0	3	2.38 (0.24) b	0.007 (0.0008) a	21.40 (1.26) a	76,67 (8.21) e	5,54 (0.52) ab

[a-d] Means with different letters within a column indicate significant differences (p= 0.05).

The values of OP of these films oscillated in a range of 7.4 to 13.1×10^{-3} cm^3 O$_2$ m^{-1} day^{-1} atm^{-1} similar to those results reported [1]. The chitosan concentration has a significant incidence to p =0.05 and as ten-

dency a decrease it can be observed with the clay presence in chitosan films. On the other hand the chitosan and clay concentration don't influence significantly on the CO_2 permeability to p =0.05 (see Table 2)

The OP of the films can be described by the following polynomial equations:

OP= 0.029 - 0.022 q -0.061 q^2 $\qquad$ p = 0.05 $\qquad$ R^2 =0.75 $\qquad$ [Equation 4]

The TS of chitosan/nanoclay films increased significantly (p<0.05) with increasing chitosan and clay concentration, while the values of E decreased slightly for high values of chitosan concentration (Table 2). These values were in good agreement with previously reported values for chitosan film [4]. The main reason for the increase in tensile strength in polymer/layered silicate clay nanocomposites in the strong interaction between polymer matrix and silicate layers via the formation of hydrogen bonds [16]. The extent of the increase in TS depends directly upon the average length of the dispersed clay particles and, hence, the aspect ratio [4].The TS and E of the films can be described by the following polynomial equations:

TS= - 133.0 + 193.4 q -48.77 q^2 $\qquad$ p = 0.05 $\qquad$ R^2 =0. 92 $\qquad$ [Equation 5]

E = 31.166 -22.765 q -6.805 c +3.154 qc. $\quad$ p = 0.05 $\qquad$ R^2 =0. 70 $\qquad$ [Equation 6]

DSC plots of chitosan films showed two endothermic peaks for all products. The first endothermic peak that occur over a temperature range (78 ^{0}C – 94^{0}C) was attributed to solvent evaporation [5, 17], while the peaks in the range of 179–190°C showed that crystallization of the chitosan was not inhibited by the nanoclays. The T_m (melting point) increased with the increase of chitosan concentration, the changes in T_m, with addition of clay, were not significant to films, similar results were report [5]. The onset temperatures of thermal degradation did not show significant variations among the chitosan and chitosan/clay films. All chitosan films were degraded at 286 ^{0}C to 297 ^{0}C which agrees well with the results reported [5, 17].

Conclusions
Tomato and carrot have low energy surfaces; their critical surface tensions were determined. The increase of the concentration of chitosan and plasticizers decreased the values of W_s and W_a. The optimum values of the spreading coefficients were experimentally obtained. The results of wettability determinations allowed adjusting a polynomial model, thus creating the basis for a future choice of the composition of the films.
The oxygen, carbon dioxide and water vapor permeability of chitosan films depend on chitosan concentrations while keeping Tween 80 concentration constant.
The chitosan-based nanocomposite films showed an improvement of the water vapour barrier property by incorporation of clay in the film matrix. The chitosan and clay concentration had incidence on the properties evaluated.

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GUILLERMO CRISTIAN MARTINEZ ÁVILA

Valorization of mandarin peels through enzymatic pectin extraction

ABSTRACT

Pectin is a polysaccharide widely distributed in many higher plants and it has many applications in the food, medical and pharmaceutical industry. Pectin can be extracted from agroindustrial by-products through chemical, physical or biotechnological methods. The aim of this study is to develop a process for pectin extraction from mandarin peels using a commercial enzyme from *Aspergillus aculeatus*. Response surface methodology was used to optimize the enzymatic pectin extraction prosess. The selected experimental design was a three factor five level central composite design for enzyme concentration, solid/liquid ratio and extraction time on the pectin yield. Second order model was used to generate three dimensional response surface plots for the independent variables and the pectin yields. Pectin content in mandarin peel extracts expressed as percent of dry weight of polysaccharide varied from 8.7 to 21.3% (w/w). Enzyme concentration showed the highest effect on the pectin yield from the mandarin peels.

TESTIMONIAL

"For me was very good experience."
"Helped me to have professional and personal growth." "It gave me the opportunity of knowing new places and to improve my English level"

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Valorization of mandarin peels through enzymatic pectin extraction

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Abstract

Pectin is a polysaccharide widely distributed in many higher plants and it has many applications in the food, medical and pharmaceutical industry. Pectin can be extracted from agroindustrial by-products through chemical, physical or biotechnological methods. The aim of this study is to develop a process for pectin extraction from mandarin peels using a commercial enzyme from *Aspergillus aculeatus*. Response surface methodology was used to optimize the enzymatic pectin extraction prosess. The selected experimental design was a three factor five level central composite design for enzyme concentration, solid/liquid ratio and extraction time on the pectin yield. Second order model was used to generate three dimensional response surface plots for the independent variables and the pectin yields. Pectin content in mandarin peel extracts expressed as percent of dry weight of polysaccharide varied from 8.7 to 21.3% (w/w). Enzyme concentration showed the highest effect on the pectin yield from the mandarin peels.

Resumen

La pectina es un polisacárido que se encuentra en la mayoría de las plantas superiores, este tipo de compuestos tienen una amplia aplicación en la industria alimentaria, médica y farmacéutica. La pectina puede ser extraída de residuos agroindustriales por métodos químicos, físicos y biotecnológicos. El propósito de este estudio fue desarrollar un proceso para la extracción de pectina de pomaza de mandarina utilizando una enzima comercial de *Aspergillus aculeatus*. Se usó la metodología de superficie de respuesta para optimizar el proceso de extracción de pectina. Se empleó un diseño de composición central de tres factores (concentración de enzima, relación sólido líquido y el tiempo de extracción) con cinco niveles para estudiar su efecto sobre el rendimiento de pectina. Un modelo de segundo orden fue empleado para generar gráficas de superficie de respuesta en tercera dimensión para las variables dependientes y los rendimientos de pectina. El contenido de pectina extraída de la pomaza de mandarina se expresó como porcentaje en peso seco del polisacárido. Los rendimientos estuvieron en un rango de 8.7 a 21.3% (w/w). El efecto lineal de la concentración de enzima mostró el efecto más significativo sobre los rendimientos de pectina de pomaza de mandarina.

Introduction

Pectic substances are important polysaccharides; they form part of the cell walls of higher plants since they are located primarily in the middle lamella of the vegetable tissue [1]. Pectins are a group of complex colloidal polymeric materials, where the predominant structure is integrated by two domains: homogalacturonan that is formed by lineal chain of α 1-4 linked D-galacturonic acid with some of the carboxyl groups esterified with methanol [2], and rhamnogalacturonan, where are the rhamnogalacturonan I and the rhamnogalacturonan II complex [3]. There are diverse alternatives for the extraction of pectic polysaccha-

rides for industrial applications such as chemical, physical and biotechnological. Chemical extraction processes are the most used and consist of using strong acids or bases [4]. Physical extraction involves the use of microwaves [5], autoclaving [6], and extrusion [7]. Biotechnological processes involve the use of enzymes for the pectin extraction process [8, 9]. Pectin composition and structure depend on their source and extraction method [10]. Most pectin products used in the food industry are prepared from citrus peels [11] and apple pomace using a chemical extraction process (acidified hot-water) [12]. Pectins in the food industry are used in the manufacture of jam, marmalades, jellies, confectionary gums, as stabilizer of fermented and acidified milks, fruit yogurts and related foods [6]. In addiction the pectins are employed in the cosmetic and medical industry where they have diverse applications [13]. Extraction and characterization of pectin from many plant materials have been studied, i.e., sunflower [14], peach pomace [15], tecojote, nopal powder and jicama [6].

Mathematical models are an important tool in the food processing area, where they can help to indentify the key parameters in a process, reduce costly experiments and save time. Mathematical models have been used to describe pectin extraction process from agroindustrial wastes [16, 17]. Response surface methodology (RSM) is a statistical and mathematical tool useful for developing, improving and optimizing processes [18]. The main advantage of RSM is the reduced number of experiments needed to evaluate multiples parameters and their interactions. RSM has been used successfully to optimize the extraction process of pectins from different sources [5, 7]. Reports were not found regarding to application of RSM to optimize the enzymatic extraction process of pectin from mandarin peels.

The aim of this study was to optimize the conditions of pectin extraction press from dry mandarin peels using a RSM to study the effects of enzyme concentration, solid/liquid ratio and extraction time on pectin yields.

Materials and Methods

Endo-polygalacturonase M2 from *Aspergillus aculeatus* was obtained from Megazyme International Ireland Ltd., Ireland.

Preparation of the pomace

Mandarins were purchased from a local market (TESCO) in Cork, Ireland. The fruit was washed and the peels were manually separated. Peels were prepared following the methodology reported by Contreras-Esquivel et al., 1999 [19]. The peels were washed three times with two volumes of ethanol 96% (w/v) during 12 hours for each of them, after the washings; the peels were dried in a oven at 50 °C during 12 hours. Then, the material was pulverized using a food mixer Moulinex (Model: DEPOSE) and sifted in a RO-TAP system. Particle size from mandarin peels used to performance the enzymatic pectin extraction was 150 μm.

Experimental design

RSM was applied to determine the work conditions on process of pectin extraction from mandarin peels. The effect of independent variables x_1 (enzyme concentration), x_2 (solid/liquid ratio) and x_3 (time, h), at three variation levels, is shown in the Table 1.

Table 1. Independent variables values in pectin extraction process and their corresponding levels

Independent variable	Symbol	Levels		
		-1	0	1
Enzyme concentration (U)	x_1	11	18	25
Extraction time (h)	x_2	18	24	30
Solid/liquid ratio (w/v)	x_3	1:30	1:35	1:40

The complete design consisted of 16 points including two replications of the center points, and the triplicates were performed at all design points in a randomized order.

Enzymatic pectin extraction and extraction recovery of pectin
Pectin extraction was developed using 50 mM citrate buffer solution (pH 5.5), and the factors enzyme concentration, extraction time and solid/liquid ratio used were according to the experimental design. Pectin extraction process was carried out in a water bath at 40 ºC and 90 rpm. After the reaction time, the flasks were cooled in an ice bath and pectin was precipitated from the filtrate adding two volumes of ethanol (96%) and incubated at 4 ºC by 12h. The alcohol-insoluble material was filtered using cheesecloth and then dried at room temperature (22-23 ºC) until constant weight. The pectin was weighted and the yields were expressed in dry basis of pectin obtained from mandarin peels.

Data Analysis
The generalized second order polynomial model used in the response surface analysis is given in following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \qquad (1)$$

where β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i, and X_j are the independent variables. Data were analyzed using Statistica Software (Release 7, 2004 Start soft, Tulsa, OK, USA).

Results and Discussion
Pectin yield extracted enzymatically from mandarin peels was in the range of 8.6±1.68 to 21.3±1.3% (w/w). Several reports in literature showed that the yields of pectin extracted enzymatically from mandarin peels were similar to the maximum obtained in this work. Shkodina et al. (1998) [8], reported pectin extraction yields of 22, 19 and 15% from pumpkin pulp using a *Trichordema viride* cellulase, *Aspergillus niger* hemicellulase and glycosidase complex from *Xanthomonas campestris*, respectively. Contreras-Esquivel (2003) [20], reported pectin yields of 27 and 17.6% when pectin was extracted from lemon peels with polygalacturonases from *Aspergillus kawachii* and *Aspergillus niger*, respectively.
A regression analysis was carried out to fit the mathematical model to the experimental data aiming at an optimal region for the responses studied. The significant fitted model for describing the extraction efficiency of pectin as a function of independent variables studied is given below:

$$Y = 0.2084 + 0.0120x_1 - 0.0221x_1^2 - 0.0348x_2^2 + 0.0327x_3^2 \quad (2)$$

The significance of each coefficient was determined using the F-test and p-value and the results are shown in the Table 2. The corresponding variables would be more significant if the absolute F-value becomes greater and the p-value becomes smaller [21]. It can be seen that the variables with the largest effect were the linear terms of enzyme concentration (x_1) followed by quadratic term of enzyme concentration (x_1), extraction time (x_2) and solid/liquid ratio (x_3).

Table 2. Analysis of variance of relationship between response variables (pectin yield) and independent variables (x_1, x_2, x_3)

Variables	Degrees of freedom	Mean squares	F-value	p-value
x_1	1	0.0059	10.15	0.0028 *
x_1^2	1	0.0135	23.29	<0.0001*
x_2	1	0.0005	0.877	0.3549
x_2^2	1	0.0337	57.93	<0.0001 *
x_3	1	0.0005	0.879	0.3541
x_3^2	1	0.0297	51.05	<0.0001 *

* Significant (P<0.01).

Analysis of response surface

The relationship between enzyme concentration (x_1) and extraction time (x_2) on the pectin yield is shown in Figure 1. It was observed that there is a maximum of pectin yield when the enzyme concentration of 18 U was used; high and low levels of enzyme had a negative effect on the pectin yield. Tono and Fujita (1980) [22], did not observe a significant effect of the enzyme concentration on pectin mandarin peels yields.
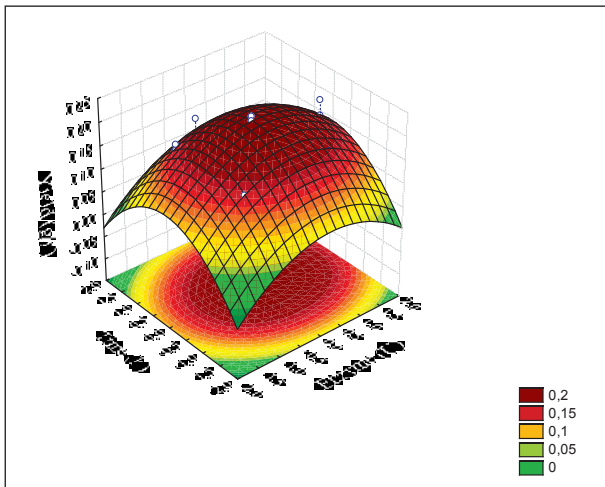
Figure 1. Response surface plot showing of effect enzyme concentration and extraction time on grams of pectin per gram of mandarin pomace

The effect of solid/liquid ratio (x_3) and extraction tiem (x_2) on the pectin yield is illustrated in Figure 2. The maximum yield of pectin was obtained when 1:35 solid/liquid ratio was used. Extraction time showed a similar trend to that of solid/liquid ratio on the pectin yields; in this case 24 h was the time need to extraction of maximum pectin yields. Lower processing times have been reported for enzymatic pectin extraction from lemon [26] and apple [23].
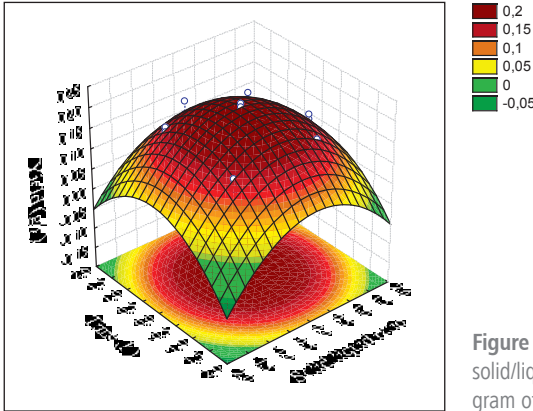
Figure 2. Response surface plot showing the effect of solid/liquid ration and extraction time on grams of pectin per gram of mandarin pomace
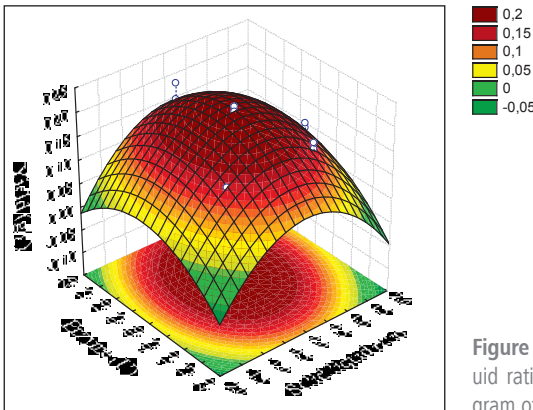
The effect of enzyme concentration (x_1) and solid/liquid ratio (x_3) on the pectin yield is shown in Figure 3. Higher pectin yield values were obtained when enzyme concentration was increased until 18 U, but higher levels of enzyme concentration decreased the pectin yield. Solid/liquid ratio showed the same pattern as enzyme concentration, increasing the solid/liquid ratio until 1:35, the pectin yield was increased, but it decreased when the solid/liquid ratio was increased. Wang et al. (2007) [5], observed that the pectin extraction yield obtained by microwaves was higher when the solid/liquid ratio increased, in contrast to the results observed in this work, where a pronounced increment in solid/liquid ratio negatively affected the pectin yield.

Figure 3. Response surface plot showing the effect solid/liquid ratio and enzyme concentration on grams of pectin per gram of mandarin pomace

Few studies are reported about the enzymatic pectin extraction, may be due to the high yields obtained with the chemical procedures; however, these methods are very harsh, non-specific and pollutants [24]. Several efforts have been conducted to apply enzymatic bioprocesses to extract pectin from different agroindustrial by-products. Zhang et al. (2005) [25], reported a value of 35.7% of pectin yield when a crude protopectinase extract from *Aspergillus sp*XZ-131 was used on citrus peels. Contreras-Esquivel et al. (2006) [26], compared the pectin extraction yields using as raw materials lemon peels, reaching values of 17.6 and 20.2 % when used an enzymatic and chemical extraction, respectively. They demonstrated that the use of fungal endo-polygalacturonase can be an attractive alternative to extract pectin from agroindustrial residues. In relation to the optimization of pectin extraction using RSM, Canteri-Schemin et al. (2005) [23], evaluated the time and acid concentration to extract pectin from apple residues; however, the pectin extraction yield obtained was lower than the value obtained in our study.

It is important to consider that the extraction temperature, the chemical composition and particle size of pectin source affect seriously the pectin extraction process. From the present study, it can be mentioned that further studies need to be developed in order to validate the best conditions founded for the enzymatic pectin extraction from mandarin peels.

Conclusions

The enzyme concentration had the strongest effect on the pectin yield followed by extraction time. Solid-liquid ratio did not show any effect on pectin solubilization. The conditions necessary for maximum pectin extraction were a concentration of 18 U of enzyme, a solid/liquid ratio of 1:35 and an extraction time of 24 hours. The extraction of pectin using an enzymatic method could be an attractive alternative to extract this polysaccharide from mandarin peels due to the fact that the pectin yields obtained in this work were similar to the reported in previous studies where chemical methods were used.

Acknowledgements

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Importance of the proteases Caspase-4 and Caspase-12 in L-histidine decarboxylase

ABSTRACT

Histamine is a mediator of various physiological processes. The formation of histamine is catalyzed by the enzyme L-histidine decarboxylase (HDC; EC 4.1.1.22). Catalytically active mammalian HDC is a homodimer containing two subunits. ER resident proteases (caspases 4 and 12) would be responsible for HDC processing. Alternatively, the 502-504 site identified as a putative cleavage site in the rat HDC protein shows significant sequence similarity to the consensus cleavage site for site 1 protease (S1P).

We attempted to develop an experimental cell model expressing high levels of unprocessed and processed forms of the enzyme so we could look at cleavage. Stably transfected HEK293 and PT67 packaging cell lines were generated. We explored the use of endogenous HDC expressing cell lines like RAW and RBL-2H3 cells, and looked at expression and processing in transiently transfected HEK293T cells. Cells were incubated in the presence or absence of a variety of drugs that we expected to affect the ER expression or processing of HDC. While we could detect expression of unprocessed ~74kDa HDC in many of these models we were not satisfied that sufficient levels of cleavage could be detected to support experimental studies specifically on processing.

Cos7 cells that were transiently transfected to express rat HDC isoforms containing 502-504, 518/519 or 553/554 mutations. Only the 502-504 mutations significantly affected processing pointing towards a role for S1P in the cleavage of rat HDC. Our data suggest that aspartate cleaving caspases are not important for rat HDC processing in Cos7 cells.

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Importance of the proteases Caspase-4 and Caspase-12 in L-histidine decarboxylase

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Abstract

Histamine is a mediator of various physiological processes. The formation of histamine is catalyzed by the enzyme L-histidine decarboxylase (HDC; EC 4.1.1.22). Catalytically active mammalian HDC is a homodimer containing two subunits.

ER resident proteases (caspases 4 and 12) would be responsible for HDC processing. Alternatively, the 502-504 site identified as a putative cleavage site in the rat HDC protein shows significant sequence similarity to the consensus cleavage site for site 1 protease (S1P).

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Cos7 cells that were transiently transfected to express rat HDC isoforms containing 502-504, 518/519 or 553/554 mutations. Only the 502-504 mutations significantly affected processing pointing towards a role for S1P in the cleavage of rat HDC. Our data suggest that aspartate cleaving caspases are not important for rat HDC processing in Cos7 cells.

Resume

La histamina es mediadora de varios procesos fisiológicos. Su formación se cataliza por la enzima L-histidina decarboxilasa (HDC; EC 4.1.1.22). Catalíticamente la HDC activa de mamífero es un homodímero que contiene dos subunidades.

Las proteasas residentes del RE (caspasas 4 y 12) serían responsables de su proceso. El sitio 502-504 identificado como un sitio putativo de corte en la proteína HDC de rata muestra una significante similitud al sitio consenso de ruptura para Sitio 1 proteasa (S1P).

Intentamos desarrollar un modelo celular experimental que expresara niveles altos de las formas procesadas y sin procesar de la enzima para observar el corte. Se generaron las lineas celulares estables HEK293 y de empaquetamiento PT67. Exploramos el uso de las líneas celulares que expresan endógenamente HDC, RAW y RBL-2H3, y estudiamos la expresión y procesado en las células transfectadas temporalmente HEK293T. Se incubaron las células en presencia o ausencia de una variedad de drogas que esperábamos afectaran la expresión o procesando de HDC. Detectamos la expresión de HDC ~74kDa en muchos de estos modelos pero no niveles suficientes de proteína procesada que pudieran apoyar los estudios experimentales específicamente en el procesado.

Células de Cos7 que eran temporalmente transfectadas para expresar en rata las isoformas de HDC conteniendo las mutaciones 502-504, 518/519 o 553/554. Sólo las mutaciones 502-504 significativamente afectaron el procesamiento que apunta hacia un papel S1P para el corte. Nuestros datos sugieren que el aspartato que cortan las caspasas no es importante para HDC de rata que se procesa en Cos7.

Introduction

The biogenic amine histamine controls a number of important biological processes, including physiological regulation of gastric acid secretion, elements of innate and adaptive immunity, and neurotransmission. It also has well described roles in a variety of human pathologies, including allergic response, and many different kinds of cancers . In mammals it is generated by a single dimeric enzyme, L-histidine decarboxylase (HDC), which decarboxylates the amino acid L-histidine .

HDC is initially translated as an inactive and unstable 74kDa protein that gets targeted to the endoplasmic reticulum. The enzyme is then proteolytically cleaved, with the production of active amino-terminal proteins, including ~54kDa and ~63kDa isoforms . These are capable of homo- (and presumably hetero-) dimerization, with production of the holoenzyme. Previous studies have looked at regulation of HDC protein at the levels of cellular localization, catalytic activity and protein degradation, but it is only recently that the rate limiting step of post-translational proteolysis has been examined.

Two studies in particular are worthy of mention. The first, which was performed on the rat HDC protein sequence identified residues, identified residues 502-504 as being of importance for production of the ~54kDa isoform. Mutating these residues disrupted processing . A second study on mouse HDC identified aspartate residues DD517/518 and DD550/551 as putative cleavage sites for caspase 9 with *in vitro* proteolysis supporting these conclusions . The human caspse 9 enzyme is localized to the mitochondrion that can become localized to the nucleus upon activation. In light of combined evidence that the 74 kDa primary translation product is localized to the ER, we considered whether ER resident proteases, such as caspase 4, might be more likely mediators of HDC processing . Caspase 4 also recognises and cleaves at aspartate residues in conserved motifs and could potentially cleave at DD517/518 and DD550/551 of the mouse HDC protein sequence (residues 518/519 and 543/544 of the rat protein sequence).

Results

At the outset of this project we successfully PCR amplified the human caspase 4 cDNA for cloning into an expression vector that could be employed to generate recombinant caspase 4 protein for use in *in vitro* proteolysis assays.

In parallel we attempted to develop an experimental cell model expressing high levels of unprocessed and processed forms of the enzyme so we could look at cleavage using siRNA knockdown of specific proteases. We wanted preferentially to work with established HDC expressing cell lines or easily transfectable human cell lines, and only with the successful establishment of a robust HDC expressing model would it be possible to explore the factors and proteases that regulate HDC processing. Stably transfected HEK293 cells were generated, as well as stably transfected PT67 packaging cell lines that we hoped could be used for virally mediated HDC protein expression.

We explored the use of endogenous HDC expressing cell lines like RAW, P815 and RBL-2H3 cells, and looked at expression and processing in transiently transfected HEK293T cells. Cells were incubated in the presence or absence of a variety of drugs that we expected to affect the ER expression or processing of HDC including; tunicamicyn (Tun - which interferes with glycosylation), thapsigargin (Thap - a tight-binding inhibitor of intracellular calcium (SERCA) pumps), dithiothreitol (DTT - a reducing agent that inter-

feres with disulfide bond formation, proteasome inhibitors lactacystin (LN) and N-acetyl-leucyl-leucyl-norleucinal (ALLN), 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 3,4-dichloroisocumarin (DCI) an inhibitor for serine proteases, alphafluoromethylhistidine (á-FMH) an histidine decarboxylase inhibitor and phorbol-myristate-acetate (PMA). While we could detect expression of unprocessed ~74kDa HDC in many of these models we were not satisfied that sufficient levels of cleavage could be detected to support experimental studies specifically on processing.

Site directed mutagenesis was used to mutate putative cleavage sites in the rat HDC protein sequence. These are the same sites that were shown to mediate cleavage of the mouse HDC enzyme in P815 cells. These constructs were transfected into Cos7 cells. These cells are monkey in origin and do not express endogenous HDC, but are nevertheless capable of processing the enzyme. As can be seen in Fig.1, the DD motifs located in rat HDC do not seem to important for post-translational processing in Cos7 cells and only the 502-504 mutant that has previously been described is showed disruption of processing.

Figure 1
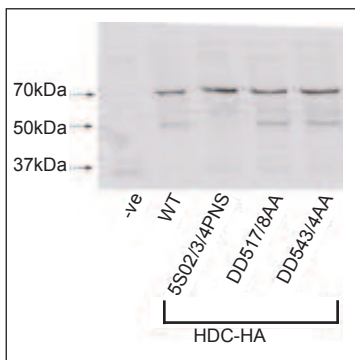
Conclusions

In this study we demonstrated that the DD motifs in the rat HDC protein sequence are not employed for posttranslational processing in Cos7 cells.

Future studies will address whether this reflects patterns of cell or species specific processing.

We additionally identified cell models that despite their limitations for studying HDC processing may nevertheless serve as a useful source of unprocessed enzyme for use in proteolysis assays.

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Research and Characterization of New Materials for the Production of Edible Coatings

ABSTRACT

Tropical fruits are subjected to great losses from harvest to consumption. The main objective of this work was to research and characterize new materials as galactomannans and determine the optimal composition of galactomannan-based coatings in view of their application to extend the shelf life of several tropical fruits. Galactomannans extracted from seeds of *Caesalpinea pulcherrima*, *Adenanthera pavonina*, *Gleditsia triacanthos* and *Sophora japonica* were characterized as coatings for five tropical fruits: acerola (*Malpighia emarginata*), cajá (*Spondias lutea*), mango (*Mangifera indica*), pitanga (*Eugenia uniflora*) and seriguela (*Spondias purpurea*). The intrinsic viscosity of galactomannans solutions with and without glycerol, were determined. The surface properties of fruits was determined, and different formulations of aqueous galactomannan solutions (0.5 %, 1.0 % and 1.5 %) with glycerol (1.0 %; 1.5 % and 2.0 %) were tested in terms of wettability on the five fruits. To analyse the capacity of the galactomannans to decrease the fungal growth, tests using four strains of fungi were performed with: *Penicillium commune*, *Penicillium crustosum*, *Penicillium roqueforti* and *Botrytis cinerea*. The galactomannan that demonstrated to provide a more significant fungal inhibition was *G. triacanthos*. The *C. pulcherrima* seed galactomannan was analysed to determine its sub-chronic toxicity in mice, measuring the evolution of weight, glycemic levels and total cholesterol levels during three months; no toxicity was observed. The weight loss from acerola and mango, coated with galactomannan from *C. pulcherrima,* during storage time was determined, giving good indications that the use of solutions of this galactomannan and glycerol as coating decreases the weight loss of the fruits. The obtained results confirmed the suitability of galactomannan-based coatings to prolong the shelf life of tropical fruits.

TESTIMONIAL

"Helped me to learn to live in a new country with different culture."
"This experience was good in all levels: personal, academic, cultural and professional."

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Research and Characterization of New Materials for the Production of Edible Coatings

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Abstract

Tropical fruits are subjected to great losses from harvest to consumption. The main objective of this work was to research and characterize new materials as galactomannans and determine the optimal composition of galactomannan-based coatings in view of their application to extend the shelf life of several tropical fruits. Galactomannans extracted from seeds of *Caesalpinea pulcherrima*, *Adenanthera pavonina, Gleditsia triacanthos* and *Sophora japonica* were characterized as coatings for five tropical fruits: acerola (*Malpighia emarginata*), cajá (*Spondias lutea*), mango (*Mangifera indica*), pitanga (*Eugenia uniflora*) and seriguela (*Spondias purpurea*). The intrinsic viscosity of galactomannans solutions with and without glycerol, were determined. The surface properties of fruits was determined, and different formulations of aqueous galactomannan solutions (0.5 %, 1.0 % and 1.5 %) with glycerol (1.0 %; 1.5 % and 2.0 %) were tested in terms of wettability on the five fruits. To analyse the capacity of the galactomannans to decrease the fungal growth, tests using four strains of fungi were performed with: *Penicillium commune*, *Penicillium crustosum*, *Penicillium roqueforti* and *Botrytis cinerea*. The galactomannan that demonstrated to provide a more significant fungal inhibition was *G. triacanthos*. The *C. pulcherrima* seed galactomannan was analysed to determine its sub-chronic toxicity in mice, measuring the evolution of weight, glycemic levels and total cholesterol levels during three months; no toxicity was observed. The weight loss from acerola and mango, coated with galactomannan from *C. pulcherrima,* during storage time was determined, giving good indications that the use of solutions of this galactomannan and glycerol as coating decreases the weight loss of the fruits. The obtained results confirmed the suitability of galactomannan-based coatings to prolong the shelf life of tropical fruits.

Resumo

Os frutos tropicais são sujeitos a grandes perdas desde a colheita até o consumo. O objectivo principal deste trabalho foi determinar a composição óptima dos revestimentos de galactomananas, tendo como finalidade aumentar o *shelf life* de frutos tropicais. Galactomananas extraídas de sementes de *Caesalpinea pulcherrima*, *Adenanthera pavonina, Gleditsia triacanthos* e *Sophora japonica* foram solubilizadas e caracterizadas como revestimento de cinco frutos tropicais: acerola (*Malpighia emarginata*), cajá (*Spondias lutea*), manga (*Mangifera indica*), pitanga (*Eugenia uniflora*) e seriguela (*Spondias purpurea*). A viscosidade intrinseca foi determinada com soluções de galactomananas com e sem glicerol. As propriedades de superfície dos cinco frutos foram determinadas, e diferentes formulações de soluções aquosas de galactomanana (0.5 %, 1.0 % e 1.5 %) com glicerol (1.0 %; 1.5 % e 2.0 %) foram testadas em termos de *wettability* nos cinco frutos. Com o objectivo de analisar a capacidade das galactomananas estudadas em decrescer o crescimento

fúngico, foram realizados testes com cinco estirpes de fungos: *Penicillium commune*, *Penicillium crustosum*, *Penicillium roqueforti* e *Botrytis cinerea*. A galactomanana que demonstrou um maior efeito na inibição no crescimento fúngico foi a *G. triacanthos*. A galactomanana de *C. pulcherrima* foi usada para determinar a toxicidade sub-crónica, avaliando a evolução dos pesos, colesterol e níveis de glicemia de ratinhos durante 3 meses. Não foi observada toxicidade para a galactomanana de *C. pulcherrima* quando comparada com o grupo de controlo. Também se avaliou a perda de massa da acerola e da manga durante o seu armazenamento, demonstrando-se que os revestimentos de *C. pulcherrima* diminuem a perda de massa dos frutos. Os resultados obtidos confirmaram a possibilidade de aplicar os revestimentos de galactomananas em frutos tropicais para lhes aumentar o tempo de prateleira.

Introduction

During the last decades, there has been an increase in the demand for fresh fruit and vegetable products that forced the industry to develop new and improved methods for maintaining food quality and extending shelf life. Great losses (from 20 to 80%) in the quality of fresh fruits occur between their harvest and consumption, and one of the most important drawbacks in fruit distribution chains is their short shelf life (1). On the other hand, consumers around the world demand food of high-quality, without chemical preservatives, and with extended shelf life. So, an increased effort has been made to discover new natural preservatives and antimicrobials (2). The main factors responsible for extending the shelf life of fruits and vegetables include: careful harvesting (as not to injure the product), harvesting at optimal horticultural maturity and good sanitation (3, 4). When these practices are applied, the implementation of optimum storage conditions through modified atmosphere can be quite effective at maximizing the shelf life and quality of the product. This is done by controlling factors such as temperature, relative humidity, gas composition, light and mechanical/physical stress. In particular, packaging plays a decisive role in the improvement of fruit shelf life and new packaging materials are expected to be developed. Most of these will be derived from renewable resources (5). The application of edible coatings to freshly harvested products offers a less expensive alternative with potentially equally beneficial outcomes. The use of coatings creates a modified atmosphere surrounding the commodity similar to that achieved by controlled or modified atmospheric storage conditions. The modified atmosphere created by edible coatings can protect the food from the moment it is applied, through transportation to its final retail destination, and in the home of the consumer (6, 7, 8). Coatings made of polysaccharides have a no oily aspect, a low caloric content and can be used to increase the shelf life of fruits, vegetables, shellfish or meat products avoiding the dehydration, the oxidative rancidity and the darkening of the surface. Their application in agriculture became popular due to their permeability to CO_2 or O_2, their more convenient color, the effect of reducing weight loss, extends shelf life and can prevents microbial spoilage of the fruits (9, 10, 11). However, the effectiveness of edible coatings for fruits preservation depends primarily on the control of the wettability of the coating followed by the permeability properties and mechanical resistance. Currently the international trends demand the introduction of alternative sources of seed gums (12) and it is therefore important to search for alternative renewable sources for e.g. the production of edible and biodegradable films and coating materials. In particular, Latin American sources of galactomannans are not well known, in spite of the rich biodiversity of the local flora and of the favourable climate for their production (13).

Materials and Methods

The **seeds** of *A. pavonina* (AP) and *C. pulcherima* (CP) were collected in Campus do Pici, Federal University of Ceará-Fortaleza (Ce-Brazil) during January 2006. The seeds of *G. triacanthos* (GT) and *S.

japonica (SJ) were collected in Botanic Garden in Porto, Portugal, during April of 2006. The **materials** used to prepare the edible coating solutions were: galactomannans, glycerol (87 %, Panreac, Spain) and distilled water. Bromonaphthalene (Merck, Germany), formamide (Merck, Germany) and ultra pure water were used to determine fruits surface properties. Acerola, pitanga, seriguela, cajá and mango were purchased from a local supermarket (Fortaleza, Ce-Brazil). All fruits were kept at 8-10 ºC until further use. The **fruits** were selected for their uniformity, size, color and absence of damage and fungal infection. Before testing, the fruits were left at room temperature (20 ºC) and their surface was cleaned with distilled water. Thin portions of the outer surface (skin) of the fruits were cut with a knife and adhered to a glass plate.

To obtain the **galactomannan** an aqueous extraction was performed. In this process, the seeds were removed from the pods, cleaned and placed in a blender, where they were mechanical broken. Following the operation the endosperm was manually separated from the germ and the hull, suspended in ethanol (purity 99.8 %, Riedel-de Haën, Germany) at 70 ºC during 15 minutes. The ethanol was decanted and distilled water was added in a proportion of 1:5 (endosperm: water), the suspension was left to rest for approximately 24 hours. Then water, in a proportion of 1:10, (suspension: water) was added and mixed in a blender for 5 min. The endosperm mixed in the blender was filtered through a nylon net followed by a centrifugation step at 3 800 g (Sigma 4K, B. Braun, Germany) during 20 minutes at 20 ºC. The precipitation of the galactomannan was achieved by adding the supernatant to ethanol (purity 99.8 %, Riedel-de Haën, Germany) at a ratio of 1:2. The ethanol was decanted and the precipitated galactomannan was lyophilized and kept in a dry place until further use.

The **intrinsic viscosity** measurements were performed in aqueous solutions prepared by stirring the mixture of galactomannans and glycerol were total solubilization was achieved. An Ubbelohde capillary viscometer of 75 mL was used. Intrinsic viscosity determinations were made in solutions of different concentrations of galactomannans and glycerol.

The **coating solutions** were prepared dissolving the galactomannans in distilled water followed by the addition of the glycerol. Each solution was stirred during 2 hours and left to stabilize during 10 minutes at room temperature.

The **critical surface tension** of the fruits as determined according to Zisman (14). In systems having a surface tension lower than 100 mN/m (low-energy surfaces), the contact angle formed by a drop of liquid on a solid surface will be a linear function of the surface tension of the liquid. The Zisman method is applicable only for low energy surfaces; therefore it is necessary to determine the surface energy of the fruits. The estimation of the critical surface tension was performed by extrapolation from Zisman plots (14). Owens and Wendt (15), Rabel (16) and Kaelble (17) demonstrated that both the tensions of liquid and solid can be separated in to polar and dispersive interactions. The liquids used to determine the surfaces properties from the fruits have: the surface tension, the dispersive and the polar component were, respectively, 72.10, 19.90 and 52.20 mN/m to water, 44.40, 44.40 and 0.00 mN/m to bromonaphtalene and 56.90, 23.50 and 33.40 mN/m to formamide (18).

When a solid is contacted by a liquid in the presence of vapour, the liquid will adhere well on the solid surface if the total free energy required for the creation of the new interface decreases. The physical significance of this energy change is the work needed to separate the solid and liquid from the solid/liquid interface, being the equilibrium the **spreading coefficient** (*Ws*). Contact angle and liquid-vapor surface tension were measured in a face contact angle meter (OCA 20, Dataphysics, Germany). The **surface tension** of the coating solution was measured by the pendant drop method using the Laplace-Young approximation (19). The samples of the coatings were taken with a 500 µL syringe (Hamilton, Switzerland), with a needle of 0.75 mm of diameter. The contact angle at the fruit surfaces was measured by the sessile drop method (20), in which a droplet of the tested liquid was placed on a horizontal surface and observed with a face contact angle meter.

The **antifungic test** was performed with four strains of fungi: *Penicillium commune*, *Penicillium crustosum*, *Penicillium roqueforti* and *Botrytis cinerea*. These standard strains were obtained from the Micoteca da Universidade do Minho (MUM), in Braga, Portugal. The stock culture was maintained at 4 ºC. In vitro antifungal activity was determined using Malt extract Agar (MEA). The fungi were cultured on MEA and its conidial suspensions were prepared by flooding a Petri dish containing a 1 to 2 week-old sporulating culture with a solution of sterile distilled water containing agar 0.2 % (w/w) and Tween 80 0.05 % (w/w). MEA was prepared and galactomannan was used to partially replace agar in a proportion of 50 % (w/w) of galactomannan and 50 % (w/w) of agar. The following procedure of autoclaving and plating was followed as before. The prepared plates containing galactomannan were inoculated with 20 µL of spore's suspension. The diameter of fungal growth was measured and used as a measure of growth inhibition during six days; the experiments were performed in triplicate. Plates containing natamicine were used as the negative control.

The **sub-chronic toxicity** has the objective of providing information of the potential risk to the health of repeated dosage of galactomannans in a limited period of time. The experiments were conduced in rats and cares were taken such as adaptation to the experimental atmosphere, and food regime. The animals were randomly distributed into three groups of five animals per sex per group and received untreated control diet mixed with control diet at dose levels 1000 mg/kg/day and 500 mg/kg/day for a period of at least three months. A control group (five individuals per sex) received untreated standard laboratory diet (control). The animals were heavy, weekly. The parameters: corporal weight, glycemic levels and total cholesterol were certain and appraised.

For the determination of the glycemic levels, the catalyzed reaction of Hexoquinase was used. The samples were picked in the anticoagulant presence. The kit of LABTEST (Centerlab, Brazil) was used and the readings made at 340 nm in a spectrophotometer. For the determination of the total cholesterol the reactions of the enzymes esterase, oxidase and peroxidase were used. The samples were collected in the presence of an anticoagulant. The kit of LABTEST (Centerlab, Brazil) was used and the readings were made in a spectrophotometer at 500 nm.

Results and Discussion

Rheological Properties

The knowledge of rheological properties, such as intrinsic viscosity, of galactomannans and their mixtures with glycerol, is important to understand the possibilities of application and the behaviour of novel galactomannan solutions as coatings. Table 1 shows the values of the intrinsic viscosity for the studied mixtures. The intrinsic viscosity increases with the decreasing degree of substitution of the backbone chain: *A. pavonina* < *G. triacanthos* < *C. pulcherrima* < *S. japonica*, which is in accordance with Cui (21). The glycerol is a small stiff molecule which will interfere with the mixture of polysaccharide and water decreasing the interactions between the polysaccharide chains (22). The synergistic interactions between the galactomannan and plasticizante decrease the intrinsic viscosity of the solutions, in the case of GT the synergistic increase the intrinsic viscosity.

Table 1 – Values from intrinsic viscosity of the galactomannans of four species of plants, with and without glycerol

Species	A. pavonina	C. pulcherrima	G. triacanthos	S. japonica
Galactomannan (no glycerol)	6.47 ± 0.45	7.22 ± 0.52	6.62 ± 0.29	9.13 ± 0.17
Galactomannan - 1 X Glycerol	3.70 ± 0.79	4.33 ± 0.67	7.31 ± 0.26	8.81 ± 0.11
Galactomannan - 4 X Glycerol	2.77 ± 0.89	3.90 ± 0.50	7.73 ± 0.20	1.88 ± 0.42

[1]Measured at the temperature of 29.06 ± 0.14 ºC (n = 5)

Surface tension and critical surface tension of fruits skins

Table 2 displays the values of the surface tension of the fruits and its polar and dispersive components. All the fruits present a higher dispersive component, which shows the ability of the fruit surface to participate in non-polar interactions. This was also demonstrated by Ribeiro et al. (8) in strawberry, where the dispersive component was higher than the polar component. Critical surface tension (Table 2) was obtained for each fruit, and varies between 9.39 and 23.92 mN/m. In all cases it is possible to conclude that the studied fruits have low energy surfaces (below 100 mN/m) meaning that the Zisman method is applicable. The obtained values are close to the critical surface tension of the apple (18.70 mN/m) and of the orange (20.00 mN/m) presented by Choi et al. (23), exception observed to acerola and pitanga that present a lower value. Also, the values of critical superficial tension must be lower than the values of superficial tension of the solid (24), which holds true for all the fruits used in this study.

Table 2 – Surface tension, polar component, dispersive component and critical surface tension of the tested fruits

Fruits	Surface tension (mN/m)	Polar component (mN/m)	Dispersive component (mN/m)	Critical surface tension (mN/m)
Acerola	27.94 ± 0.03	4.35 ± 0.01	23.59 ± 0.02	9.389 ± 0.001
Cajá	30.15 ± 0.02	2.29 ± 0.01	27.86 ± 0.01	23.923 ± 0.001
Mango	29.04 ± 0.02	1.47 ± 0.01	27.57 ± 0.01	22.678 ± 0.002
Pitanga	26.95 ± 0.02	3.07 ± 0.01	23.88 ± 0.01	13.419 ± 0.001
Seriguela	31.48 ± 0.05	4.59 ± 0.03	26.89 ± 0.02	19.622 ± 0.002

[1]Measured at the temperature of 21.27 ± 0.08 ºC (n = 20)

Wettability

Wettability determinations were performed with different galactomannan concentrations for varying plasticizer concentrations. The wettability was studied by determining the values of the spreading coefficient (*Ws*). The values of the spreading coefficient from the galactomannans of AP, CP, GT and SJ when applied on each fruit were analysed and are presented below. To GT and SJ were tested only in mango. For each fruit, the best (higher) value of *Ws* for the respective galactomannan was determined (Tukey test, p < 0.05). The best values are filled in gray. The results show that the values of *Ws* are quite dependent on both the source and concentration of galactomannan, and the fruit tested. In table 3, the values of *Ws* obtained using the galactomannan of AP are displayed.

Table 3 – Spreading coefficient (*Ws*), obtained for solutions of AP galactomannan and glycerol on the analysed fruits

Gal. (w/v)	Glycerol (v/v)	Acerola	Cajá	Mango	Pitanga	Seriguela
0.5	1.0	-29.92 ± 2.10 [a]	-36.50 ± 3.05 [a]	-30.97 ± 2.17 [ade]	-28.17 ± 7.27 [a]	-29.15 ± 2.78 [a]
0.5	1.5	-36.35 ± 3.95 [b]	-35.32 ± 3.74 [a]	-31.40 ± 2.97 [ade]	-31.71 ± 6.11 [a]	-23.72 ± 2.01 [b]
0.5	2.0	-42.38 ± 3.58 [c]	-27.84 ± 2.89 [b]	-37.91 ± 3.80 [b]	-39.13 ± 7.15 [bcd]	-28.95 ± 3.74 [a]
1.0	1.0	-42.11 ± 3.03 [c]	-30.80 ± 2.96 [b]	-34.37 ± 2.43 [abc]	-38.53 ± 3.91 [bcd]	-31.11 ± 2.79 [ad]
1.0	1.5	-46.71 ± 2.91 [d]	-36.96 ± 4.37 [a]	-30.93 ± 3.62 [ade]	-38.18 ± 3.78 [bcd]	-37.46 ± 2.65 [c]
1.0	2.0	-47.09 ± 4.54 [d]	-32.00 ± 3.44 [c]	-35.39 ± 1.75 [bc]	-44.22 ± 6.98 [c]	-36.86 ± 3.15 [c]
1.5	1.0	-41.57 ± 5.04 [c]	-32.85 ± 2.67 [c]	-29.18 ± 3.57 [e]	-26.45 ± 4.58 [a]	-32.35 ± 2.96 [d]

[1]Measured at the temperature of 29.06 ± 0.14 ºC (n = 5)

| 1.5 | 1.5 | -41.06± 3.04 [c] | -31.24 ± 2.91 [bc] | -33.71 ± 2.95 [acd] | -32.75 ± 3.27 [ca] | -32.54 ± 3.70 [d] |
| 1.5 | 2.0 | -42.68 ± 1.41 [c] | -31.54 ± 3.21 [bc] | -30.38 ± 2.39 [ade] | -35.15 ± 2.68 [cd] | -37.90 ± 2.26 [c] |

[a-e] Means (n = 10) in same column with different superscript are significantly different ($p < 0.05$).

When the galactomannan of CP was used, the values of Ws (table 4) present statistically significant differences for each fruit. In this case, a single solution that has the lower value of Ws was found. In all cases, with the exception of mango (the best Ws value was obtained with 1.5 % of galactomannan) the best value of Ws was obtained with values of 0.5 % of galactomannan.

Table 4 – Spreading coefficient (Ws), obtained for solutions of CP galactomannan and glycerol in the analysed fruits

Gal. (w/v)	Glycerol (v/v)	Acerola	Cajá	Mango	Pitanga	Seriguela
0.5	1.0	-42.68 ± 6,50 [a]	-27.69 ± 3.73 [a]	-51.29 ± 4.14 [a]	-40.76 ± 4,61 [a]	-40.57 ± 3.10 [a]
0.5	1.5	-39.83 ± 5,95 [a]	-31.85 ± 2.37 [b]	-65.48 ± 4.57 [b]	-35.54 ± 5,39 [b]	-36.33 ± 3.39 [b]
0.5	2.0	-32.59 ± 4,65 [b]	-38.86 ± 5.14 [ce]	-49.73 ± 6.09 [a]	-39.70 ± 3,71 [a]	-40.66 ± 2.82 [a]
1.0	1.0	-44.70 ± 4,42 [a]	-45.34 ± 4.46 [d]	-68.82 ± 6.38 [c]	-47.91 ± 6,25 [c]	-45.27 ± 3.17 [c]
1.0	1.5	-43.47 ± 3,37 [a]	-45.08 ± 3.54 [d]	-77.83 ± 5.87 [d]	-50.01 ± 4,73 [c]	-51.16 ± 3.82 [d]
1.0	2.0	-41.36 ± 3,32 [a]	-47.48 ± 4.31 [d]	-66.24 ± 7.72 [ec]	-57.02 ± 2,86 [d]	-49.87 ± 3.51 [d]
1.5	1.0	-42.38 ± 6,32 [a]	-40.06 ± 6.04 [ce]	-64.36 ± 7.84 [ec]	-41.88 ± 4,30 [a]	-41.44 ± 4.72 [a]
1.5	1.5	-40.60 ± 3,77 [a]	-37.55 ± 2.59 [c]	-62.81 ± 4.26 [e]	-43.01 ± 5,46 [a]	-42.29 ± 3.37 [a]
1.5	2.0	-58.65 ± 5,65 [c]	-43.58 ± 3.72 [ced]	-45.20 ± 4.49 [f]	-58.83 ± 5,31 [d]	-47.81 ± 3.59 [d]

[a-e] Means (n = 10) in same column with different superscript are significantly different ($p < 0.05$).

The wettability of galactomannans solutions from GT and SJ were tested on mango surface. The values of Ws (table 5) to GT solution present the best value to 1.0 % of galactomannan and 2.0 % of glycerol presenting statistically significant differences for the other solutions. In SJ case, the solution of 1.0 % of galactomannan and 1.5 % of glycerol has the lower value of Ws (statistically significant difference from other solutions). The SJ solutions of 1.5 % were not tested, the high viscosity of the solutions do not allow the application of the wettability procedure.

Table 5 – Spreading coefficient (Ws), obtained for solutions of GT and SJ galactomannan and glycerol in mango.

Gal. (w/v)	Glycerol (v/v)	*G. triacanthos*	*S. japonica*
0.5	1.0	-64.79 ± 2.72 [abc]	-56.45 ± 6.28 [a]
0.5	1.5	-66.89 ± 5.14 [ab]	-65.49 ± 5.21 [b]
0.5	2.0	-64.15 ± 2.46 [abc]	-57.97 ± 8.65 [ab]
1.0	1.0	-60.79 ± 5.13 [cd]	-61.23 ± 4.69 [ab]
1.0	1.5	-68.46 ± 1.75 [b]	-41.66 ± 4.72 [c]
1.0	2.0	-52.24 ± 4,26 [e]	-50.36 ± 6.14 [d]
1.5	1.0	-61.38 ± 3.54 [acd]	-
1.5	1.5	-57.98 ± 5.35 [d]	-
1.5	2.0	-64.34 ± 1.00 [abc]	-

[a-e] Means (n = 10) in same column with different superscript are significantly different ($p < 0.05$).

Antifungic tests

Some of the galactomannan present inhibitory effects in fungal growth. In the negative control no fungal growth happens. The galactomannan of GT presents the most favourable results. With *P. commune* the best results happen with GT. Exist statistically difference between the results for plates with galactomannan from GT and the positive control. In the other and, do not exist statistically difference with the negative control. Also to CP exist statistically difference with positive control. To *P. crustosum* the best results also happens to *G. triacanthos* and *C. pulcherrima,* existing statistically difference between there results and the positive control. To *P. roqueforti,* only GT present good results, presenting statistically difference with the positive control. In the case of *B. cinerea* GT galactomannan have the best results with statistically difference with positive control and without statistically difference with negative control, to all days of test.

**Table 6 –** Mean zone inhibition for the five types of plates to the four fungi's used.

	Mean zone inhibition (cm)									
	Positive Control		Negative control		*A. pavonina*		*G. triacanthos*		*C. pulcherrima*	
Time (days)	3	6	3	6	3	6	3	6	3	6
P. commune	1.9 [a]	4.0 [b]	0.5 [c]	0.5 [c]	1.9 [a]	3.5 [d]	1.0 [c]	2.3 [e]	1.5 [f]	3.1 [d]
P. crustosum	2.3 [ab]	4.4 [c]	0.5 [d]	0.5 [d]	2.2 [ae]	4.1 [c]	1.5 [f]	2.6 [b]	1.8 [ef]	3.0 [g]
P. roqueforti	2.7 [a]	6.1 [b]	0.5 [c]	0.5 [c]	2.9 [a]	6.4 [b]	1.5 [c]	4.2 [d]	2.4 [a]	5.2 [e]
B. cinerea	2.0 [a]	5.4 [b]	0.5 [c]	0.5 [c]	2.6 [ad]	10.0 [e]	0.5 [c]	1.1 [c]	2.4 [d]	4.5 [b]

[a-e] Means (n = 10) in same column with different superscript are significantly different ($p < 0.05$).

Sub-chronic toxicity

The sub-chronic was tested to the galactomannan from CP. Figure 1 presents the mean body weight of males during the three months of treatment. Comparing the means of the three groups by the Tukey test it is possible to conclude that there is no statistically difference between the three groups. No mortality occurred during this study in the case of the males.

In the group of females (Figure 1 (b)) treated with 500 mg/kg/day of CP, two have died, one at day 80 and the other at day 89. Tukey test demonstrates that there was no statistically difference between the three groups. The results show that the inclusion of galactomannan in the rats' diet does not influence their body weight.
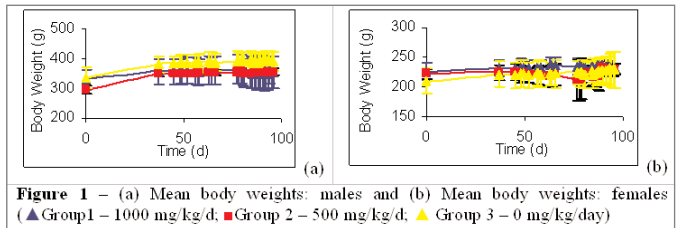
Figure 1 – (a) Mean body weights: males and (b) Mean body weights: females (▲Group1 – 1000 mg/kg/d; ■Group 2 – 500 mg/kg/d; ▲ Group 3 – 0 mg/kg/day)

Glycemic and cholesterol levels

The glycemic and cholesterol levels were measured with the galactomannan from CP. Figure 2(a) shows that the values of glucose for both groups having a daily amount of galactomannan of 500 and 1000 mg/Kg decrease.

In the case of the females (Fig. 2(b)), those eating a daily amount of galactomannan of 500 mg/kg show an increase of the glycemic levels comparing with the control group. In the group fed with 1000 mg/kg/day of galactomannan the value decrease. In cholesterol levels there was an increase (Fig. 2(c)) in the

groups to which the galactomannan has been fed, being the group fed with 500 mg/kg//day the one which shows a higher level of total cholesterol. Figure 2(d) show that also the females display an increase in the total cholesterol levels. In both cases (males and females) the level of total cholesterol is higher for the group that was feed with 500 mg/kg/day of galactomannan.

Figure 2 – Glycemic levels of the males (a) and of the females (b); Total cholesterol levels of the males (c) and of the females (d) (■Group1 – 1000 mg/kg/d; ■Group 2 – 500 mg/kg/d; ■ Group 3 – 0 mg/kg/day)
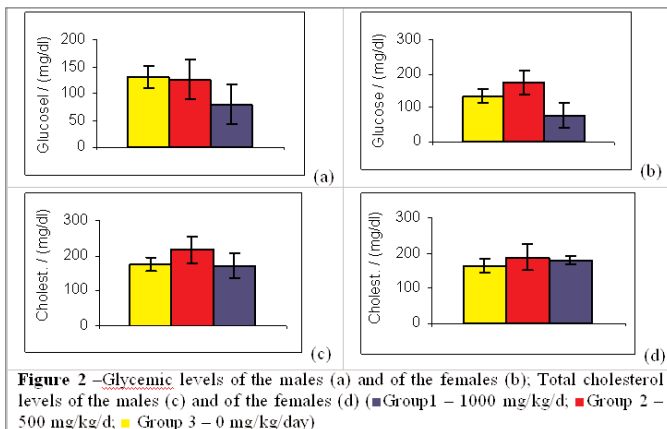
Application of the coating (CP) in acerola and mango fruit

After the optimization by th *Ws* of the solution of galactomannan from *C. pulcherrima*, the solution with 0.5 % galactomannan and 2.0 % of glycerol was applied on acerola. The fruit weight was measured during two days after the application of the solution of galactomannan and glycerol (respectively) when the fruit was stored at the temperature of 25.5ºC, and during six days under the storage temperature of 8.1 ºC. At a temperature of 25.5 ºC and after 2 days the mass of acerola without coating was decay 3.40 g and with coating 2.56 g. The same experiment was repeated under the temperature of 8.1 ºC in order to study the effects temperature. Also in the case that the temperature is 8.1 ºC the fruits with the coating have less lost of the weight that the fruit without coating (control). With those results we can say that at this temperature the coating go to influence the decrease of the weight from acerola. The same solution of CP was applied in mango fruits, in this case two techniques were tested; in the first the fruits were merged in CP solution and in the second case the fruits were painted. The fruit weight was measured sixteen days after the application during the storage at 25 ºC. The control and the merged fruits do not have statistically difference, on the other hand the painted show a decrease of weight loss. The visual appearance of the mango presents a big difference, with the fruits which were treated with coatings showing a much better appearance.

Conclusions

The first conclusion drawn from this study is that galactomannans can be extracted from seeds in a fast and cheap way and that they can be applied as coatings. The intrinsic viscosity of solutions from different galactomannans and glycerol were determined using a glass capillary viscometer, of Ubbelohde type. The values to the four galactomannans: *Adenanthera pavonina, Caesalpinia pulcherrima, Gleditsia triacanthos and Sophora japonica,* were respectively: 6.47 cm^3/g, 7.22 cm^3/g, 6.62 cm^3/g, 9.13 cm^3/g. The capacity of the seed galactomannans to decrease the fungal growth has been evaluated and there was an observable decrease in growth, especially in the presence of the galactomannan from seeds of *G. triacanthos*. The subchronic toxicity analyse from the *C. pulcherrima* galactomannan in rats, measuring the body weights, glycemic levels and total cholesterol levels, have demonstrate that this galactomannan don't have toxicity in rats. The fruit surface was characterized, being the surface tension and the critical surface measured for acerola, cajá, mango, pitanga and seriguela. The surface properties of the galactomannans were analysed in different fruits, and the mixture galactomannan/glycerol for the coating of a given fruit was optimized.

The best values of wettability were obtained for the coating compositions under study, depending on the origin of the galactomannan also on the fruit. The best coatings for the fruits were those corresponding to best values of the spreading coefficients (*Ws*). The study of weight variation of acerola during time for two different storage temperatures was performed using the best coating (0.5 % galactomannan and 2.0 % of glycerol) from *C. pulcherrima* as coatings. There was a decrease of weight loss using coatings for both temperatures tested. The study of weight variation of mango (1.5 % galactomannan (CP) and 2.0 % of glycerol) showed that the weight loss observed for different application methods of coatings does not make a significant difference, but the visual appearance of the mango presents a big difference, with the fruits which were treated with coatings showing a much better appearance. The obtained results confirmed the suitability from galactomannan based coatings to be applied in tropical fruits; further studies have to be made to study the permeability and mechanical properties from these coatings as the differences in respiration from coated and uncoated fruits.

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MÁRIO ALBERTO CRUZ-HERNANDEZ

Preliminary culture conditions for *Aspergillus niger* GH1 tannase production in submerged culture

ABSTRACT

Nowadays, tannase is produce by submerged culture (SmC) being the enzyme obtained in an intracellular form resulting, as a consequence, in high production costs. To demonstrate that fungal tannase excretion is strongly influenced by the culture conditions, tannase production by *Aspergillus niger* GH1 on SmC using different bioreactors was evaluated. Two sets of experiments were conducted using in a first step a 1.5 L bioreactor and in a second step 100 mL erlenmeyer flasks. Modifications to culture conditions previously reported allowed to obtain positive results for tannase production in SmC, being the selected conditions: initial pH 5, temperature 35°C, initial substrate concentration 25g/L and agitation 200 rpm.

TESTIMONIAL

"Was extremely important at a professional point of view, giving me the opportunity to meet experienced people in my research area." "I also had the chance of understand some of the cultural differences between Portugal and my home country, which helped me to adapt and enjoy myself."

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Preliminary culture conditions for *Aspergillus niger* GH1 tannase production in submerged culture

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Abstract

Nowadays, tannase is produce by submerged culture (SmC) being the enzyme obtained in an intracellular form resulting, as a consequence, in high production costs. To demonstrate that fungal tannase excretion is strongly influenced by the culture conditions, tannase production by *Aspergillus niger* GH1 on SmC using different bioreactors was evaluated. Two sets of experiments were conducted using in a first step a 1.5 L bioreactor and in a second step 100 mL erlenmeyer flasks. Modifications to culture conditions previously reported allowed to obtain positive results for tannase production in SmC, being the selected conditions: initial pH 5, temperature 35°C, initial substrate concentration 25g/L and agitation 200 rpm.

Resumo

A cultura submersa (SmC) é o processo empregado para produzir comercialmente a enzima tanase, po-rém neste sistema da cultura, a tanasa é expressada principalmente de maneira intracelular o que represen-ta custos de produção elevados. Para demonstrar que a excreção da tanasa do fungo está fortemente in-fluenciada pelas condições da cultura, a produção da enzima tanase por *Aspergillus niger* GH1 em SmC foi avaliada. Dois conjuntos de experiências foram conduzidos usando em uma etapa um fermentador de 1.5 L e em uma segunda etapa matrazes (erlenmeyers) de 100 mL. As modificações às condições da cul-tura previamente descritas permitiram a obtenção de resultados positivos para a produção do tannase neste modo de produção, tendo sido definidas as seguintes condições de cultura: pH inicial 5, concentração ini-cial de substrato 25g/L, temperatura 35°C e agitação 200 rpm.

Introduction

Tannase hydrolyzes the molecule of tannins by its esterase activity, giving gallic acid and glucose as pro-ducts [1]. Tannase can be obtained from animal, plant and microbial sources being this the most important, because the enzyme produced by the microbial route is more stable that the analogous obtained by other sources. Also, microorganisms can produce larger amounts using friendly techniques of fermentation that coupled with genetic manipulation result in an increment in the activity of the produced enzyme [2]. The production on commercial scale of tannase is through submerged fermentation – SmC - using filamentous fungi in which the enzyme is expressed in an intracellular form being located in cell periplasm [3]. The enzyme is retained in the cell wall of the fungus requiring, as a consequence, an efficient recovery and purification so that an enzyme extract with high activity is obtained [4]. Tannase has many applications in the food industry, in particular in the juice industry. The production system to obtain this enzyme has been

intensively studied and with the aim of developing an efficient processes new culture conditions, new reactors and new strains have been investigated. Solid state fermentation – SSC - has been reported as an attractive process alternative [5] as, in this system, tannase is excreted from the hyphae in higher levels then in SmC and has been reported as more stable in a wide range of temperatures and pH values.

Results on the effect of agitation, temperature, air flowrate and substrate concentration on tannase production by *Aspergillus niger* GH1 in submerged fermentation are presented.

Materials and Methods

Microorganism

The fungus obtained from a lyophilized stock was hydrated with sterile distilled water. The obtained sample was inoculated in a plate with potato dextrose agar (PDA) and incubated at room temperature during one week. In the next step, the fungus was inoculated in other PDA containing plate and the obtained pure culture was distributed in three parts - one to be stored, one to be used as a working culture and another for the fermentation experiments.

Submerged culture

The effect of temperature (25 and 35ºC) on the *Aspergillus niger* GH1 biomass production was evaluated in a 1.5L bioreactor, at an agitation rate of 200 rpm. An initial tannic acid concentration of 25 g/L on a Czapek dox medium and a pH of 5.5 were used. The spore´s concentration was $1*10^6$ spores/mL. In the experiments using 100 mL Erlenmeyer flasks, the effect of pH on tannase production was evaluated for initial pH values of 3 and 5. The use of mycelia or spores as alternative inoculation processes was also considered.

Tannase assay

One unit of tannase was defined as the amount of substrate (methyl gallate) consumed per minute under assay conditions. The used protocol is as follows: the blank contained 100ì L buffer acetate 50 mM pH 5 and 100ì L of methyl gallate 0.1M; the sample was formed by 100 ì L of extract and 100 ì L of methyl gallate and the control by 100 ì L of buffer with 100 ì L of extract; solutions were incubated at 30°C for 30 minutes and the reaction stopped by the addition of 200 ì L of 2M HCl.

Analytical methods

The consumed substrate was evaluated using the phenol sulphuric acid method (Dubois 1954), the protein was determined with the Bradford method and the biomass concentration by dry weight. Intracellular extracts were obtained by disrupting the cells with liquid nitrogen and the proteins ressuspended with acetate buffer.

Results and Discussion

Fig. 1 shows the main fermentation results for the experiments carried in the 1.5 L agitated tank, for two temperatures – 25 and 35 ºC. Samples were taken each 8 h in a total operation time of 120 and 90 hours. The results presented in Fig. 1A show that biomass concentration decreases during the first 48 h while, for the higher temperature (Fig. 1B) this decrease is prolonged till 60 hours of fermentation.
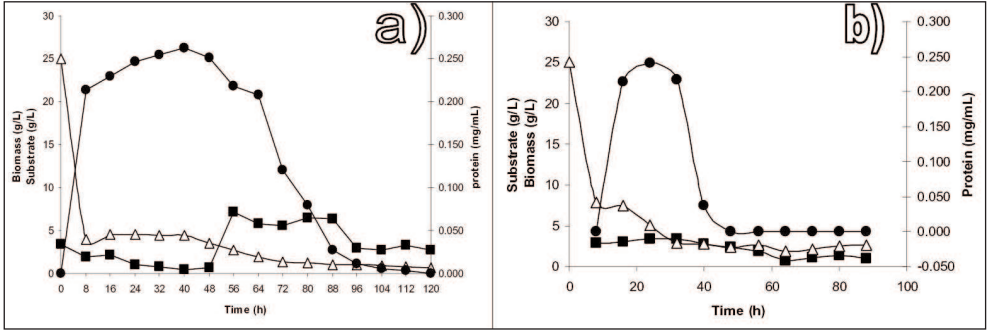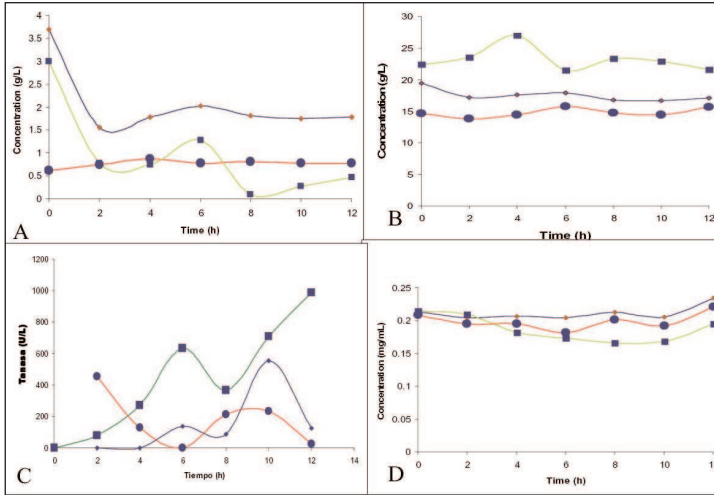
Figure 1. Substrate degradation (Δ), protein production (●) and biomass production (■) at 25°C (A) and 35°C (B) for the fermentation experiments in a 1.5 L bioreactor

Following the results obtained in the 1.5 L bioreactor, it was necessary to have a more detailed evaluation of the fermentation behaviour due to the fast uptake of substrate that occurs during the first 12 hours. The same trend has not been observed for biomass production and has been different according to the used temperature.

More detailed information concerning biomass formation was obtained by carrying fermentation experiments in shake flasks. Experiments were done using two types of innoculum – spores and homogenous fungal mycelium - as enzyme production might reduce the lag phase for microbial growth when mycelium is used. These experiments also aim at giving relevant information for the establishment of the best fermentation parameters. Figure 2A shows the amount of biomass produced in the fermentation process mentioned above during the first growth stages. The production of biomass during this analysis presented a constant performance in the experiments with spores and mycelium.

Concerning substrate degradation, (Figure 2B) no major change was observed due to the short fermentation time. The fungus had not enough time for developing remaining in the lag phase, although a small reduction in substrate concentration was observed. Protein content in the extracellular space increased slightly as the fungus is still starting the biosynthesis of the enzymes production system after 12h of fermentation. Although protein concentration is a reference parameter for tannase production, no tannase was measured in the extract after 12 hours of fermentation. Figure 2D shows a similar performance concerning protein in the culture medium when different types of inoculums are used. The intracellular tannase activity was also measured for these fermentations, being observed that the innoculum properties affect the intracellular enzyme production. After 12 hours, a higher tannase concentration was obtained for the experiment done using spores as innoculum. The amount of enzyme was clearly smaller for the other types of innoculum (Figure 2C). The obtained results require that longer fermentations are done to have a correct evaluation of the expression of tannase, both intracellular and extracellular.

Figure 2. A) Biomass production, B) Substrate degradation, C) Tannase production
D) Protein production using different types of innoculum.
*(□) first fermentation using spores, (◇) Fermentation using spores (○) Fermentation using fungal mycelium

Production of tannase can be done by various methods of fermentation as liquid surface, submerged, modified solid-state fermentation and solid-state fermentation. The use of submerged fermentation is advantageous because of the easy sterilization and the better process control process in these systems. Also, recovery of the products is easier.

Finally, several fermentations running for a longer time were done. The first variable evaluated was substrate degradation being observed that substrate concentration starts to diminish after 8 hours of fermentation achieving the lowest concentration at 48 h. At pH 5, almost complete substrate consumption is obtained. No relation could be established between substrate consumption and the production parameters. In what concerns biomass, a reduction in biomass concentration is observed, when most of the substrate consumption occurs, instead of the expected increase. The amount of protein also shows a decrease as fermentation proceeds and no protein is detected at the end of the process. Two different initial pH values were evaluated as formation of a precipitate was observed when 0.1 M NaOH was added to the fermentation medium in order to increase the initial pH. Figure 3A shows the fermentation behaviour at pH 3 and pH 5 - pH 3.0 is the standard value in the culture medium and the pH 5.0 is the adjusted pH. The results show that substrate degradation is higher when the medium pH is adjusted to 5, although a similar degradation profile is observed for both pH values. The rate of tannase production was slow being a significant increase in intracellular tannase concentration observed at 96 h of fermentation for pH 5.0. Results presented in Figures 3A and 3B clearly demonstrate the importance of adjusting the initial pH to 5 in order to obtain higher values of tannase concentration.
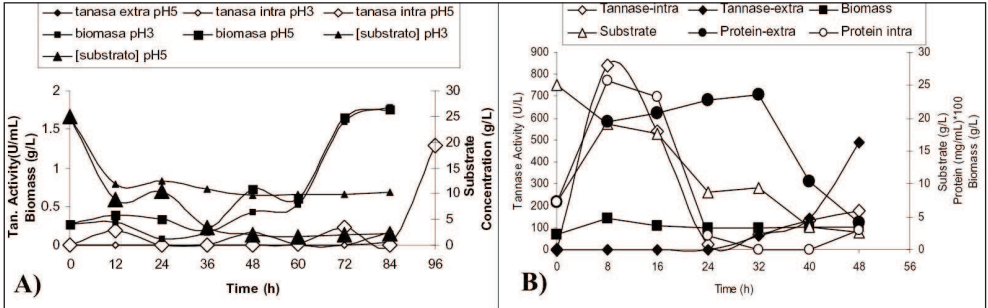
Figure 3. Time evolution of the fermentation parameters at pH 5 and pH 3 (a) and a detailed presentation of the fermentation parameters at pH 5 (b).

As the several fermentation runs were being carried and the obtained values for the fermentation parameters were not satisfactory, an optimization of the used analytical techniques was done and several improvements dealing with biomass, protein and tannase activity determination implemented. New procedures included dialysis of the samples, use of polyethylene glycol for samples concentration, cellular rupture using liquid nitrogen and measurement of tannase activity products by HPLC.

The application of these improved analytical procedures allowed for the obtention of the values represented in Figure 4. A clear biomass growth is observed in parallel with a decrease in substrate concentration and an increase in enzyme concentration.
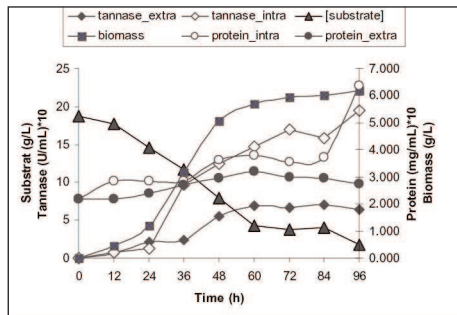
Figure 4. Time evolution of the fermentation parameters at pH 5, using the optimized analytical procedures

It is also shown that the intracellular tannase production is higher what may constitute a major disadvantage for this fermentation process as, usually, complicated recovery techniques are needed. Nevertheless, we must take in account that extracellular tannase production was also detected.

Conclusions

The best fermentation conditions for tannase production using submerged fermentation were: initial pH 5.0, temperature 35°C, initial substrate concentration 25g/L and agitation 200 rpm. In these conditions, most of the produce tannase was intracellular.

In order to correctly evaluate the evolution of the fermentation experiments, improvements in the used analytical techniques were developed and implemented.

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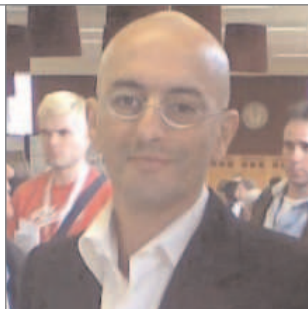
Microalgae Photobioreactor Development

ABSTRACT

This study evaluated the efficiency of a photobioreactor that has been developed, using commercial saline solution sterile plastic bags. *Nannochloropsis oculata* (Eustigmatophyceae) growth performance on batch cultivation was similar between treatments with a specific growth rate (μ_{max}) of 0.12 h^{-1} and 0,13 h^{-1} for MCT (marine water) and SST (saline solution + commercial marine salt) respectively. The cell production achieved for MCT was 5.47×10^7 mL^{-1} cells after 84h and for SST was 3.61×10^7 mL^{-1} . The production of microalgal pigments under conditions of culture medium starvation assay showed a decline in the level of Astaxanthin, zeaxanthin and Lycopene content. These results encourages future research effort to optimize the mass transfer and design of the photobioreactor proposed.

TESTIMONIAL

"Was an opportunity to up grade all aspects of my life-career, cultural, friendliness."
"I quote Fernando Pessoa ...'Sinto-me nascido a cada momento para a eterna novidade do mundo'... "

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Microalgae Photobioreactor Development

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Abstract

This study evaluated the efficiency of a photobioreactor that has been developed, using commercial saline solution sterile plastic bags. *Nannochloropsis oculata* (Eustigmatophyceae) growth performance on batch cultivation was similar between treatments with a specific growth rate (μ_{max}) of 0.12 h^{-1} and 0,13 h^{-1} for MCT (marine water) and SST (saline solution + commercial marine salt) respectively. The cell production achieved for MCT was 5.47×10^7 mL^{-1} cells after 84h and for SST was 3.61×10^7 mL^{-1} . The production of microalgal pigments under conditions of culture medium starvation assay showed a decline in the level of Astaxanthin, zeaxanthin and Lycopene content. These results encourages future research effort to optimize the mass transfer and design of the photobioreactor proposed.

Resumo

O presente estudo avaliou a eficiência do fotobioreator que vem sendo desenvolvido utilizando soro fisiológico comercial. Em sistema de batelada a microalga *Nannochloropsis oculata* (Eustigmatophyceae) apresentou performance de crescimento similar entre tratamentos com uma taxa de crescimento específico (μ_{max}) de 0.12 h^{-1} e 0,13 h^{-1} para MCT (água marinha) e SST (soro fisiológico + sal marinho comercial) respectivamente. A produção cellular alcançou 5.47×10^7 mL^{-1} para MCT após 84h enquanto para SST foi 3.61×10^7 mL^{-1} . Testando a produção de pigmentos em condiçãoes de restrição de nutrientes no meio de cultura observou-se o declínio nos níveis de Astaxantina, Zeaxantina e Licopeno. Estes resultados encorajam futuras pesquisas no sentido de otimizar a transferência de massa e o design do fotobioreator proposto.

Introduction

Microalgae are recognized for their potential in many industrial applications such as: production of vitamins, pharmaceuticals, natural dyes, as a source of fatty acids, proteins and other biochemicals in health and food products. Microalgae are effective in aquaculture feed, and as bio-fertilizers, for removal nitrogen, phosphorus and toxic substances in sewage treatment. Microalgae are phototrophic with high growth rate and yield when compared to crop plants, which has led to the promotion of microalgae as an alternative for both biofuel production and CO_2 sequestration (1,2,3,4). Research efforts are underway to overcome constraining biotechnological, technological and economic factors to develop more cost-effective large scale microalgal production systems (4,5,6). A variety of apparatus and methods have been employed on compositions and systems relating to open and closed bioreactors, photobioreactor designs, harvesting

and scale-up process of production to achieve viable commercial-scale microalgal production systems(1,3). This preliminary study aimed to evaluate the efficiency of a cheap and practical photobioreactor that has been developed, for batch cultivations of *Nannochloropsis oculata* (Eustigmatophyceae), using commercial plastics bags and marine water replacement. As a validation step for the culture system, we investigated the production of microalgal pigments under culture medium starvation.

Materials and Methods

The microalgae *Nannochloropsis oculata* strain CCAP 849/1 used in this study was obtained from the Culture Collection of Algae and Protozoa - CCAP/UK. Cells were maintained and cultured in F/2 medium. The experiments were carried out in the Genetics & Biotechnology Lab, UCC/Ireland. A Microclima 1000-E plant culture chamber (Snijeders scientific) was adapted with an aeration system composed of 4L compressor, carbon activated and glass fibre, biologic and particulate air filters (Whatman) respectively and a flowmeter (KI 10LPM). The plastic bag photobioreactor assays were performed under controlled environmental conditions: 26º C , 4 vvm air flow, pH 8.5 ± 0.2, with 4.5 Klux of light intensity for an illumination scheme of 16-14 h light/dark photoperiod. The inoculum of 8 X 10^6 cells was obtained from 36 h pre-culture in 1 litre Erlenmeyer flasks grown under the same parameters described above. A batch culture using 400-mL volume (WV) was performed in triplicate. Both F/2 medium and commercial marine salt (Instant Ocean) was added to sterile 9% NaCl saline solution plastic bags (SST), to achieve the salinity of the control. Plastics bags containing marine water whose salinity was 35 ‰ (measured by refractometer ATC-S/MILL-E) were used as control (MCT). Samples were collected at 12 h intervals over 84 h growth experiments. Cells were counted with a Neubauer ultraplane hemacytometer. The assay of metabolic behaviour of *N. oculata* under medium starvation was carried out in cell culture flask (50 mL WV) with F/2 medium at the same temperature, pH, light intensity and illumination scheme. As inoculum, 10% v/v of pre-culture in log phase was added to fresh medium. The strain was maintained for 30 days and the control was fed every 5 days. Carotenoid content was determined by the methodology applied in Lyons *et al*., 2002 (7).

Results and discussion

The *N. oculata* strain CCAP 849/1 growth performance was similar between treatments (fig.1), without a significant lag phase. The apex of the log phase was reached around the first 15 hours of culture, achieving a μ_{max} (specific growth rate) of 0.12 h^{-1} and 0,13 h^{-1} for MCT and SST respectively. After 36 hours of culture the death phase to SST treatment began, while the MCT maintained its growth on the stationary phase. The maximum cell production obtained on MCT was 5.47×10^7 mL$^{?1}$ cells after 84h and SST achieved 3.61×10^7 mL^{-1} after 48 h of culture. Durmaz, 2007 (8) demonstrated vitamin E (α-tocopherol) production by *N. oculata* under nitrogen limitation obtaining 5.2±0.3×10^7 cells ml^{-1}, verifying that decreasing N concentrations led to an increase in α-tocopherol accumulation, but reduced the growth rate. *N. oculata* under feed starvation showed a change of colour from dark green to light brown, probably due to the carotenoid metabolism change and the loss of chlorophyll pigments. Flynn *et al*., 1993 (2) grew *Nannochloropsis sp*. for two generations after exhaustion of the N-source, and verified that the increase in the cell C/N mass ratio lead to the storage of C excess as fatty acids, in addition to a significant decline of intracellular concentrations of carotenoids and *Chl a*. The carotenoid extraction method applied for this work was crude and not specific for microalgae samples, and future quantification will be more accurate. However, as shown in fig. 2, the decline in the level of astaxanthin, zeaxanthin and lycopene content was relevant under nutrient starvation. Our results agree with Zhekisheva *et al*., 2005 (9) where the high irradiance or nitrogen starvation severely inhibited the astaxanthin accumulation, however, the fatty acid synthesis was not

proportionally decreased in *Haematococcus pluvialis* (*Chlorophyceae*). Changes in NaCl concentration increased the lipid but decreased the cell concentration of *Dunaliella*, as reported by Takagi *et al,* 2006 (10).

Fig. 1 – *N. oculata* growth in plastic bags, F/2 medium,400 mL, 4 vvm, pH 8.5±0.2, 26° C, 4.5 klux, 16-14 h l/d. ◆ SST - physiologic solution added salt marine; ▲ MCT - marine water.
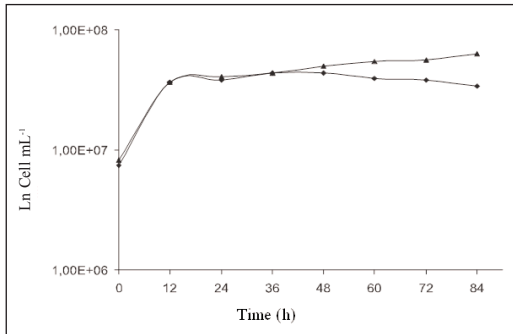
Fig. 2 – *N. oculata* carotenoid. Starvation assay, pH 8.5±0.2, 26°C, 4.5klux, 16-14h l/d. 30d., 50mL, F/2 ctrl fed every 5d. Saponified(S), unsap.(U): ▨ Astaxanthin; ▥ Lutein; ▮ Zeaxanthin; ▩ Lycopene; ⊟ β-carotene.
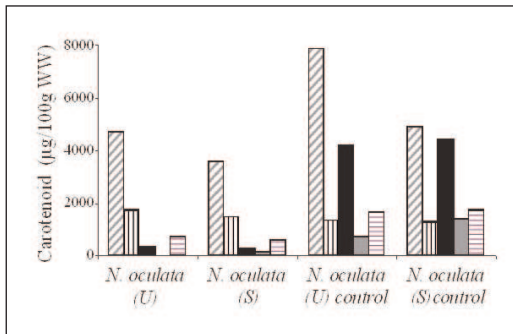
Conclusion

The cell growth obtained (MCT 5.47×10^7 mL^{-1} and SST 3.61×10^7 mL^{-1}) encourages future research effort to optimize the mass transfer and design of the photobioreactor proposed. The metabolic behaviour *N. oculata* under culture medium starvation suggests a change of carotenoid metabolism and fatty acid accumulation, and provides a basis for further more in-depth investigations.

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JUAN PABLO FUCIÑOS GONZÁLEZ

Conformational flexibility of a *Candida rugosa* lipase estudied by electronic spectroscopies techniques

ABSTRACT

We have used second-order orthogonal designs to obtain empirical models that describe the influence of pH and temperature on the lipase from C*andida rugosa* secondary structure. Important variations in the the secondary structure was found with the different assayed media. Contrary, minor changes were observed with the incubation time. This behaviour suggest that the modifications in the secondary structure of the protein are consequence of conformational changes induced by the media and not the result of a progressive denaturation. In addition, the thermal unfolding of lipase at different pH values was followed by measuring the circular dichroims signal as function of temperature over a temperature range of 20–80°C. The results have showed a melting temperature at pH 5.5 ($T_m = 62.1°C$) significantly higher than the measured at pH 7.0 ($T_m = 55.0°C$), in which the lipase maintain the maximum residual activity.

TESTIMONIAL

"I feel that the time went by very fast." "Their friendly people, stunning landscapes and incredible cuisine made my experience abroad very pleasant." "It has been an unforgettable experience marked mainly by the people I met there."

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Conformational flexibility of a *Candida rugosa* lipase estudied by electronic spectroscopies techniques

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Abstract

We have used second-order orthogonal designs to obtain empirical models that describe the influence of pH and temperature on the lipase from C*andida rugosa* secondary structure. Important variations in the the secondary structure was found with the different assayed media. Contrary, minor changes were observed with the incubation time. This behaviour suggest that the modifications in the secondary structure of the protein are consequence of conformational changes induced by the media and not the result of a progressive denaturation. In addition, the thermal unfolding of lipase at different pH values was followed by measuring the circular dichroims signal as function of temperature over a temperature range of 20–80°C. The results have showed a melting temperature at pH 5.5 ($T_m = 62.1°C$) significantly higher than the measured at pH 7.0 ($T_m = 55.0°C$), in which the lipase maintain the maximum residual activity.

Resumen

Se ha empleado un diseño de segundo orden para obtener modelos que describan la influencia del pH, temperatura sobre la estructura secundaria de la lipasa Lip1 producida por *Candida rugosa*. Importantes variaciones en la estructura secundaria fueron encontradas con el tiempo de incubación. Este comportamiento sugiere que la modificaciones en la estructura secundaria es consecuencia de cambios conformacionales inducidos por el medio y no por progresiva desnaturalización. La desnaturalización termina de la lipasa fue seguida por m medidas de dicroismo circular y emisión de la fluorescencia nativa de la enzima. Se encontró un valor de T_m de 62,1 °C para la enzima a pH 5.4, mientras que a pH 7,0 se observo una disminución significativa del T_m a 55,0°C.

1. Introduction

Lipases are enzymes that catalyze the hydrolysis of carboxyl esters and even synthesis of these ones in environments with low water content. This feature has increased the biotechnological interest on these enzymes for a number of industrially significant biotransformations. Potential applications include modification of sugars, synthesis of flavour esters for the food industry, the resolution of racemic mixtures and the bioful obtention. Their activity drastically increased upon binding to the interface, a phenomenon known as interfacial activation [1]. This process has been associated with a conformational change in which a flap consisting of at least one alpha helice opens by rotating around its hinge regions. Various fungal species are efficient producers of lipases. From an industrial standpoint, fungi are more interesting sources of these enzymes than animals or plants. Fungal lipases are multiform enzymes, and the mechanisms by which they are produced vary widely. Fungal lipases may be closely related or may be charac-

terized by quite different isoforms. In the case of the yeast *Candida rugosa*, a family of functional genes codes for several isoenzymes that have closely related sequences [2]

In this work, we have used the Response-Surface-Methodology joined to circular dichroism, fluorescence and quenching of the native fluorescence techniques to monitoring conformational changes induced in the lipase Lip1 from *Candida rugosa* by the combined effects of temperature and pH during a 60 minutes incubations.

2. Materials and Methods

2.1. Chemical: Candida rugosa lipase Type VII and pnitrophenyl butyrate (PNPB) were obtained from Sigma (St. Louis, MO).

2.2. Purification of Candida rugosa lipase: was carried out according to a method previously reported [3].

2.3. FCP effect on the emission of the native protein fluorescence: It was analysed obtaining the fluorescence emission spectrum of the protein in a buffer phosphate 50mM medium, pH 7.0 at different temperatures. The scanning rate was 1 nm/min and the data acquisition was each 0.2 nm with a slit of 0.2 nm. The fluorescence spectra were obtained in a Amico Browman spectrofluoromether Serie 2000 using a thermostatized cuvette of 1cm path length and were corrected using a software given by the instrument manufacturer.

2.4. FCP effect on the native protein fluorescence quenched by acrylamide: the quenching of the protein tryptophan residues (TRP) fluorescence was carried out by titration with acrylamide in the presence and absence of FCP. The data were analyzed using the Stern Volmer equation [4]:

$$\frac{F_0}{F_i} = 1 + K_Q\,[Q]\;(1)$$

where Fo and Fi are the protein fluorescence excited at 280 nm in the absence and presence of a quencher, respectively, K_Q being the Stern Volmer constant related to the lifetime of the fluorophore and the bimolecular quenching constant, [Q] the quencher concentration.

2.5. Temperature effect on the protein secondary structure: Circular dichroism (CD) scan of Lip1 was carried out using a Jasco spectropolarimether, model J-815. The ellipticity values [θ] were obtained in milidegrees (mdeg) directly from the instrument. A 0.1cm cell pathlength was used for the spectral range 200–250nm. In all cases, five scans were made and the non-protein spectrum was subtracted. The faction of α-Helix, β-sheet, random and turn content in Lip1 were calculated using the program CONTINLL .

Thermally induced unfolding was monitored by measure the CD signal at 222 nm, as it was previously reported [5, 6]. The analysis of the data was made assuming an approximation of a two-state model of denaturation where only the native and unfolded states were significantly populated. We have used non-linear least squares to fit the absorbance versus temperature (T) data, the temperature at the mid-point of unfolding (Tm) was determined, the unfolded protein fraction (α) was calculated from:

$$\alpha = \frac{\theta_i - \theta_N}{\theta_D - \theta_N}$$

(2) where θ_N, and θ_D are the molar ellipticity of the native and the unfolded states respetively at some reference temperature (0ºC) and and θ_i the ellipticity at a given temperature. From Eq. (2) the equilibrium constant for the unfolded process temperature dependence can be calculated:

$$K = \frac{\alpha}{1 - \alpha}$$ (3) the free energy (ΔG°) was calculated from Eq. (4) as $\Delta G^{\circ} = -RT \ln K$ (4). From a plot of ΔG° versus T, the entropic unfolded (ΔS°) was calculated according to Eq. (5):

$$\frac{\partial \Delta G}{\partial T} = -\Delta S^{\circ}$$

The enthalpy change (ΔH°) was calculated from the equation:

$$\frac{\Delta T - \Delta H}{T} = -\Delta S^{\circ}$$ (6)

3. Results

3.1 Effect of pH and temperature on the fluorescence native emission of Lip1: Fig 1-A shows the native fluorescence emission spectra of Lip1 (while exciting at 280 nm) at increasing concentration of iodide. A pH 7.0 and 30 °C, a decreased in the emission band whiout modification on its position occur, which remain around 320 nm. This finding suggests that under this experimental condition, none modification of the tryptophan environment is induced by the iodide attack. It has been informated that the temperature affect the conformational flexibility of Lip1, however at 30 °C Lip1 remain its close structure evading in this way the solvent access to the tryptophan enzyme. Then, the quenching by iodide was assayed at pH 8.6 and 47°C, a next shift of the emission band from 320 nm for Lip1 alone to 336 nm at higher Lip1/ iodide ratio can be observed as shows Fig. 1-B, which is consistent with a lost of the hydrophobicity tryptophan residues environment. The fluorescent emission at 320 nm in the absence and presence of increasing iodide concentration was measured and expressed as a Stern Volmer plot, as shown Fig. 2. A non lineal plots were obtained in agree with two o more tryptophans populations with different accessibility to the quencher.

At pH 8.6 and 48°C, a poor quenching effect is produced by iodide, while at pH 7.0 and 30 °C an important quenching effect was observed, suggesting under this last condition a significant conformational change of Lip1 is produced which expounds the tryptophan residues of Lip1 to the solvent attack.
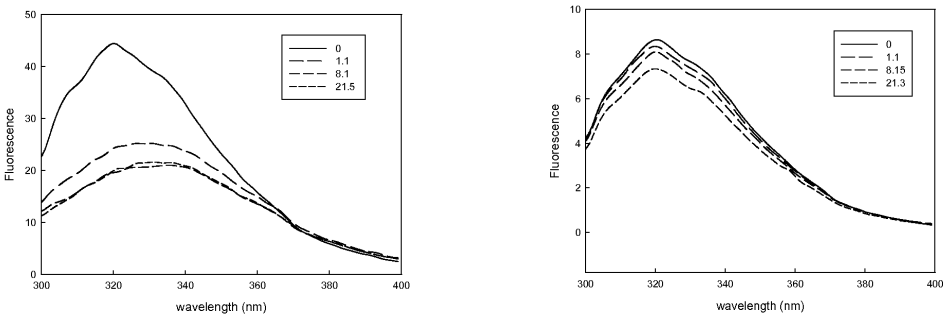
Figure 1 Fluorescence emission spectrum of Lip1 at pH 7,0 (left) and 30°C and pH 8.6 (right) and 48 °C at increasing NaI concentration (indicate in the right corner) in μM. Lip1 concentration 0.18 10 μM. Medium sodium phosphate buffer 50 mM.
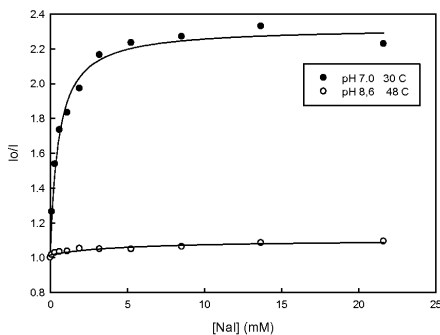
Figure 2 Stern Volmer plots for fluorescence quenching emission of PL1 by iodure. Medium sodium phosphate buffer 50 mM, Lip1 concentration 0.18 μM

3.2 CD spectrum of Lip1: Fig 3 shown the DC spectrum of Lip1 in the absence as presence of the quencher iodure at pH 7.0 and 37°C, a lost of the secundary structure is inducing in agree with the quenching effect observed in Fig. 2.
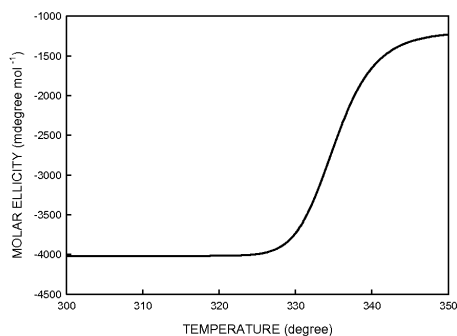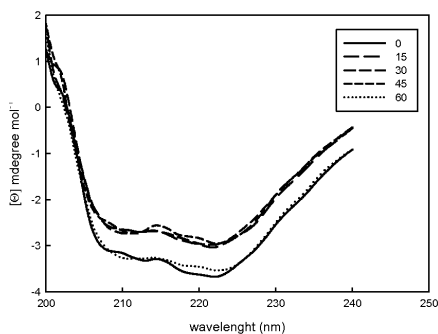
Figure 2 (left) Circular dichroims spectrum of Lip1 at increasing concentration of NaI. Medium conditions: buffer sodium phosphate 50 mM, pH 7.0 Temperature 30°C. Lip1 concentration 0.16 μM. In the insert the incubation times
Figure 3 (right): molar ellipticity at 222nm of Lip1 vs. the temperature at pH 5.4 in a medium sodium phosphate 50 mM.

To analyze the thermodynamical stability of Lip1 a thermal patter of the enzyme was obtained by a heating of it, calculating its unfolding fraction vs temperature, them by non linear fitting of the data the middle point temperature of the transition was calculated (as shown Table I) and applying the equations 3, 4 and 5 the ΔH and ΔCp of the unfolding process was determined, as shown Table I.

Table I
Thermodynamic function values for the Lip1 thermal unfolding

Condition	Tm (°C)	ΔH (kcal /mol)	ΔCp
pH 5.5	62.1 ± 0.2	81.5 ± 0.1	4.08 ± 0.09
pH 7.0	54.9 ± 0.1	49.7 ± 0.2	3.3± 0.1

A significant decrease in the Tm value until 54.9 °C is observed for the Lip1 at pH 7.0 in agree with a lost of its thermodynamically stability under this condition. The low ΔH and ΔCp observed at pH 7.0 is suggesting a previous break of bounds in the Lip1 with results in a minor secondary structure [7].

To obtain a major information of the about the Lip1 stability, the α-helix, β-sheet, random and turn fractions were calculated at pH 5.4, 7.0 and 8.6 (at 48 °C) in function of the time, such as shown Fig 4. At pH 5.4 the Lip1 showed to be stable because the alpha an beta helix fraction did not vary during one hour.

A pH 7.0 a significant lost in the alpha helice content of about 50% of the Lip1 is observed and in parallel an increase in the beta helix is observed, this finding is in agree with the thermodynamical parameters found for the enzyme (see table I). A pH 8.6 an increasing in the alpha helix fraction is observed with the time, while the turn fraction decreased in agree with a stabilization of the protein structure.
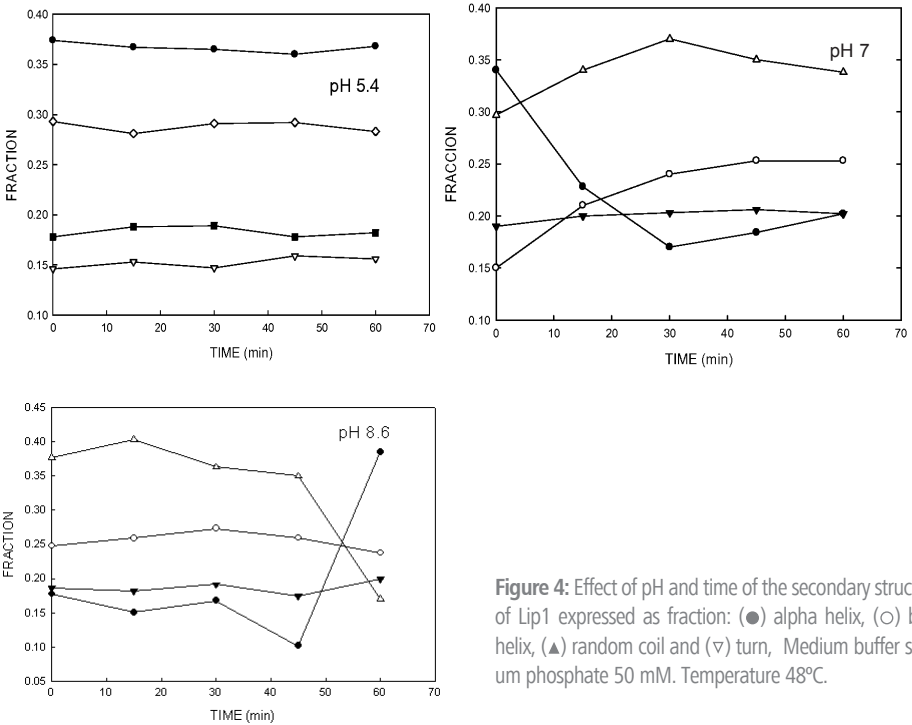
Figure 4: Effect of pH and time of the secondary structure of Lip1 expressed as fraction: (●) alpha helix, (○) beta helix, (▲) random coil and (▽) turn, Medium buffer sodium phosphate 50 mM. Temperature 48ºC.

Conclusions:

In this work, we have studied the thermodynamical stability of Lip1 under different condition of pH and the effect of the temperature influence on the conformational structure of this protein. It was found that the medium condition has important effect on the Lip1 structure, thus at pH 7.0 the enzyme showed to lost a very important fraction of its alpha helice content while at pH 8.6 part of this structure y recovery, while at pH 5.4 showed that the medium and a temperature of 48 °C, did not influent on the protein structure. Also the time was an important factor to determine the conformational structure of Lip1, thus at 48 °C during one hour the enzyme remained its secondary structure without any significant variation in it, while at pH 7.0 a significant lost of the conformational structure was found. This behaviour suggest that the mod-

ifications in the secondary structure of the protein are consequence of conformational changes induced by the media and not the result of a progressive denaturation. In addition, the thermal unfolding of Lip1 at different pH values was followed by measuring the CD signal as function of temperature over a temperature range of 20–80°C.

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LUIS S. JOVA

Design and Evaluation of Fermented Non-Alcoholic Functional Cereal-Base Beverage

ABSTRACT

Research on fermented non alcoholic functional cereal beverages is a promising way to offer production diversification to industrial sectors based on fermented alcoholic cereal beverages aiming to extend their market. Three fermented non-alcoholic functional cereal beverage prototypes, two from traditional recipes, were developed as part of this study. Fermentation was carried out at ~42 °C for up to 5h and those prototypes showing superior behaviour progressed to sensory analysis. Results showed that samples prepared with brown rice, 5% Lala yoghurt as inoculum and 1% of Megamix 40® had a better performance regarding lactic acid production. Milk as ingredient boosted fermentation. However, Megamix 40® showed a better result in this aspect. Total sugars content increased during the first 3 hours of fermentation. Microbial growth kinetic curves were well fitted by a Weibull model. Flavour (sour and sweet), colour and viscosity were the factors with greater influence on the general sensory acceptance of the samples. The Kansei engineering questionnaire was an effective tool for determining panelists' emotions linking general acceptance with sensations of taste and health perception, physical aspect and consistency and sense of novelty and innovation.

TESTIMONIAL

"Studying or working abroad is always exciting."
"There was also time for making friends and visiting some interesting places."

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Design and Evaluation of Fermented Non-Alcoholic Functional Cereal-Base Beverage

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Abstract

Research on fermented non alcoholic functional cereal beverages is a promising way to offer production diversification to industrial sectors based on fermented alcoholic cereal beverages aiming to extend their market. Three fermented non-alcoholic functional cereal beverage prototypes, two from traditional recipes, were developed as part of this study. Fermentation was carried out at ~42 °C for up to 5h and those prototypes showing superior behaviour progressed to sensory analysis. Results showed that samples prepared with brown rice, 5% Lala yoghurt as inoculum and 1% of Megamix 40® had a better performance regarding lactic acid production. Milk as ingredient boosted fermentation. However, Megamix 40® showed a better result in this aspect. Total sugars content increased during the first 3 hours of fermentation. Microbial growth kinetic curves were well fitted by a Weibull model. Flavour (sour and sweet), colour and viscosity were the factors with greater influence on the general sensory acceptance of the samples. The Kansei engineering questionnaire was an effective tool for determining panelists' emotions linking general acceptance with sensations of taste and health perception, physical aspect and consistency and sense of novelty and innovation.

Keywords: functional drink, fermented cereal beverage, sensory analysis and Kansei Engineering

Resumen

El desarrollo de bebidas funcionales no-alcohólicas fermentadas basadas en cereales podría ser una interesante manera de diversificación de la producción para el sector industrial basado en bebidas alcohólicas a partir de cereales con vista a una extensión de su mercado. Tres prototipos fueron desarrollados, dos a partir de recetas tradicionales, como parte de este estudio. La fermentación se llevo a cabo a ~42 °C durante unas 5h y los prototipos con mejor comportamiento se utilizaron para un análisis sensorial. Las muestras preparadas con arroz integral, 5% yogurt Lala (inoculum) y 1% de Megamix 40® tuvieron mejor desempeño en cuanto a producción de acido láctico. Utilizar leche potencia la fermentación. Sin embargo, Megamix 40® mostró un mejor desempeño en este aspecto. Los azúcares totales aumentan durante las primeras 3 horas de fermentación. La cinética de crecimiento microbiano ajusta bien a un modelo tipo Weibull. La aceptación sensorial de las muestras estuvo influenciada mayormente por factores como los aromas acido y dulce, el color y la viscosidad. El cuestionario El cuestionario Kansei fue efectivo para la determinación de las emociones en los panelistas que vinculan la aceptación general con las sensaciones de sabor y percepción de producto saludable, el aspecto físico y la consistencia, y el sentido de novedad e innovación.

Palabras claves: bebidas funcionales, bebida de cereal fermentada, análisis sensorial e Ingeniería Kansei

Introduction

One of the most important industrial sectors of the Irish agro-food industry is based on fermented alcoholic cereal beverages: for i.e. beer (including the well known Irish stouts) and whiskey. However, the potential alcoholic beverage market is limited by health and social connotations and therefore research on non-alcoholic fermented cereals functional beverages would be an interesting way to offer to this industrial sector a new market opportunity.

Consumers are increasingly concern with what they eat and drink being natural and healthy according to their perception and system of values and beliefs. Therefore, researchers have increased their search for data providing evidence of health benefits from "functional" foods. Cereal grains constitute a major source of dietary nutrients all over the world. Although, cereals crops are deficient in some basic nutritional components (e.g. essential amino acids), poor protein quality is often the main concern in cereal products. Several methods have been developed to improve their nutritional quality; however, fermentation [1] seems to be the most simple and economical way. Several conventional foods and beverages are largely produced from cereals in the Western world; while currently there is a trend for developing new foods/beverages with enhanced health properties [2]. Thus, nutritional and health research has led to an increased interest in specific fermented cereal beverages, such as those based on soy, buckwheat, barley and oats. Researchers consider that besides the nutritional contribution, high fibre content (often related to a development of a healthy gut flora) and cholesterol-lowering properties, health benefits of these products can be enhanced by using probiotic strains which also have a strong association with gut health. Sensory impact is however an issue, and therefore the development of a complex recipe can help to develop products of high sensory quality by masking or compensating synergistically the off-flavours released by the probiotic. There are many indigenous fermented cereal products, some of which are taken as beverages, and some are not alcoholic (this depends to a large extent on the microbial strain(s) that dominate the fermentation process). The sensory profiles generated are a function of the microbial strains involved in the fermentation as well as the mix of ingredients and process techniques. However, most of these traditional recipes remain at household level and only some of them have been converted to an industrial scale. Since fermentation at household level is time consuming and not always safe, these products provide a fertile ground for improvement.

Traditional sensory analysis which aims to be an analytical evaluation of food sensory quality by means of the human senses, has been considered the main tool for the consumers characterization of food products since it appeared during the Second World War as a way to bring adequate sensory quality to soldier's food rations [3]. However, circumstances and consumers have evolved and the development of new food products is, nowadays, basically looking for products with added value to be offered in a strong competitive environment where companies' value margins are reduced because of similarity between products and high product development cost. Therefore, it is evident that continuing with a strong product development activity will require minimizing development costs and maximizing the product features which sustain its higher added value and will underpin product's success. A consumer-oriented product design engineering system [4] will be the ideal approach to achieve these goals. Kansei [5] engineering is a promising design engineering system, which may be used for the development of new food products, based on the development of functional models that relate product design factors and options with consumer perception. The most basic *kansei* engineering methodology would be comparable to conjoint analysis (used in marketing studies), with the difference that instead of asking consumers rational answers to rational questions, the method will attempt to extract product descriptors from stated feelings and emotions (*kansei*, pronounced as "cansay", is a Japanese word meaning ill-defined feelings or emotions) [6]. However, Kansei has been rarely used for designing new food products so there are some aspects on Kansei application which still need to be addressed.

The objective of this work was to design a fermented non-alcoholic functional cereal beverage by using a multiple approach including fermentation studies and a sensory evaluation where a kansei engineering approach would also be used besides a traditional one sensory analysis.

Materials and Methods

2.1. Fermentation Screening

Three fermented non alcoholic cereal beverages were produced according to recipes obtained in a previous work: Boza, Pozol (names taken from traditional Turkish and Mexican beverages respectively) and an Oat-rice beverage. The main difference amongst the recipes in that in Boza cooked rice is the base for the recipe while a mix of cooked rice with nixtamalized maize flour or with oats is the base for Pozol and Oat-rice beverage; respectively. An experiment was designed to study the fermentation stage for these three recipes. Samples, in duplicate, were prepared with white rice (WR) or brown rice (BR), as stated in the recipe; placed in closed bottles and three different lactic acid bacterial strains from commercial registered yoghurts (Soful, Lala and Yoplait) were added as fermentation inoculum (3% w/w) at a first stage. A second study was performed to further improve the fermentation on the recipe that showed the best result (Boza) however, Rice Flour (RF) was used instead of rice, Lala as inoculum (3 and/or 5% w/w), evaporated milk (1% w/w inoculum) and/or a flour improving ingredient called Megamix 40® (1% w/w flours) were also added according to the experimental design. Fermentation was always carried out by storing the samples at ~42°C for up to 5 hours (Felisa Oven). Sampling was made every 45 min and opened bottles were eliminated from the study. Lactic acid production by titrable acidity [7], changes in pH (pH meter-Thermo Orion Model 420) using pH 4 and 7 buffer solutions at 25 °C [8] and total sugars [9] were followed.

Additional experiments were performed in order to study the total sugar content and fermentation kinetics in some Boza samples prepared with WR or BR, Lala (5%) as inoculum and Megamix 40® (1% w/w flours) (recipe that showed the best fermentation performance in the RF study) . Fermentation and sampling were carried out as described above. Sampling collection was made in 1 ml eppendorf tubes which were kept frozen till cell counts were carried out at the end of the experiment. The cell yield was determined by using the Neubauer camera [10].

2.2. Sensory analysis and Kansei Engineering Test

Thirty-seven participants (about 2/3 females, aged 18-27, untrained) received a set of six different samples and were asked to evaluate them in a seven-point scale for a sensory analysis and a kansei test. A typical kansei questionnaire consists of 40 to 90 adjectives that describe the product. Each word is placed against its antonym in a five or seven point semantic differential scale. In this study, 34 pairs of adjectives were used in a seven point scale.

Samples were made according to three recipes previously developed: Boza, Pozol and Oats-rice beverage (using WR or BR as part of the recipe was the main difference between them). Fermented fresh samples (unpasteurized) were randomly coded and given for testing in clear 35 ml plastic cups. Assessors were told about the main objective of the test and also a brief explanation of how to answer the Sensory and Kansei Questionnaires.

All participants evaluated all drinks. The number of was set to less than 7 in order to minimize potential inconsistencies with continuous testing of too many samples. Assessors were asked to evaluate samples in the same order given to them and answer both questionnaires when evaluating each sample.

Statistical analysis with ANOVA, Factor Analysis and mathematical model building were performed with the Statistica Software (release 7, 2004, Stat soft, Tulsa, Oklahoma, USA).

Results and Discussion

3.1. Fermentation study

It can be seen that Lala and BR led to bigger pH drop and this combination also showed larger lactic acid production as can be observed in figures 1 and 2. Brown rice gave better result on pH decrease and lactic acid production when Lala and Yoplait was used as inoculum compared to white rice (significant difference in a Tukey test), however, it did not help when Soful was used. Furthermore, Soful gave the worst result for these two aspects in all three recipes developed (Figure 1 and 2). Figure 3 shows larger lactic acid production for samples based on RF fermenting with 5% inoculum rather than 3% w/w. It also can be seen that adding milk and/or Megamix 40® may improve lactic acid production. Megamix 40® gave a better result that only milk within samples with 5% inoculum and also compared to samples with 3 % inoculum-Milk-Megamix 40® which may suggest that the combination (5% inoculum-Megamix 40®) may be used when milk is not expected to be included in the recipe. Then, this combination (5% inoculum-Megamix 40®) was used for fermenting samples of Boza prepared with WR or BR and total sugar and microbial growth kinetics during the fermentation stage were followed. It was found that total sugars increases its quantity during the first 3 hours of fermentation (Figure 4) while microbial growth kinetic curves were well fitted by a Weibull model with a shape parameter **B** of 1.426 (WR) and 1.699 (BR)(Figure 5).
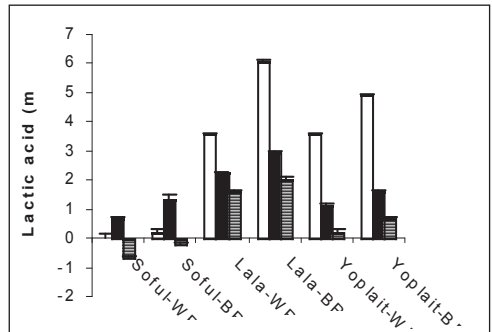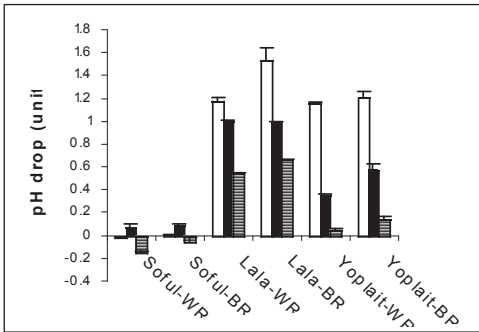
Fig. 1: pH drop units during fermentation after 225 min (3% w/w inoculum). WR means white rice; BR represent brown rice; White fills means Boza; Black fills Pozol and Horizontal striped fills Oats-rice beverage.

Fig. 2: Lactic acid production during fermentation after 225 min (3% w/w inoculum). WR means white rice; BR represents brown rice; White fills means Boza; Black fills Pozol and Horizontal striped fills Oats-rice beverage.
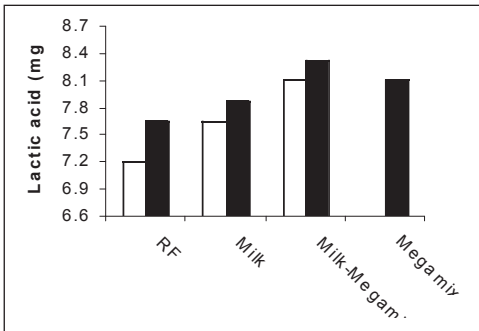
Fig. 3: Lactic acid production during Boza samples fermentation after 270 min. RF means Rice Flour, White fill means 3% and Black fills means 5% Lala inoculum.
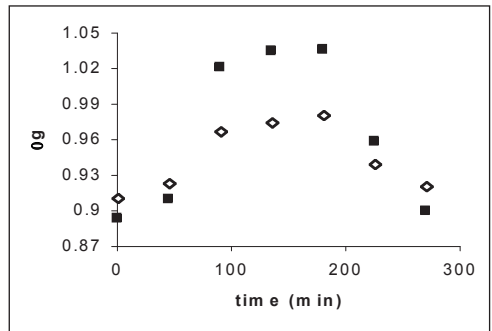
Fig. 4: Total sugar content during fermentation up to 270 min. ■ means Brown rice and ◇ means White rice.
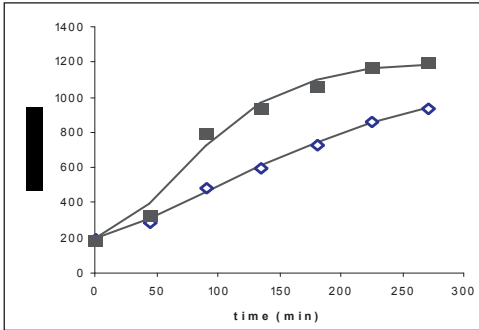
Fig. 5: Kinetics of microbial growth up to 270 min fermentation fitted by a Weibull model. ■Means Brown rice (Cells= 188*e$^{-(t/105.875)1.699}$ and ◇ means White rice (Cells= 200*e$^{-(t/197.636)1.426}$)
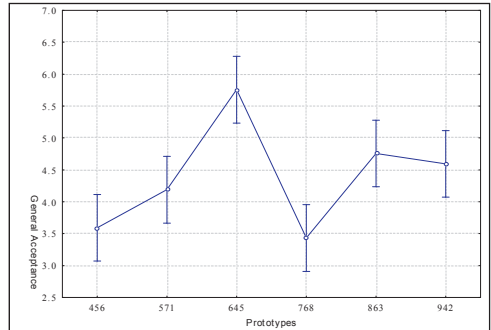
Fig. 6: General acceptance of recipes (Box and Whisker Plot). 456 mean Pozol (WR); 571 Rice Oats Drink (BR); 645 Boza (WR); 768 Pozol (BR); 863 Boza (BR); 942 Rice-Oats Drink (BR); (WR) mean White rice and (BR) Brown rice

3.2. Sensory analysis and Kansei Engineering Test

Figure 6 and table 1 show the general acceptance of the samples. It can be seen that sample coded 645 (Boza prepared with WR) had the higher preference while sample coded 768 (Pozol prepared with BR) had the worst result (significant difference for p<0.05).

Table 1: Summary of the Tukey Test on General Acceptance.
Means with similar letters are not significantly different (p<0.05)

Prototype	General Acceptance Means
768	3.43 a
456	3.59 a,b
571	4.18 a,b,c
942	4.59 b,c
863	4.75 c,d
645	5.75 d

In order to identify which sensory factors might have a greater influence in explaining the general acceptance scores, the sensory questionnaire further requested participants to rate aspects of odour, flavour, aspect and mouth feeling. Taking general acceptance as a response and the sensory descriptors as factors, an ANOVA was applied to the data. The contribution of each factor was determined by removing the effects of all other factors. The results are shown in fig. 7 as a pie chart of the sums of squares explained by each factor. The remaining to the total sum of squares is the contribution of error, which pools pure error (variability between panellists), unaccounted factors and interactions between factors. The sum of squares is a measured of the observed spread of data, with the variance being equal to the sum of squares divided by the number of degrees of freedom (6 for each factor and 167 for the error). The coefficient of determination was low (0.419), which is usual in sensory analysis due to the variability between different individuals. Notwithstanding, the results showed that sour flavour, sweet flavour, colour and viscosity affected the general acceptance score with statistical significance (at 95% confidence level). To represent the influence of these factors in a concise manner, a principal component analysis (PCA) with Varimax normalised rota-

tion was applied to all sensory scores. The PCA combined all 9 sensory descriptors with only 2 Principal Components (PC), which combined explained 80.5% of the variance of the data. Sour flavour, sweet flavour and viscosity were the main contributors to PC1, while colour, granular appearance, cereal flavour and sweet odour had the statistically significant loading factors in PC2, the first one being the only factor that is known to have statistical significance on the overall results. Fig. 8 a shows the correlation plot of the two PCs together with the values of general acceptance. It is evident that the lower the value of PC1 the better, and that generally the lower the value of PC2 the better also. Fig. 8b shows that lowering the value of PC1 is achieved primarily by increasing sweet flavour and viscosity and decreasing sour flavour, while and lowering the value of PC2 mostly by increasing colour.
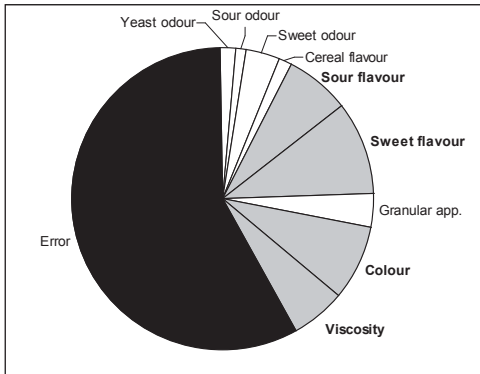
Fig. 7: Percentage of the sum of squares of the data explained by each factor. Those with statistical significance are shown with grey background. Error refers to the sum of squares unaccounted for, due to possible interactions between factors, unaccounted factors and pure error (variability between panelists)
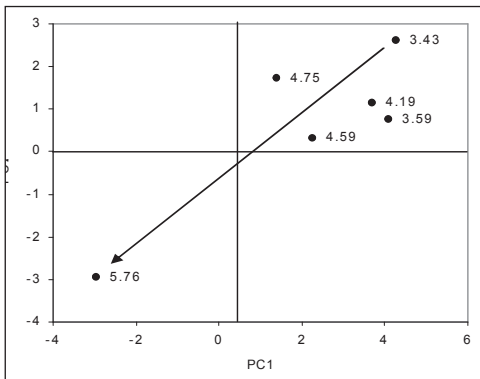
a)
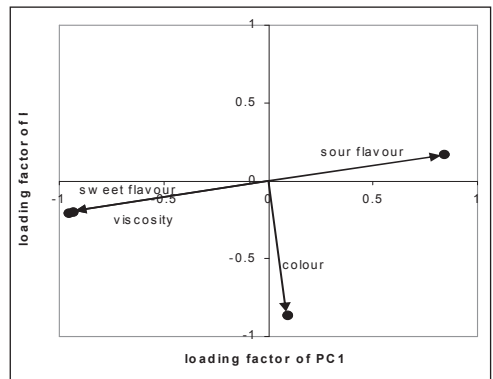
b)

Fig. 8: Graphical results of the PCA of the sensory descriptors. a) Correlation plot between the first two principal components. The labels indicate the average general acceptance score of each sample and the arrow the general direction of product improvement. b) Locations of the loading factors of the first 2 PCs only for the statistically significant factors. The arrows show the influence of increasing the sensory descriptor indicated.

The Kansei questionnaire results were analyzed by applying a principal component analysis (PCA) to the scores of the 34 pairs of adjectives. The first 3 principal components were sufficient to explain 89.51% of the variance of the data. By noting which adjective clustered in each PC according to the highest loadings, it was found that PC1 expressed sensations of taste and health perception, PC2 related to a well balance physical aspect and consistency and PC3 quantified a sense of novelty and innovation.

Three emotions were then quantified using only the loading factors in each PC that were statistical significant, that is, clustering the adjectives according to the PC's and using the normalized loading factors as weights to calculate each emotion (E). Figure 9a shows the plot between E1 and E2 where the general acceptance scores are also indicated. It is very clear that the overall sensory score is higher the higher the intensity of both emotions 1 and 2 (taste/health and aspect/consistency). Then emotions already account for more than 80% of the results but a three dimensional space can be generated with E3. Figure 9b shows the sense of novelty helps to improve the score, as it justifies a better score for 863 compared to 942 and 571, in spite of lower PC1 and PC2, as increasing the intensity of E3 also greatly increases the score. Moreover, the sample of lowest score also had a high E3 intensity.
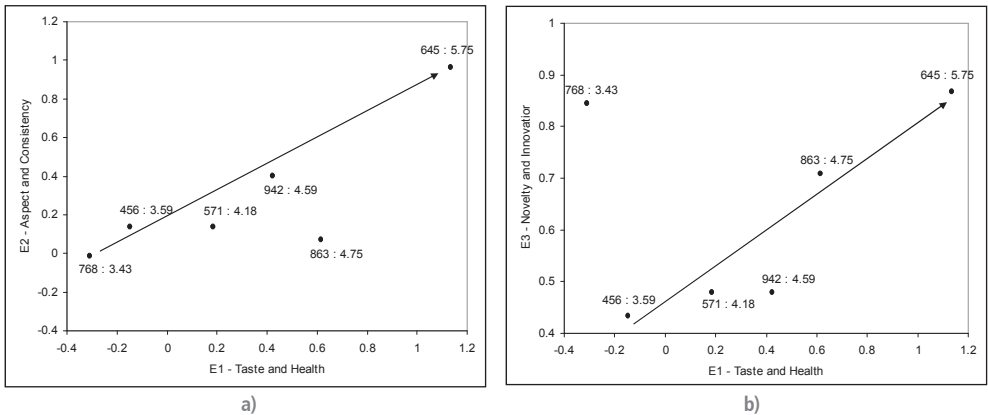
Fig. 9: Correlation plots between the Kansei quantification of emotions 1 and 2 (a) and 1 and 3 (b). Near each point, the first number indicates the sample reference and the second number is the average general sensory score. The arrow shows the general direction of product improvement for each pair of emotion

4. Coclusions

Brown rice, 5% Lala as inoculum and 1% of Megamix 40® showed a better performance for fermentation regarding lactic acid production. Milk as ingredient also improved the fermentation. However, Megamix 40® showed a better result. Total sugar content increased during the first 3 hours of fermentation.

Microbial growth kinetic curves fits were well fitted by a Weibull model. Samples prepared with brown rice had the wider deviation from exponential first order behaviour and also the higher rate of growth of micro-organisms compared to samples prepared with white rice.

Flavours (sour and sweet), colour and viscosity were the sensory factors with the most significant effect on the general acceptance of the samples. The Kansei engineering questionnaire was an effective tool for determining panelists' emotions linking general acceptance with sensations of taste and health perception (explaining 59.6% of the variance of the data), physical aspect and consistency (23.5%) and sense of novelty and innovation (6.3%).

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Influence of galactomannans/collagen edible coatings in gas transfer rates in fruits

ABSTRACT

One of the most important problems in fruit conservation is their short shelf life. Shelf-life can be extended by reducing respiration rates. This is done by controlling factors such temperature, relative humidity, gas composition (ethylene, O_2 and CO_2) and light. An important strategy to control some of these factors is the use of modified atmospheres, obtained using galactomannan coatings, those show low caloric contents. Collagen coatings have already been used on meats and sausages to reduce gas permeability and/or water vapor permeability (WVP). The objective of this work was to produce new edible coatings, based on the mixture of galactomannan, collagen and glycerol, and study their influence in gas transfer rates in mangoes and apples. The coatings presenting the best values of wettability were tested in relation to their gas permeability properties (CO_2, O_2 and H_2O). Mangoes coated with a solution of *Adenanthera pavonina* galactomannan, collagen and glycerol, and the gas transfer rates compared with mangoes without coating. The gas transfer rate was calculated. A 28% less O_2 consumption and 11% less CO_2 production were observed in coated mangoes when compared with mangoes without coating. The same procedure was done in apples (in this case using *Caesalpinia pulcherrima* galactomannan). The CO_2 production and the O_2 consumption is approximately 50% lower in apples with coating than in apples without coating. Results suggest that the coatings can reduce gas transfer rates in these fruits, and can be important tools to extend the shelf-life of fruits.

TESTIMONIAL

"The the Alpha-Valnatura Project was a fantastic experience, which transformed me in a better scientist, a better person. It was a unique opportunity to live with new cultures, exchange experiences of life, and above all, exchanging knowledge"

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Influence of galactomannans/collagen edible coatings in gas transfer rates in fruits

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Abstract

One of the most important problems in fruit conservation is their short shelf life. Shelf-life can be extended by reducing respiration rates. This is done by controlling factors such temperature, relative humidity, gas composition (ethylene, O_2 and CO_2) and light. An important strategy to control some of these factors is the use of modified atmospheres, obtained using galactomannan coatings, those show low caloric contents. Collagen coatings have already been used on meats and sausages to reduce gas permeability and/or water vapor permeability (WVP). The objective of this work was to produce new edible coatings, based on the mixture of galactomannan, collagen and glycerol, and study their influence in gas transfer rates in mangoes and apples. The coatings presenting the best values of wettability were tested in relation to their gas permeability properties (CO_2, O_2 and H_2O). Mangoes coated with a solution of *Adenanthera pavonina* galactomannan, collagen and glycerol, and the gas transfer rates compared with mangoes without coating. The gas transfer rate was calculated. A 28% less O_2 consumption and 11% less CO_2 production were observed in coated mangoes when compared with mangoes without coating. The same procedure was done in apples (in this case using *Caesalpinia pulcherrima* galactomannan). The CO_2 production and the O_2 consumption is approximately 50% lower in apples with coating than in apples without coating. Results suggest that the coatings can reduce gas transfer rates in these fruits, and can be important tools to extend the shelf-life of fruits.

Resumo

Um grande problema na conservação de frutos é a sua curta vida de prateleira. A vida desses frutos pode ser prorrogada reduzindo as taxas respiratórias. Isso pode ser feito controlando fatores como a temperatura, umidade relativa, composição de gases (etileno, O_2 e CO_2) e luz. Uma estratégia importante para controlar alguns desses fatores é o uso de atmosferas modificadas, obtidas usando revestimentos de galactomanana, que possuem baixo teor calórico. Revestimentos de colágeno têm sido usados em carnes e salsichas para reduzir a permeabilidade a gases e/ou ao vapor de água. O objetivo do trabalho foi produzir filmes comestíveis, baseados na mistura de galactomanana, colágeno e glicerol, e estudar sua influência nas transferências de gases em mangas e maçãs. Os filmes que apresentaram melhores valores de molhabilidade foram testados em relação as suas permeabilidades a gases . Mangas foram revestidas com solução de galactomanana de *Adenanthera pavonina*, colágeno e glicerol, e a taxa de transferência de gases comparada com mangas sem revestimento. A taxa de transferência de gases foi calculada. Um consumo 28% menor de O_2 e produção 11% menor de CO_2 observou-se em mangas revestidas quando comparadas com mangas sem revestimento. O mesmo procedimento foi feito em maçãs (neste caso usando

galactomanana de *Caesalpinia pulcherrima)*. A produção de CO_2 e consumo de O_2 é aproximadamente 50% menor em maçãs revestidas que em maçãs sem revestimento. Os resultados sugerem que estes revestimentos podem reduzir as taxas de transferência de gases,e podem ser ferramentas importantes para prolongar vida de prateleira de frutos.

Introduction

Great losses in quality and quantity of fresh fruits occur between the harvest and the consumption. Estimates show that 25 to 80 % of losses of fresh fruits are due to of their putrefaction. One of the most important problems, in transport, storage and commercialization of fruits and vegetables, is their senescence. When the fruit is harvested, there is an interruption in the gaseous balance, occurring a high influx of oxygen with the proportional loss of carbon dioxide. In this new condition the cells are not renewed and the respiration rate increases, causing a metabolic loss and taking the fruit to a gradual maturation and eventual senescence. The respiration rate depends both of internal and external factors. The internal factors include the species, the cultivar, and the growth state, while the external factors include the atmospheric composition O_2, CO_2 and ethylene rates, the temperature and stresses [1].

The films act as semipermeable barriers which are able to assure the quality of the food. Some of them, besides being biodegradable, offer alternative packaging systems which cause reduced environmental damages. The modified atmosphere created by the cover, generates a physical capture of CO_2 inside the fruit and a partial occupation of the pores, reducing in such a way the gaseous exchange and reducing the respiration rate. If the permeation of oxygen (O_2) to the fruit´s interior is reduced, a prolongation of the maturation time occurs [2]. Film formation and properties of several polysaccharide materials such as starch and starch derivatives, alginates, cellulose derivatives, carrageenan, various plant and microbial gums, chitosan and pectinates have been studied in the last years. In general, due to their hydrophilic nature, polysaccharide films generally exhibit limited water vapor barrier ability. However, certain polysaccharides, applied in the form of high moisture gelatinous coatings, can retard moisture loss from coated foods by functioning as sacrificing agents rather than moisture barriers [3]. In accordance with Banker [4] an inverse relation between the permeability to water vapor and the permeability to oxygen, is sometimes observed. Galactomannans as reserve carbohydrates are found in cell wall storage polysaccharides of various albuminous or endospermic seeds. The physicochemical and conformational properties of the galactomannans are related with the ratio mannose/galactose (M/G) and the distribution of galactose residues throughout the main chain. The polar nature of proteins confers to protein films the property of being excellent barriers to oxygen (apolar), possibly due to their impermeability to apolar substances and the high value of cohesive energy that they contain. Collagen is an abundant protein constituent of connective tissue in vertebrate (about 50 % of total human protein) and invertebrate animals [5].

Blending has acquired importance in improving the performance of the polymeric materials. It has become an economical and versatile way to obtain materials with a wide range of desirable properties [6]. Biodegradable protein and polysaccharide films with satisfactory mechanical properties and good appearance are potential and ecological alternatives for substituting synthetic packaging in pharmaceutical and food applications. The formation of protein–polysaccharide complexes has been related to enhancements of the functionality of proteins adsorbed at the fluid interfaces [7]. Protein–polysaccharide interactions are sensitive to details of protein and polysaccharide structures as well as to pH [7, 8].

Having for base this type of knowledge, a good strategy to be evaluated is the development of films produced from blends of galactomannans and collagen, with the purpose of improving coating properties through the possible synergism between them. The objective of this study was to produce new edible coatings, based in the mixture of novel galactomannans (*Adenanthera pavonina and Caesalpinia pulcherri-*

ma), collagen and glycerol, to characterize the coatings with the best wettability values in terms of their physical-chemical properties and to evaluate the use of this coatings in some fruits (apple and mango).

Materials and Methods

The **seeds** of *A. pavonina* and *C. pulcherima* were collected in Fortaleza, Ceará (Brazil) during June 2006 and kept in a dry place until further use. The soluble anionic **collagen** was prepared by alkaline treatment of bovine intestinal submucosal tissue, at 20 °C for a period of 72 h, followed by homogenization in 0.5 mol L^{-1} acetic acid solution and brought to a final collagen concentration of 10 g L^{-1} [9]. The **galactomannan extraction** was performed with ethanol and distilled water. In this process the seeds are removed from the pods, cleaned and put in a blender. The endosperm is separated from the cotyledon, peeled and suspended in previously warmed ethanol (70 °C) during 15 minutes (to inactivate enzymes that could degrade the polysaccharide). The ethanol is decanted and distilled water is then added in the proportion of 1:100. This mixture is left during approximately 1 hour and then mixed in a blender during 5 min. The purification of the galactomannan is achieved by filtering it through nylon, followed by centrifugation at 3200 g during 30 minutes. The galactomannan is precipitated in ethanol with the proportion 2:1 (ethanol – galactomannan solution). In the end of this process the precipitated galactomannan is lyophilized, and kept in the freezer until further use. The **coating solutions** (blends) were prepared dissolving the lyophilized galactomannans in distilled water followed by the addition of the collagen solution and the plasticizer (glycerol). Each blend was homogenized during 5 minutes at room temperature (21 °C) and left to stabilize during 10 more minutes at the same temperature. This study was preceded by a ''screening'' with 12 different ratios of collagen, galactomannan and glycerol. The **critical surface tension** of the fruits was determined according to Zisman [10]. In systems having a surface tension lower than 100 mN/m (low-energy surfaces), the contact angle formed by a drop of liquid on a solid surface will be a linear function of the surface tension of the liquid. The Zisman method is applicable only for low energy surfaces; therefore it is necessary to determine the surface energy of the fruits. The estimation of the critical surface tension was performed by extrapolation from Zisman plots [10]. The liquids used to determine the surfaces properties from the fruits have: the surface tension, the dispersive and the polar component were, respectively, 72.10, 19.90 and 52.20 mN/m for water, 44.40, 44.40 and 0.00 mN/m for bromonaphtalene and 56.90, 23.50 and 33.40 mN/m for formamide [11].

When a solid is contacted by a liquid in the presence of vapour, the liquid will adhere well on the solid surface if the total free energy required for the creation of the new interface decreases. The physical significance of this energy change is the work needed to separate the solid and liquid from the solid/liquid interface, being the equilibrium the **spreading coefficient** (*Ws*). Contact angle and liquid-vapor surface tension were measured in a face contact angle meter (OCA 20, Dataphysics, Germany). The **surface tension** of the coating solution was measured by the pendant drop method using the Laplace-Young approximation [12]. The samples of the coatings were taken with a 500 μL syringe (Hamilton, Switzerland), with a needle of 0.75 mm of diameter. The contact angle at the fruit surfaces was measured by the sessile drop method [13], in which a droplet of the tested liquid was placed on a horizontal surface and observed with a face contact angle meter.

Oxygen permeability ($O_2 P$) was determined based on the ASTM (2002) method. A film was sealed between two chambers; having each one two channels. In the lower chamber O_2 is supplied at a controlled flow rate to keep its pressure constant in that compartment. The other chamber was purged by a stream of nitrogen, also at a controlled flow. This nitrogen acted as a carrier for the O_2 and the flow leaving this chamber was connected to an O_2 sensor. The flows of the two chambers were connected to a manometer to ensure the equality of pressures between both compartments. As the O_2 was carried continuously by

nitrogen flow, it was considered that O_2 partial pressure in the upper compartments is null, therefore ΔP is equal to 1 atm. **Carbon dioxide permeability** (CO_2P) was determined based on the ASTM (2002) method. The films were sealed between two chambers, having each one two channels. In the lower chamber CO_2 is supplied at a controlled flow rate to keep its pressure constant in that compartment. The other chamber was purged by a stream of nitrogen, also at a controlled flow. This nitrogen acted as a carrier for the CO_2 and the flow leaving this chamber was collected for CO_2 quantification. The flows of the two chambers were connected to a manometer to ensure the equality of pressures between both compartments. As the CO_2 was carried continuously by nitrogen flow, it was considered that CO_2 partial pressure in the upper compartment is null, therefore ΔP is equal to 1 atm. To determine CO_2 concentration 1 mL of sample was injected in a gas chromatograph (Chrompack 9001, Middelburg, Netherlands) at 110 ºC with a column Porapak Q 80/ 100 mesh 2 m x 1/8" x 2 mm SS, using a flame ionization detector (FID) at 110 ºC. Helium at 23 mL/min was used as carrier gas. A standard mixture containing 10 % CO_2, 20 % O_2 and 70 % N_2 was used for calibration. The measurements were repeated three times for each film. The **water vapor permeability** (*WVP*) of the films was determined gravimetrically based on ASTM E96-92 method [14,15]. The test film was sealed on the top of a permeation cell containing distilled water (100 % RH; 2.337 x 10^3 Pa vapor pressure at 20 °C), placed in a desiccator which was maintained at 20 °C and 0 % RH (0 Pa water vapor pressure) with silica gel. The water transferred through the film and adsorbed by the desiccant was determined from weight loss of the permeation cell. The cups were weighed at intervals of 2 hours during 10 hours. Steady-state and uniform water pressure conditions were assumed by keeping the air circulation constant outside the test cup by using a fan inside the desiccator [14]. The slope of weight loss versus time was obtained by linear regression. The measured (*WVP*) of the films was determined as follows:

WVP = (WVTR. L) / Δ P

where *WVTR* is the measured water vapor transmission rate (g /m^{-2}.s^{-1}) through a film, *L* is the mean film thickness (m), and *Δ P* is the partial water vapor pressure difference (Pa) across the two sides of the film. For each type of film, *WVP* measurements were replicated three times.

The **O_2 and CO_2 consumption/production in apple and mango** were measured by placing fruits inside a hermetic jar and closing it. The air circulation was promoted inside the jar by using a miniature fan. The atmosphere inside the jar was measured by drawing the gas samples with a 1 mL syringe through a septum fitted in the jar lid. The O_2 and CO_2 content in the jar was determined using a gas chromatograph (Chrompack 9001, Middelburg, Netherlands) at 110 ºC with a column mol.sieve 5A 80/ 100 mesh 1 m x 1/8" x 2 mm to separate the O_2 and a column Porapak Q 80/ 100 mesh 2 m x 1/8 "x 2 mm SS to separate the CO_2 using a flame ionization detector (FID) at 110 ºC. Helium at 23 mL/min was used as carrier gas. A standard mixture containing 10 % CO_2, 20 % O_2 and 70 % N_2 was used as standard for calibration.

Results and Discussion

Critical Surface Tension

Table 1 displays the values of the surface tension of the fruits, and their two components. The estimated values of the polar and dispersive components of the surface tension, are 1.71 and 24.77 mN/m, respectively for the mango and 0.68 and 27.13 mN/m, respectively for the apple, being the surface tensions of the mango and apple the sum of the two components (26.48 and 27.81 mN/m). Both vegetables are therefore, low energy surfaces. This type of surface interacts with liquids primarily through dispersion forces [16].

Table 1 - Surface tension from mango and apple, at the temperature of 20 °C

Fruit	Surface tension / (mN/m)	Polar component / (mN/m)	Dispersive component / (mN/m)
Apple	27.81 ± 0.03	0.68 ± 0.01	27,13 ± 0.02
Mango	26.48 ± 0.02	1.71 ± 0.01	24,77± 0.01

Once both values of the surface tension are lower than 100 mN/m the Zisman method can be applied to estimate the critical surface tension by extrapolation from the corresponding Zisman plot.

Wettability

Wettability determinations were performed with different galactomannan, collagen and glycerol concentrations. The wettability was studied by determining the values of the spreading coefficient (*Ws*). The values of the spreading coefficient from the galactomannans on each fruit were analysed and are presented below. The best values are filled in red. The best wettability values obtained for apple are found for the coating with no glycerol. This fact is probably associated to the particularity that apple surface presents a high dispersive component, denoting a predominance of apolar forces, once glycerol is a polar substance, probably this can explain the observed results. The optimum values of the spreading coefficients, in mango, were obtained with blends of 0.5 % galactomannan of *A. pavonina*, 1.5 % collagen and 1.5 % glycerol. The optimum values of the spreading coefficients, in apple, were obtained with blends of 0.5 % galactomannan of *C. pulcherrima*, 1.5% collagen and no glycerol.

Table 2 - Values from the spreading coefficient (Ws) for different collagen, galactomannan and glycerol blends (95% of confidence level – Tukey Test).

Galactomannan/ Collagen/Glycerol	Apple C. pulcherrima	A. pavonina	Mango C. pulcherrima	A. pavonina
0.5% - 1.5% - 0%	-42.79[h]	-50.01[g]	-49.85[c]	-35.87[g]
0.5% - 1.5% - 0.5%	-49.36[ef]	-56.22[de]	-49.31[c]	-36.73[fg]
0.5% - 1.5% - 1%	-45.46[g]	-53.21[ef]	-36.60[d]	-38.61[f]
0.5% - 1.5% - 1.5%	-47.49[fg]	-55.21[de]	-38.38[d]	-29.07[h]
1% - 1% - 0%	-50.12[def]	-64.80[a]	-52.45[b]	-53.09[a]
1% - 1% - 0.5%	-57.78[b]	-60.26[b]	-56.73[a]	-41.63[e]
1% - 1% - 1%	-49.32[ef]	-56.55[d]	-48.20[c]	-48.42[c]
1% - 1% - 1.5%	-51.48[de]	-61.76[b]	-52.10[b]	-53.38[a]
1.5% - 0.5% - 0%	-55.18[c]	-52.11[fg]	-47.85[c]	-45.20[d]
1.5% - 0.5% - 0.5%	-63.51[a]	-57.50[cd]	-58.59[a]	-50.01[bc]
1.5% - 0.5% - 1%	-52.26[cd]	-59.53[bc]	-59.63[a]	-49.33[bc]
1.5% - 0.5% - 1.5%	-54.19[c]	-62.00[ab]	-57.15[a]	-51.72[ab]

Water Vapor, Oxygen and Carbon dioxide permeabilities

Figure 1 shows the differences of oxygen permeability (O_2P), carbon dioxide permeability (CO_2P) and water vapor permeability (*WVP*) between the samples with best wettability values. The sample with 0.5 % of *A. pavonina* galactomannan; 1.5 % of collagen and 1.5 % glycerol is less permeable to oxygen (O_2P) than the sample with 0.5 % *C .pulcherrima* galactomannan; 1.5% of collagen and no glycerol. The addition of plasticizer decreases the presence of cracks and pores, improving the dispersion and decreasing the gas permeability [17]. Similar results were obtained to carbon dioxide permeability (CO_2P). The film with 0.5 % of *A. pavonina* galactomannan; 1.5 % of collagen and 1.5 % glycerol is approximately 18 times less permeable to CO_2 than the one with 0.5 % of *C.pulcherrima* galactomannan; 1.5% of collagen and no glycerol. In the water vapor permeability (*WVP*) the opposite occurs as observed for CO_2 and O_2 permeability. The coating with 0.5 *% C.pulcherrima* galactomannan; 1.5 % collagen and no glycerol is approximately 60 % less permeable to water vapor than the coating with 0.5 % *A. pavonina* galactomannan; 1.5 % collagen and 1.5 % glycerol. The plasticizer decreases the intermolecular attractions between polymeric chains, facilitating the penetration of water vapor molecules [3]. Glycerol is a hydrophilic molecule (polar) and its increase causes an amplification on water vapor mass transfer.
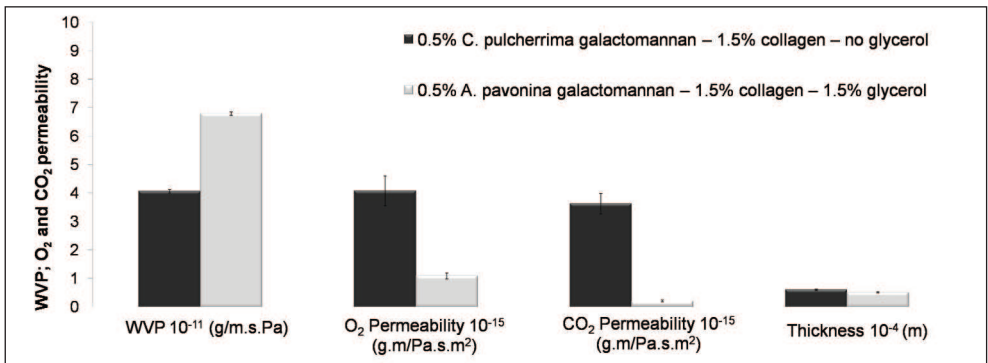
Figure 1 - Water Vapor Permeability (*WVP*), Oxygen Permeability (O_2P) and Carbon Dioxide Permeability (CO_2P) properties of coatings based on galactomannan-collagen blends, and the respective standard deviations.

O_2 and CO_2 consumption/production in apple and mango

Apples were coated using a solution with 0.5 % of *C. pulcherrima* galactomannan, 1.5 % of collagen and no glycerol and its O_2 and CO_2 transfer rates were compared with some apples without coating. The gases were measured during 60 hours and the gas transfer rate was calculated and the results are presented in Figure 2a. The coated apple permits a lowest gas exchange. The CO_2 production and the O_2 consumpttion is approximately 50 % lower in apples with coating than in apples without coating. The rate of CO_2 production is higher than that of O_2 consumption. Mangoes were coated using a solution with 0.5 % of *A. pavonina* galactomannan, 1.5 % of collagen and 1.5 % of glycerol and its O_2 and CO_2 transfer rates were compared with mangoes without coating. The gases were measured during 120 hours and the gas transfer rate was calculated and the results are presented in Figure 2b. The coated mango permits lowest gas exchange. A 28 % less O_2 consumption and 11 % less CO_2 production is observed in coated mangoes when compared with mangoes without coating.
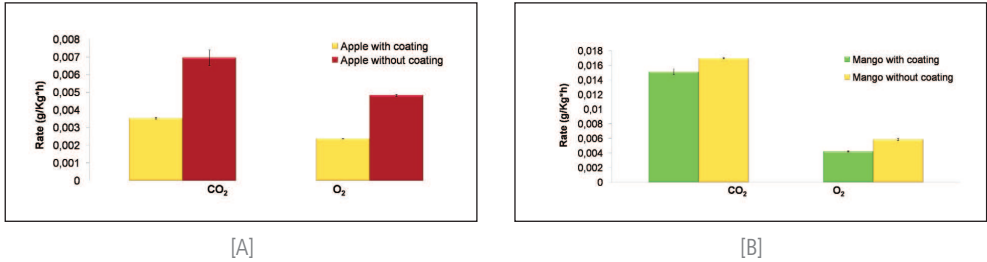
Figure 2 – O_2 and CO_2 consumption/production in apple (A) and mango (B)

Conclusions

Mango and apple have low energy surfaces with a surface tension of 26.48 and 27.81 mN/m, respectively and a polar and dispersive component of 1.71 and 24.77mN/m for the mango and 0.68 and 27.13 mN/m for the apple, respectively. The critical surface tensions are 19.5 and 25.4 mN/m, respectively. The optimum values of the spreading coefficients, in mango, were obtained with blends of 0.5 % of galactomannan of *C. pulcherrima*, 1.5 % of collagen and 1 % of glycerol. When the galactomannan used was the *A. pavonina* the best values were obtained with blends of 0.5 % of galactomannan of *A. pavonina*, 1.5 % of collagen and 1.5 % of glycerol. The optimum values of the spreading coefficients, in apple, were obtained with blends of 0.5 % of galactomannan of *C. pulcherrima*, 1.5 % of collagen and no glycerol. When the galactomannan used was the *A.. pavonina* the best values were obtained with the same ratios of 0.5 % of galactomannan of *A. pavonina*, 1.5 % of collagen and no glycerol.

The coatings presenting the best values of *Ws* were tested in relation to their gas permeability properties. The coating with 0.5 % *C. pulcherrima* galactomannan, 1.5 % collagen and no glycerol shown that it is less pemeable to water vapor. The coating with 0.5 % *A. Pavonina* galactomannan, 1.5 % collagen and 1.5 % glycerol shown that it is less permeable to O_2 and CO_2.

A 28% less O_2 consumption and 11% less CO_2 production were observed in coated mangoes when compared with mangoes without coating. The same procedure was done in apples (in this case using *C. pulcherrima* galactomannan). The CO_2 production and the O_2 consumption is approximately 50% lower in apples with coating than in apples without coating. Results suggest that these coatings can reduce gas transfer rates in these fruits, and can be important tools to extend shelf-life of fruits.

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ANA IOLANDA MAIA DE OLIVEIRA

Biological treatment of solid wastes from the tobacco industry for enzyme production

ABSTRACT

Aiming at the production of enzymes using solid wastes from the tobacco industry, the solid fermentation kinetics of *Aspergillus niger* and *Aspergillus terreus* using waste of dark tobacco and Virginia tobacco as substrate were characterized.

The efficiency of the fermentation process was evaluated by determining the enzymatic activity of the three enzymes that constitute the cellulose enzymatic system (CMCase, PFase and Xylanase).

The results obtained led to the establishment of the best initial conditions of fermentation and the selection of the most efficient microorganism for enzyme production. The best results were obtained with *Aspergillus terreus* for both tobacco residues. In the case of black tobacco, the best incubation temperature was 31 ºC for the enzymes CMCase and Xylanase and 36 ºC for the PFase and initial pH 5.5 for the three enzymes. For the Virginia tobacco, the best incubation temperature and initial pH are the same for the three enzymes, 36 ºC and 5.5 respectively.

The biological activity of the fermented tobacco residues was evaluated being the highest rate of inhibition of microbial growth - 72% - obtained with the residue of Virginia tobacco treated with *Aspergillus niger.*

TESTIMONIAL

"It was a great experience, both personally and professionally."
"On the return luggage brought in many lessons, particularly the desire to go back and review all good friends who have left!"

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Biological treatment of solid wastes from the tobacco industry for enzyme production

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Abstract

Aiming at the production of enzymes using solid wastes from the tobacco industry, the solid fermentation kinetics of *Aspergillus niger* and *Aspergillus terreus* using waste of dark tobacco and Virginia tobacco as substrate were characterized.

The efficiency of the fermentation process was evaluated by determining the enzymatic activity of the three enzymes that constitute the cellulose enzymatic system (CMCase, PFase and Xylanase).

The results obtained led to the establishment of the best initial conditions of fermentation and the selection of the most efficient microorganism for enzyme production. The best results were obtained with *Aspergillus terreus* for both tobacco residues. In the case of black tobacco, the best incubation temperature was 31 ºC for the enzymes CMCase and Xylanase and 36 ºC for the PFase and initial pH 5.5 for the three enzymes. For the Virginia tobacco, the best incubation temperature and initial pH are the same for the three enzymes, 36 ºC and 5.5 respectively.

The biological activity of the fermented tobacco residues was evaluated being the highest rate of inhibition of microbial growth – 72% - obtained with the residue of Virginia tobacco treated with *Aspergillus niger.*

Key words: Biological treatment of solid wastes, *Aspergillus niger*, *Aspergillus terreus,* dark tobacco and Virginia tobacco, solid state fermentation, enzymatic system cellulase (CMCase, PFase and Xilanase).

Resumo

De forma a atingir os objectivos propostos neste trabalho nomeadamente o tratamento biológico de resíduos da indústria tabaqueira para a produção de enzimas, foi realizado um estudo da cinética de fermentação em estado sólido dos resíduos de tabaco negro e rubio pelos microrganismos *Aspergillus niger* e *Aspergillus terreus*.

Para testar a eficiência dos processos de fermentação foi determinada a actividade enzimática através da aplicação de técnicas específicas para cada uma das três enzimas que constituem o sistema enzimático cellulase (CMCase, PFase e Xilanase).

Os resultados obtidos ao longo do estudo levaram ao estabelecimento das melhores condições iniciais de fermentação e qual o microrganismo que permite a obtenção de uma maior actividade enzimática. O microrganismo que obteve melhores resultados foi o *Aspergillus terreus* para ambos os resíduos tabaqueiros, no caso do tabaco negro com temperatura de incubação igual a 31 ºC para as enzimas CMCase e Xilanase, 36 ºC para a PFase e pH inicial de 5.5 para as três enzimas; relativamente ao tabaco rubio a temperatura de incubação e o pH inicial são o mesmo para as três enzimas sendo iguais a 36 ºC e 5.5 respectivamente.

Os resíduos tabaqueiros depois de tratados biologicamente foram submetidos a testes, no Instituto Cubano

de Investigação do Derivados de Cana-de-açúcar (ICIDCA), para verificar se possuíam actividade biológica, tendo a maior taxa de inibição de crescimento microbial (72%) sido conseguida com o resíduo de tabaco rubio tratado com *Aspergillus niger.*

Palavras-chave: Tratamento biológico de resíduos, tabaco negro e rubio, fermentação em estado sólido, *Aspergillus niger*, *Aspergillus terreus,* sistema enzimático cellulase (CMCase, PFase, Xila-nase).

Introduction

The production of enzymes is one of the most important industrial biotechnological processes. In most of the cases, enzymes are produced from a microbial source due to the high diversity that is possible to obtain from this mode of production and also due to the operational and economical difficulties that the enzyme extraction process from animal and vegetable tissues presents [1].

The application of enzymes in industries such as chemical, pharmaceutical and feed, replacing the conventional chemical catalyst, is becoming more frequent. This can be explained by the several advantages of this processes, such as higher efficiency under moderated temperature and pressure conditions, environmental pollution reduction and less secondary products formation, as result of their selectivity. About 400 companies all over the world are involved in the production of enzymes, Europe being the main producer (approximately 60 %) followed by United States of America and Japan [1].Worldwide enzyme market moves about 1.38 billions of Euros per year, with an annual increase rate of 8 % to 10%.

Nowadays, cellulase, hemicellulase and pectinase enzymes represent about 20 % of the enzymes production all over the world [2], most of them from *Trichoderma* and *Aspergillus* species [1].

The high cost and low yield of the production process of these enzymes are the major problems for their industrial application. In order to get over these difficulties several microbial strains with a high production yield are needed together with fermentation parameters optimization.

Among the several materials used as biomass, lignocellulosic biomass has been receiving a particular interest due to the reduced price and great availability. The solid state fermentation offers advantages when compared with submerged fermentation in what concerns the obtention of biomolecules from these materials.

The Cuban industries of tobacco produce annually about 4000 metric tons of solid residues rich in cellulose, hemicelluloses and lignin, that can't be used in the production of "torcidos" and "cigarrilhos". These residues can be subjected to several treatments to increase the tobacco economical value and avoid the deteriorating oh the environment [3] [4].

Material and Methods

Microorganism

The microorganisms used in this work were *Aspergillus niger* specie J-1, isolated from sugar cane bagasse, from the Collection of Chemical Engineering Faculty of the ISPJAE and *Aspergillus terreus* specie H/6.39.3, from the collection of the ICIDCA.

Aspergillus niger was maintained in sterilized malt agar and *A.terreus* in Czapck-Dox agar. The microorganisms were inoculated in erlenmeyers and incubated during 7 days at 30ºC for the obtention of the spores solution for further inoculation.

Fermentation conditions

4 black tobacco fermentations with *Aspergillus niger* were done with an initial moisture content of 70%, pH 5.5 and incubation temperature 37ºC. These conditions were selected taking in account the study carried out by Quesada C.(2004)[3].

The study by Quesada C. (2004) [3] was also used as the reference work for the fermentation experiments of both residues by *Aspergillus terreus*. Fermentations were carried out with the following initial conditions: 70 % of moisture and pH 7 or 5.5 with incubation temperatures of 31 ºC and 36 ºC.

Enzymatic activity determination

1 U of CMCase, defined as the amount of enzyme that liberates 1 μmol of reducing sugar per minute, was determined under the following conditions: 1% solution of carboxymetilcelulose in a 0.1 M sodium citrate buffer, temperature of 50 ºC, pH of 4.8 and time of reaction equal to 30 minutes.

1 U of Pfase defined as the amount of enzyme that liberates 1 μmol of reducing sugar per minute, was determined in the following conditions: 50 mg of filter paper Whatman #1 in a 0.075 M sodium citrate buffer solution, temperature of 50 ºC, pH of 4.8 and time of reaction equal to 60 minutes.

1 U of Xylanase defined as the amount of enzyme that liberates 1 μmol of reducing sugar per minute , was determined in the following conditions: 1.0 g of xylan in 500 ml of 0.1 M NaOH, temperature of 50 ºC, pH of 4.8 and time of reaction equal to 20 minutes.

Biological activity determination

The determination of the percentage of inhibition of pathogenic mold growth, in this in case *Alternaria solani*, was done as follows: the mold was incubated in a Petri plate in 15 ml of Potato Dextrose Agar PDA) that has been added 10 mL of the fermentation sample (after filtration through a 0,2 μm membrane). A control experiment, in the absence of the fermentation sample, was also made.

Results

In all the fermentations of black tobacco dust by carried *Aspergillus niger*, the obtained values for the assayed enzymatic activities were very low, even when the black tobacco dust was treated according to the procedure described by Dustet (1999) [5] .

In what concerns the fermentation experiments of the black tobacco and Virginia tobacco dust by *Aspergillus terreus*, residual values for enzymatic activity were obtained for both substrates at pH 7 and T 31 ºC. It is interesting to notice that a CMCase value of 0.2128 U/mL was obtained at the third day of fermentation, when using Virginia tobacco dust.

When the initial conditions were pH 5.5 and T 31 ºC, the obtained PFase enzyme activity values are very low for both wastes, even if there is a significant improvement in the case of black tobacco. In what concerns CMCase and xylanase activities a significant increase also occurs. The obtained maximum values of enzymatic activity using the black tobacco dust were 0.0154 U/mL at 72 hours of fermentation for the PFase, 0.1488 U/mL at 72 hours of fermentation for xylanase and 0.4163 U/mL at 96 hours of fermentation for CMCase. For the Virginia tobacco waste, the obtained maximum values of enzymatic activity were 0.3729 U/mL and 0.1148 U/mL for CMCase and xylanase, respectively, at 120 hours of fermentation and 0.0281 U/mL at 72 hours for the PFase.

Using as initial conditions pH 5.5 and T 36 ºC, the maximum values of enzymatic activity for the black tobacco dust are 0.0304 U/mL at 144 hours of fermentation for PFase, 0.1464 U/mL at 120 fermentation hours for xylanase and 0.3829 U/mL at 72 hours of fermentation for the CMCase. For the Virginia tobacco dust the maximum values of enzymatic activity registered are 0.5019 U/mL and 0.1647 U/mL, in the case of the CMCase and xylanase enzymes, respectively, at a fermentation time of 72 hours and 0.0344

U/mL for the PFase with a fermentation time of 96 hours.

The antimicrobial activity of the fermented tobacco wastes was evaluated and the results are presented in Table 1.

Table 1. Percentage of microbial growth inhibition by the treated tobacco wastes with *Aspergillus terreus* and *Aspergillus niger*.

	% of inhibition
Virginia tobacco – *Aspergillus terreus*	5.8
Black tobacco – *Apergillus terreus*	2.4
Tobacco enzymatic solution	8.6
Tobacco enzymatic solution	8.6
Black tobacco – *Aspergillus terreus*	25
Virginia tobacco – *Aspergillus niger*	71
Virginia tobacco – *Aspergillus niger*	72
Virginia tobacco – *Aspergillus niger*	8.6

All samples display antimicrobial activity, being the Virginia tobacco sample treated with the microorganism *Aspergillus niger* the one that presents a bigger percentage of microbial growth inhibition. These results indicate that bioactive metabolites are formed during the fermentation process.

Conclusions

The results obtained demonstrate that both tobacco wastes, black and rubio tobacco dust, can be biological degraded. The best results for enzymatic activity were obtained when fermentations were carried with *Aspergillus terreus* and Virginia tobacco dust was used as substrate.

The tests for biological activity evidence the formation of bioactive metabolites during fermentation.

Acknowledgements

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PRISCILA MARIA DE BARROS RODRIGUES

Production and characterization of protease from *Penicillium aurantiogriseum* URM 4622

ABSTRACT

Proteases with new properties are required due to their increasing industrial importance. In this work, the optimal fermentation conditions for the production of a protease from Penicillium aurantiogriseum dierchx (URM-4622) are presented together with partial characterization of the protease catalytic properties. The batch fermentation conditions that allow for the highest specific proteolytic activity are 26 ºC, pH 7.0, and 25 % saturation dissolved O_2 concentration. The obtained protease is stable over a wide range of pH (5.8 to 9.5) and temperature (25 to 40 ºC) values. In the presence of Zn^{2+} a 26 % reduction in the enzyme proteolytic activity occurs and, in contrast, Mn^{2+} enhances its activity by 28.9%. 96.2% and 70.8% of the protease activity are maintained after 90 min incubation in 5 and 10% (v/v) H_2O_2 aqueous solutions, respectively. PMSF inhibition reveals that this enzyme is a serine protease. Protease is able to hydrolyze different proteins.

TESTIMONIAL

"Ter participado do projeto alfa foi uma experiência maravilhosa, um crescimento profissional e pessoal fantástico."

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Production and characterization of protease from *Penicillium aurantiogriseum* URM 4622

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Abstract

Proteases with new properties are required due to their increasing industrial importance. In this work, the optimal fermentation conditions for the production of a protease from *Penicillium aurantiogriseum* dierchx (URM-4622) are presented together with partial characterization of the protease catalytic properties. The batch fermentation conditions that allow for the highest specific proteolytic activity are 26 ºC, pH 7.0, and 25 % saturation dissolved O_2 concentration. The obtained protease is stable over a wide range of pH (5.8 to 9.5) and temperature (25 to 40 ºC) values. In the presence of Zn^{2+} a 26 % reduction in the enzyme proteolytic activity occurs and, in contrast, Mn^{2+} enhances its activity by 28.9 %. 96.2 % and 70.8 % of the protease activity are maintained after 90 min incubation in 5 and 10 % (v/v) H_2O_2 aqueous solutions, respectively. PMSF inhibition reveals that this enzyme is a serine protease. Protease is able to hydrolyze different proteins.

Keywords: *Penicillium aurantiogriseum*; production; characterization; protease; bacth; bioreactor; detergent.

Indroduction

Proteases, also known as peptidyl-peptide hydrolases, are important industrial enzymes, which are responsible for approximately 60% of all enzyme sales, and are extensively used in a variety of industries, including foods, pharmaceuticals, leathers and detergents [1,2].

Microbes represent an excellent source of enzymes, including proteases, because of their broad biochemical diversity [3,4]. Microbial proteases can be produced from bacteria, fungi and yeast using solid-state fermentation as well as submerged fermentation [5]. Fungi as enzymes producers have many advantages, considering that the produced enzymes are normally extracellular, making easier its recuperation from the fermentation broth [6].

Any fermentation process is significantly influenced by physical and chemical parameters and different results can be obtained as we move from shake flask to large bioreactor fermentation as different mixing and mass transfer patterns may occur as scale is increased [7]. It is well known that extracellular protease production by microorganisms in bioreactors is greatly influenced by medium components, physical factors such as aeration, agitation, temperature, inoculum density, dissolved oxygen and incubation time. Industrial fermentation is moving away from traditional and largely empirical operation towards knowledge based and better-controlled process [5]. This work addresses the optimization of the fermentation conditions for protease production by *Penicillium aurantiogriseum* dierchx (URM 4622) and the characterization of the enzyme catalytic properties.

Materials and methods

Microorganism and culture medium

The *Penicillium aurantiogriseum* dierchx (URM 4622) was supplied by the Micoteca of Micology Department of the Universidade Federal de Pernambuco (UFPE). The strain was maintained at 28ºC in malt extract agar, consisting of: 2% (w/v) malt extract, 0.1% (w/v) peptona, 2% (w/v) glucose and 1.5% (w/v) agar. Soy flour medium (SM), as described by Porto *et al.* [8], was used for protease production and it is composed of: 1% (w/v) filtered soy flour, 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4.7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose and 0.8 mL mineral solution. The composition of the mineral solution, per 100 mL of distilled water, is: 100 mg of $FeSO_4.7H_2O$; 100 mg of $MnCl_2.4H_2O$; 100 mg of $ZnSO_4.H_2O$; 100 mg of $CaCl_2.H_2O$. The fermentation medium was sterilized in autoclave at 121 ºC, for 20 minutes.

Production of protease

The inoculum was prepared in test tubes, containing 10 mL of a solution of 0.9% (w/v) NaCl and 0.01% (v/v) Tween 80 sterilized previously at 121ºC, for 20 minutes. The solution of spores was prepared so that a final concentration of 10^6 spores.mL^{-1} was obtained. Fermentations were realized using 18 Erlenmeyer flasks (50 mL) each containing 10 mL of the Soy flour medium with 10^6 esporos.mL^{-1} of the inoculum. Flasks were incubated at 28ºC, with constant shaking at 150 rpm for 96 hours. Two samples were taken every 12 h for the determination of biomass concentration, pH and protease activity.

Bioreactor studies

Protease production in bioreactor was evaluated using a statistical design. A 2^3 full design was carried out to verify the effects and interactions of pH, temperature and aeration rate on the protease production. In this design, a set of 11 experiments, with three replicates at the central points, was performed. The range and levels of the components (factors or independent variables) under study are given in Table 1. The production of protease of *Penicillium aurantiogriseum* was carried out in a stirred tank reactor (Fermenter RALF 2.0 L) with 1.5L working volume, equipped with temperature, pH and dissolved oxygen measurement and control. The pre-inoculum was prepared in Erlenmeyer flasks (250 mL) each containing 50 mL of the Soy flour medium with 10^6 esporos.mL^{-1} and incubated at 28ºC, 150 rpm for 24 h. After this, the culture was added in bioreactor containing 1.45 L of the Soy flour medium previously sterilized. The pH of the medium was adjusted with NH_3 (6 % v/v) and/or H_3PO_4 (21% v/v). Samples were collected at regular intervals of 12 h for determinations of the biomass, protease activity and total protein concentration.

Analitical methods

The growth curve of the *Penicillium aurantiogriseum* was accompanied by measuring biomass dry weight. The fermented broth was filtered through previously weighted 0.45 µm porosity membranes (after drying at 80ºC, for 1 hour). The membranes, after the filtration of the fermented broth, were again dried at 80ºC for 2 hours. The dry weight of the samples was obtained by the difference of the weight of the membranes, before and after filtration.

The protein concentration was determined according to Bradford [9], using bovine serum albumin (BSA) as standard. The protease activity was determined according to Leighton *et al.* [10] using 1% (w/v) azocasein as substrate in a 0.1 M Tris-HCl pH 7.2 buffer. One unit of protease activity was defined as the amount of enzyme required to produce an absorbance variation of 1 for 1 hour and was expressed in U/mL.

Effect of pH and temperature on the activity and stability of the protease

The optimum pH of protease activity was determined with 1% (w/v) azocasein as substrate dissolved in different buffers. The buffers used were: 0.1 M sodium-phosphate (pH 5.8, 6.2 and 7.2); 0.1 M Tris-HCl (pH 7.2, 8.6 and 9.0) and 0.1 M glycine-NaOH (pH 8.6, 9.0 and 9.5). The pH stability of the protease was determined by incubating the enzyme solution with the different buffers mentioned in a proportion of 1:1 (v/v) during 120 min at 25ºC. Samples were collected each 30 min, for the determination of the protease activity.

The protease activity was assayed at various temperatures (25 - 70ºC) to determine the optimum temperature, using 1 % (w/v) azocasein as substrate in a 0.1 M Tris-HCl pH 9.0 buffer. For determination of thermostability, the enzyme was pre-incubated at different temperatures for 120 min, being samples taken every 30 min for determination of the protease activity. The shelf life at low temperatures (-20ºC and 4ºC) was determined after 30 days of incubation.

Table 1. Factor levels used in the 23 full design used for studying of protease production by P. aurantiogriseum.

Variables	Levels		
	Lower (-1)	Center (0)	Higher (+1)
Temperature (ºC)	22	26	30
pH	5.0	7.0	9.0
O_2 (%)	10	25	40

Effect of metal ions, inhibitors and oxidizing agent on activity

The effects of metal ions were investigated using: $CaCl_2$, $AlCl_3$, $LiCl$, $ZnCl_2$, $MnCl_2$, KCl, and NaCl in the concentration 1mM. The substrate 1% (w/v) azocasein was prepared with different metal ions and dissolved in 0.1 M Tris-HCl pH 9.0. Enzyme activities in the presence of metal ions were compared with the control (without metal ions).

To determine the type of the protease, enzyme was pre-incubated for 15 min at 37ºC with different specific protease inhibitors at a 0.1 M concentration and protease activity was determined. Tested inhibitors were: PMSF, EDTA and iodoacetic acid. Activity in the presence of inhibitors was compared with the control (without inhibitors).

The stability of enzyme activity in the presence of hydrogen peroxide was studied by incubating the protease for 120 min at 40ºC with different concentrations of hydrogen peroxide from 5 to 15% (v/v). Samples were collected each 30 min for determination of the protease activity.

Protease activity on different substrates

The proteolytic activity assay was applied to other protein substrates: bovine serum albumin (BSA), hemoglobin, and ovalbumin according to a modified method described by Pokorny et al [11], azocasein according to Leighton et al. [10] and azocoll, according to Chavira et al. [12].

Results and discussion

The time variation of biomass concentration, pH and protease activity in shake flask culture by *P. aurantiogriseum* is shown in Fig. 1. Maximum biomass production (2.7 g/L) occurred at 48 h, while maximum

enzyme production was observed at 84 h when the medium pH was 8.0. The growth curve shows a clear exponential phase, with a 48 h lag phase. After 60 h (end of exponential phase) the biomass starts to decrease, probably as a consequence of the depletion of nutrients into the culture medium. Studies on the production of alkaline protease by *Aspergillus tamarii* concluded that protease production occurred at 100 h of growth, when the medium pH was 9.0 [13].
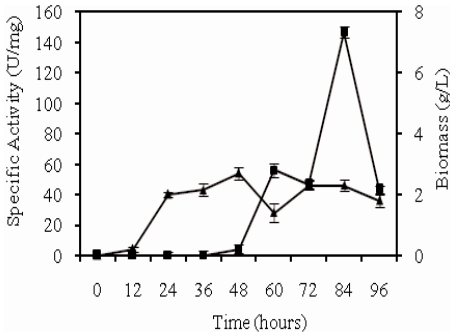
Fig. 1. Growth curve (▲) and protease production (■) of *P. aurantiogriseum.*

The results obtained with the 2^3 full factorial design are show that the central point of the experimental design (26ºC, pH 7 and 25% saturation dissolved oxygen concentration) was the best condition for the protease production, showing the highest values of specific activity 44.48, 41.39 and 44.55 (U/mg). The analysis of the Pareto chart (Fig. 2), demonstrates that only the pH value was significant for enzyme production and cell growth. A pH significant negative effect was observed, suggesting (inside the appraised conditions) that the decrease of the pH value improves the protease production and cell growth, observing that the pH 7 was the best condition. Similar results were obtained by Çalik *et al.* [14] when investigating the influence of controlled-pH and uncontrolled-pH conditions together with the initial pH in serine alkaline protease production by recombinant *Bacillus licheniformis.*
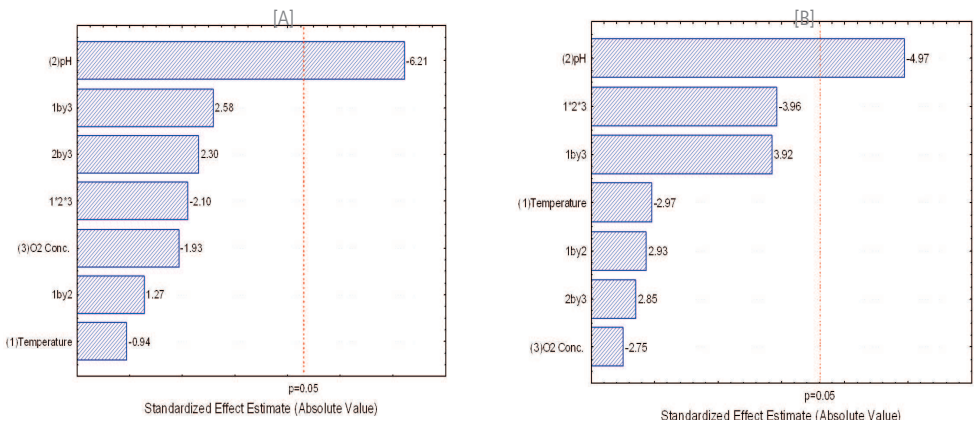
Fig. 2. *Pareto* charts for the effects of the variables temperature (*1*), pH (*2*) and O_2 concentration (*3*) on the (a) Biomass (mg/L) and (b) specific activity (U/mg).

The relative activities at various pH values (5.8 to 9.5) are shown in Fig. 3a. Regarding the effect of the buffers themselves, differences in the activity were observed with different buffers. The protease exhibited a maximum activity at pH 9.0 in buffer Tris-HCl 0.1 M. More than 90% of the maximum activity was detected for the protease between pH 7.2-9.5, which is a typical characteristic of alkaline proteases. The results are in accordance with several earlier reports showing a pH optimum of 9.0 and 8.5 for protease from *Penicillium* sp. and *Aspergillus tamari* [15,13]. Protease was very stable in a broad pH range from acid to basic, maintaining over 90% of its initial activity between pH 5.8 and 9.5, after incubation for 2h (Fig. 3b), indicating its potential for practical use in industrial purposes which require stability over wide pH ranges. Similar results were obtained with protease produced by *Penicillium* sp. [6].

The optimum temperature for the protease was found to be 50ºC (Fig. 4a). The protease was stable between 25 and 40ºC after 2 h incubation retaining above 100% of the activity, which allows lower wash temperatures when added to detergents. More than 25% of maximal activity was maintained after 30 min pre-incubation at 50ºC but total inactivation occurred above 60ºC (Fig. 4b). These results are in accordance with those obtained by Tunga *et al.* [16] and Azeredo *et al.* [17] for a protease produced by *Aspergillus parasiticus* and *Streptomyces* sp. At low temperatures (-20 and 4ºC), the protease retained 54.5% of its activity after 1 month storage. The protease from *Streptomyces* sp. was retained 75% of its activity after 2 months at low temperatures (-20 and 4ºC) [17].

The effect of metal ions on the activities of the protease is shown in Table 2. The proteolytic activity decreased about 26% in the presence of Zn^{2+} ion and increased 28.9% in the presence of Mn^{2+}. The presence of Li^+, Ca^{2+} and K^+, resulted in a discrete increase in the proteolytic activity. Similar effects of Mn^{2+} on the activity of protease were found by Agrawal *et al.* [15], when studying the production of alkaline protease by *Penicillium* sp.

The ability to hydrolyze several protein substrates is a criterion for the potential application of the enzyme [18]. In this study, the enzyme was examined for the ability to hydrolyze several proteins (Table 2). Protease activity on bovine serum albumin (BSA) and hemoglobin was 26 and 29%, respectively, as compared to its activity on azocasein. The protease showed the best activity against azocool (144%). The broader specificity of the protease from *Penicillium aurantiogriseum* may be advantageous for its use in detergents against a wide variety of stains.

The nature of the protease, enzyme activity was measured in the presence of different protease inhibitors (Table 2). No inhibition was detected when the cystein type inhibitor, i.e. iodoacetamide was added. Almost no inhibition was observed with EDTA. In contrast, protease was strongly inhibited by the serine protease inhibitor PMSF (0.1 M). This finding was similar to those of Germano *et al.* [6] and Anandan, *et al.* [13], for proteases from *Penicillium* sp. and *Aspergillus tamarii*, respectively. This result shows that *P. aurantiogriseum* secreted a serine type protease during submerged fermentation.

The results obtained in the stability of the protease in the presence of hydrogen peroxide (Fig. 5) showed that at concentrations of 5, 10 and 15% (v/v) the protease retained 96.7, 81.2 and 67.35% of its activity, respectively, after 60 minutes of incubation. After 120 minutes incubation, the protease retained 77.3, 46.5 and 27.9% of its activity for hydrogen peroxide concentrations of 5, 10 and 15%, respectively. In the detergent industry, several oxidizing agents like sodium perborate used as detergent compositions, which may release hydrogen peroxide and hence bleach stable enzymes are prepared for detergent industry [19].The protease from *Aspergillus parasiticus* [16] was not influenced by hydrogen peroxide, while the protease from *Penicillium* sp. [6] showed a good stability for a concentration of 5% (v/v) for 1 h of incubation.

Fig. 3. Effect of pH on the activity (a) and stability after incubation for 120 min (b) of the protease production by *P. aurantiogriseum*. Buffers: (■) 0.1M Sodium-phosphate; (▲) 0.1M Tris-HCl; (●) 0.1M Gliycine-NaOH.

[A]

Relative activity (%)

pH

[B]

Relative activity (%)

pH
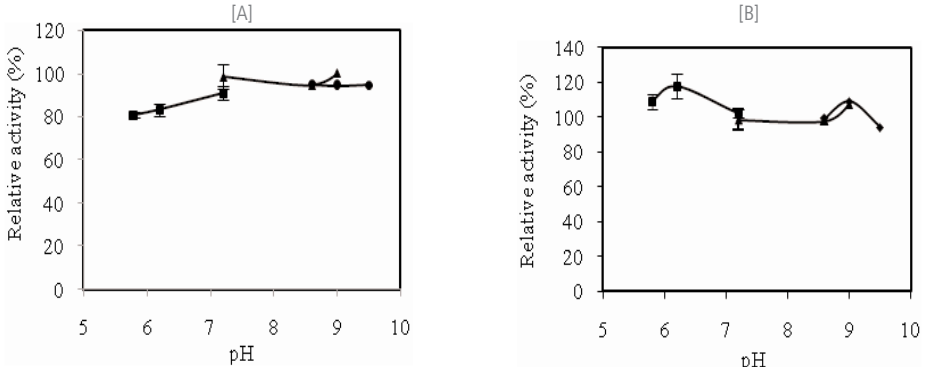
Fig. 4. Effect of temperature on the activity (a) and stability (b) of the protease production by P. aurantiogriseum. Temperatures: 25°C (◆); 30°C (■); 40°C (▲); 50°C (○); 60°C (□) and 70°C (△).

[A]

Relative Activity (%)

Temperature (°C)

[B]

Relative Activity (%)

Time (minutes)
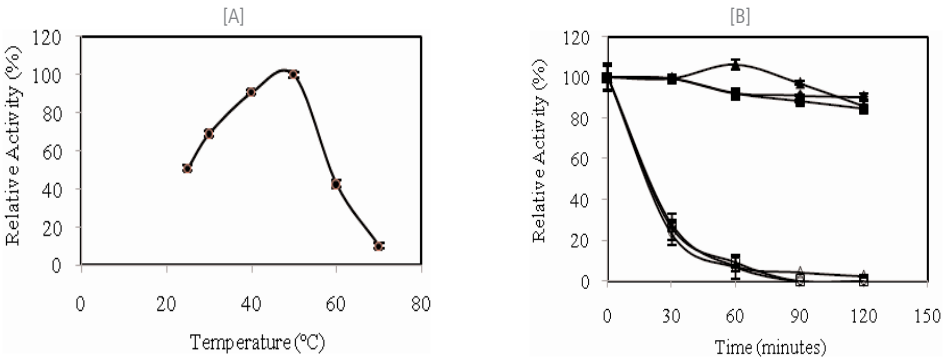
Fig. 5. Effect of oxidant agent H_2O_2 on the stability of the protease. Concentrations: (○) 5 %; (■) 10 % and (▲) 15%.

[A]

Relative Activity (%)

Time (minutes)
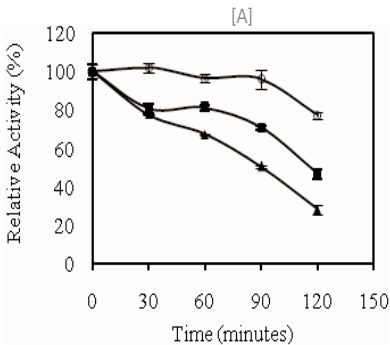
Table 2. Effect of various reagents on activity of protease

Agent	Concentration	Relative Activity (%)
$AlCl_3$	0.1 M	99.4
$MnCl_2$	0.1 M	128.9
LiCl	0.1 M	104.5
$CaCl_2$	0.1 M	104.8
KCl	0.1 M	102.9
NaCl	0.1 M	100.0
$ZnCl_2$	0.1 M	74.0
BSA	1 %	25.9
Hemoglobin	1 %	29.4
Ovoalbumin	1 %	12.1
Azocoll	0.2 %	143.8
Azocasein	1 %	100.0
EDTA	0.1 M	94.8
PMSF	0.1 M	7.0
Iodoacetic acid	0.1 M	94.9

Conclusions

The alkaline protease produced by *P. aurantiogriseum* dierchx (URM 4622) is a serine type peptidase, stable over a wide range of pH (5.8 to 9.5) and temperature (25 to 40 ºC) values. Furthermore, the presence of Mn^{2+} enhances its activity, it is not inhibited by high concentrations of H_2O_2 and is able to hydrolyze different proteins. These properties make this protease useful for application in detergent industries.

Acknowledgements

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ANDRÉA DE FÁTIMA SILVA SANTOS

Immobilization of lectin preparations from *Moringa oleifera* seeds in inert supports for water purification

ABSTRACT

Humic acids are linked to the formation of carcinogenic disinfection by-products upon chlorination of drinking water. In this work the first focus was to characterize the affinity of protein preparations obtained from *Moringa oleifera* seeds (extract, E, fraction, 0-60F and *M.oleifera* lectin, MoL) to bind humic acids. The second focus was to select a suitable support to immobilize MoL and to assess humic acid removal from water in a packed bed column. Specific hemagglutinating activity (SHA) decreased by 94 % for both E and MoL and by 50 % for 0-60F in the presence of a commercial humic acid. Humic acid-MoL precipitation bands were observed in the diffusion gel. Both results indicate humic acid-lectin binding. Carbohydrates, potassium and calcium ions as well as pH va-lues affected the SHA of MoL. A humic acid removal of 30.4 mg/g (expressed as mass of humic acid per mass of support) was obtained in a column packed with sepharose immobilized MoL receiving a 20 mg/L of carbon humic acid solution.

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Immobilization of lectin preparations from *Moringa oleifera* seeds in inert supports for water purification

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Abstract

Humic acids are linked to the formation of carcinogenic disinfection by-products upon chlorination of drinking water. In this work the first focus was to characterize the affinity of protein preparations obtained from *Moringa oleifera* seeds (extract, E, fraction, 0-60F and *M.oleifera* lectin, MoL) to bind humic acids. The second focus was to select a suitable support to immobilize MoL and to assess humic acid removal from water in a packed bed column. Specific hemagglutinating activity (SHA) decreased by 94 % for both E and MoL and by 50 % for 0-60F in the presence of a commercial humic acid. Humic acid-MoL precipitation bands were observed in the diffusion gel. Both results indicate humic acid-lectin binding. Carbohydrates, potassium and calcium ions as well as pH va-lues affected the SHA of MoL. A humic acid removal of 30.4 mg/g (expressed as mass of humic acid per mass of support) was obtained in a column packed with sepharose immobilized MoL receiving a 20 mg/L of carbon humic acid solution.

Keywords: lectin, *Moringa oleifera*, immobilization, humic acid, water treatment.

1. Introduction

A lectin is a type of receptor protein of non-immune origin that interacts with carbohydrates without modifying them. These proteins recognize and bind carbohydrates with specific characteristics and have the ability to induce cell agglutination phenomenon. The presence of these proteins is detected by a hemagglutination assay performed by serial dilution of lectin and incubation with human or animal red blood cells [1]. Lectins have mainly been obtained from seeds of leguminous plants, but also from many other plant and animal tissues. They are valuable tools in biotechnological research and biomedical applications. An important application of immobilized lectins [2] is the isolation of biomolecules [3].

Moringa oleifera is a plant of the Moringaceae family and seeds have been used in water treatment [4]. Aqueous extract from *M. oleifera* seeds contains a flocculating protein that works as a clarifying agent of turbid water [5] and lectins [6; 7]. Shelled *M. oleifera* seeds have also been used for decontamination of water containing arsenic [8].

Organic compounds of biological origin are found in all surface waters. They are referred as natural organic matter (NOM) and are divided into hydrophilic (mainly carboxylic acids, carbohydrates and proteins) and hydrophobic (humic substances, HS) fractions [9]. HS are divided in two fractions, humic acids and

fulvic acids [10]. Humic acids are heterogeneous mixtures of organic compounds containing several chemical groups. The molecular sizes of humic acids are reported to range from several hundred to several hundred thousand daltons and the chemical structures show no repetitive pattern [11]. NOM causes odor, taste and color in water and support bacterial growth [12]; it is linked to the formation of disinfection by-products upon chlorination of drinking water. Trihalomethanes (THM) constitutes the most commonly observed of these by-products; they may originate adverse health effects in animals and humans [13]. For this reason, several strategies have been investigated for the removal of humic substance from water.

The aim of the present study was to evaluate a new process for humic acid removal from water. Important goals were to characterize the affinity of protein preparations obtained from *M. oleifera* seeds (extract, fraction and lectin) to bind a commercial humic acid, to select a suitable support to immobilize the *M. oleifera* lectin, MoL, previously called SSMoL [14] and to assess humic acid removal from water in a bed column packed with the selected support.

2. Materials and methods

2.1 Lectin isolation
M. oleifera seeds were ground to flour that was extracted with 0.15 M NaCl for 6 h at room temperature (25 °C) and a saline extract (E) was obtained. Proteins present in E were precipitated with ammonium sulphate (60 %, w/v) for 4 h at room temperature (25 °C). The fraction (0-60F) obtained was chromatographed (10 mg of protein) on a guar gel column (10 x 1.0 cm) previously equilibrated (20 mL/h flow rate) with 0.15 M NaCl. The lectin (MoL) was eluted with 1.0 M NaCl.

2.2 Hemagglutinating activity and inhibition hemagglutinating activity assays
The hemagglutinating activity assay (HA) was performed in microtiter plates [15]. The inhibition assay (IHA) followed the same protocol as HA with the exception of an incubation step with inhibiting substance for 15 min at room temperature (25 ºC), before addition of erythrocyte suspension. The following substances were tested: i) humic acid (Sigma Aldrich 53680), a solution of 10 mg/L of carbon prepared in 0.1 M NaOH; ii) carbohydrates, aqueous solutions of D(+)-glucose, D(+)-galactose, L(+)-arabinose and, D(-)-galacturonic acid (0.2 M, 0.1 M, 0.05 M, 0.025 M and 0.0125 M**)**; iii) glycoprotein, solution of azocasein 5 mg/L; iv) halogenated organic compounds, aqueous solutions of dichloroacetic acid (5.6 µg/L) and trichloroacetic acid (56 µg/L), as well as chloroform (56 µg/L).

2.3 Effect of pH values and concentration of calcium and potassium on lectin hemagglutinating activity
The effect of pH in an interval of 7.5 to 10 on lectin HA was assessed in the presence of humic acid according to the procedure previously described to HA. The pH was adjusted with phosphate buffer. The effect of potassium and calcium in the lectin HA was tested in the presence of the humic acid. A humic acid solution of 100 mg/L of carbon was diluted (1:10) with $CaCl_2$ or KCl_2 solutions, 5 and 10 mM, prepared in 10 mM sodium phosphate buffer. Before the addition of erythrocyte suspension, lectin was incubated with the humic acid in the presence of K^+ and Ca^{2+} for 15 min at room temperature (25 ºC).

2.4 Protein evaluation
The protein was estimated according to Lowry *et al.* [16]. A calibration curve was prepared using bovine serum albumin (BSA) as standard in a range between 0 µg and 400 µg.

2.5 Single radial diffusion assay

A diffusion assay was carried out in agarose gel formed in a Petri dish. The gel (1%, w/v) was prepared in 0.15 M NaCl. A humic acid solution (30 µL), with a concentration of 100 mg/L and 200 mg/L of carbon, was placed in a central well; peripheral wells were occupied with 15 µL (1 mg/mL) of E, 0-60F and MoL. Assay was also carried out with azocasein (0.5 mg/mL) incubated with each protein preparation for 15 min at room temperature (25 ºC). Diffusion experiments were performed in a humid chamber at 4 °C for 48 h. Gels were exhaustively washed with 0.15 M NaCl and stained for 2 h with 0.1% (w/v) Coomassie Brilliant Blue, prepared in a mixture of ethanol 45 % (v/v) and acetic acid 10 % (v/v).

2.6 Humic acid characterization

The elemental composition of humic acid was determined with an elemental analyzer (Carlo Elba EA 1108) and on a mass basis was 48.36 % C, 26.91 % O, 4.24 % H, 0.78 % N and 0.78 % S.

2.7 Total organic carbon

Total organic carbon (TOC) was measured spectrophotometrically at 600 nm using the Method 10129 from Hach Lange GmbH (0.0 mg/L to 20.0 mg/L of carbon). Organic carbon was oxidized with persulphate in the presence of acidic conditions and the carbon dioxide formed was captured by and indicator solution that changes color proportionally to the amount of organic carbon originally present in the sample. Results were expressed in mg/L of carbon.

2.8 MoL immobilization in silica, clay and cellulose

Silica previously treated with (3-aminopropyl) triethoxysilane (APT-silane), 6 g, was added to 120 mL of APT-silane solution 10 % (p/v) in distilled water (pH 3.0) and mixture was agitated (2 h, 75 ºC). APT-silane solution was removed by centrifugation and distilled water washing (5 times). Silica was dried at 110 ºC during 17 h. Pretreated silica, clay and cellulose were activated with 2.5 % (v/v) glutaraldehyde solution in 5 mM sodium phosphate buffer (pH 7.0) and incubated during 1 h at room temperature; glutaraldehyde was removed and supports were washed with the buffer. MoL immobilization (1.9 mg/L) was performed with 50 mg of activated supports mixed with 1.5 mL of 5 mM sodium phosphate buffer (pH 7.0) for 17 h at 4 ºC. Supports were washed with the buffer (4 times) and 2 M urea (once). Supernatant was removed, volume was measured and protein was determined by Lowry.

2.9 Lectin immobilization in sepharose and agarose

Cyanogen bromide-activated sepharose 4B (0.25 g) and cyanogen bromide-activated agarose (0.25 g) were used for MoL immobilization [17]. Supports were washed with 0.5 M NaCl, pH 2.5, followed by 0.1 M NaHCO$_3$/0.5 M NaCl, pH 8.2. Incubation (24 h, 4 C) was performed with MoL (0.6 mg/L). After filtration and washing with NaHCO$_3$ solution, ethanolamine was added to a final concentration of 1 M.

2.10 Removal of humic acid from water in a packed bed column

A humic acid solution of 20 mg/L of carbon was applied to 1 mL column (0.68 x 4 cm) containing MoL immobilized in sepharose at a flow rate of 12 mL/h. The assay was carried out at room temperature and 5 mL samples were collected at 20 min intervals. TOC and protein were determined.

3. Results and discussion

The HA were normalized by the amount of protein used in each assay (specific hemagglutinating activity, SHA), detected in E (SHA: 825.1), 0-60F (SHA: 9351.6) and MoL (SHA: 3969.0) obtained from *M. oleifera*

seeds (figure 1A); in the presence of humic acid, SHA decreased by 94 % for both E (SHA: 51.5) and MoL (SHA: 248.0) and by 50 % for 0-60F (SHA: 4675.8).

Carbohydrates are present in surface water originating from the degradation of organic compounds namely, lignin, cellulose, hemicellulose and proteins [18]. Inhibition hemagglutinating activity assays were carried out in the presence of carbohydrates to assess their potential to interfere with humic acid binding to the MoL. D(+)-Galactose (0.2 M) and L(+)-arabinose (0.0125 M) reduced MoL SHA from 3969 to 496 (87.5 %). This result might be explained by the fact that D(+)-galactose and L(+)-arabinose compete with the carbohydrates on the surface of the erythrocytes to bind the MoL. Reduction in the percentage of SHA in the presence of humic acid was slightly higher than that obtained with the aforementioned carbohydrates, 94 % and 87.5 %, respectively. Nevertheless, considering that about 75 % of the dissolved organic carbon in rivers consists of humic substances [19] competition constitutes a minor problem for a future application of lectins to water treatment. D(+)-Glucose and D(-)-galacturonic acid had no effect on MoL HA at all tested concentrations.

Haloacetic acids and trihalomethanes are disinfection by-products formed in the reaction of chlorine with natural organic matter present in water and have adverse environmental and health effects [13]. Trichloroacetic acid, dichloroacetic acid, and chloroform had no effect in MoL activity under assayed concentrations.

The SHA of the MoL assessed in the presence of the humic acid decreased by 50 % with an increase in pH from 7.5 to 8.0. A further increase in pH in the range of 8.0 to 10 had no effect on MoL SHA. The later observation can be explained by assuming that pH increases the solubility of humic acid and thus its capability to compete with MoL for the carbohydrates on the surface of the erythrocytes.

The effect of mono- and divalent-cation in the SHA of MoL in the presence of humic acid is depicted in Figure 1B. The experimental results showed that K^+ (5 mM and 10 mM KCl) enhanced the interaction MoL-humic acid, since SHA was lower than the one determined in absence of potassium and magnesium, 124 and 248, respectively. An opposite result was obtained with Ca^{2+} (5 mM and 10 mM $CaCl_2$) that increased SHA to 992; calcium may decrease the availability of humic acid to compete with MoL for carbohydrates on erythrocyte's surface. Zhou *et al.* [20] suggested that calcium is able to form a metal bridge or salt linkage among carboxyl groups of humic acids which result in formation of macromolecules and might have a lower ability to bind carbohydrates than single humic acid molecules.
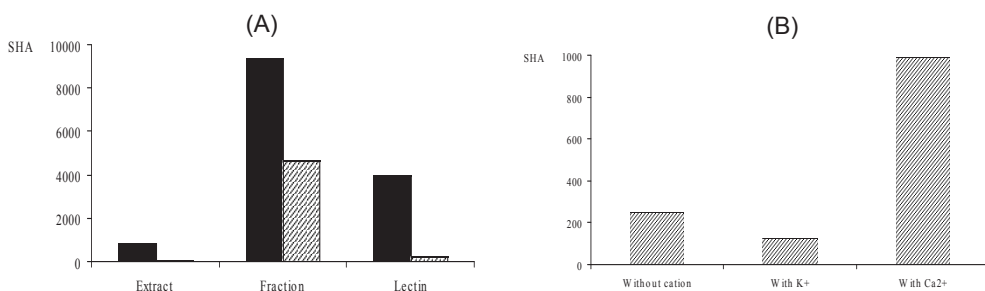
Figure 1. Specific hemmaglutinating activity (SHA) of E, 0-60F and MoL without (black bars) and with (black and white bars) humic acid (A) and MoL SHA plus humic acid (B) without and with cations (10 mM $CaCl_2$ or 5 mM and 10 mM KCl_2). SHA was determined dividing HA by protein concentration. Values represent the mean of three assays (± standard deviation): significant differences between groups were determined at ñ < 0.05.

The single radial diffusion gel showed precipitation bands indicating that E, 0-60F and MoL did bind to humic acid (100 mg/L and 200 mg/L of carbon); results are in agreement with those obtained in HA

(Figure 2). Precipitation bands were also observed with azocasein, a glycoprotein that completely inhibited MoL HA. These results suggest that affinity of humic acid functional groups might not be related to MoL azocasein binding sites, which are occupied by this glycoprotein.
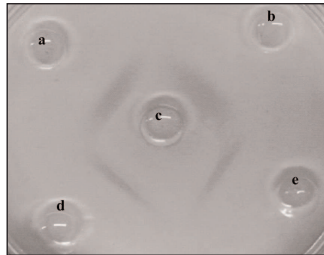
Figure 2. Precipitation bands observed in agarose gel corresponding to interaction among humic acid (c) and E (a), 0-60F (b), as well as MoL (d, e).

Immobilization of lectins in a matrix generates a new surface from the combination of physical and chemical properties of lectin and carrier. In ideal cases, surfaces with hydrophilic character (free of hydrophobic binding sites) are considered optimal to minimize non-specific interactions with protein samples and also to maximize stability. Another consideration is the type of linkers to be used to attach lectins to surface of stationary phase. Good matrices allow immobilized lectins to act in a similar way to that in nature, to recognize and subsequently to bind biomolecules of interest without steric hindrance [21]. Most reactive groups used for immobilizing proteins (glutaraldehyde, cyanogen bromide, etc.) are able to yield very stable enzyme-support bonds under mild immobilization conditions, such as neutral pH values [22]. The support with the highest amount of MoL immobilized was sepharose, 2.4 mg/g, followed by agarose, 0.2 mg/g, expressed as mass of protein per mass of support. MoL was not immobilized in silica, clay or cellulose. Figure 3 presents the breakthrough curve of a humic acid in solution (20 mg/L of carbon) obtained in a column containing immobilized MoL on sepharose. The considerable removal of humic acid (30.4 mg/g, expressed as mass of humic acid per mass of support) in the column indicates that the proposed process might be an interesting alternative to the existing ones.
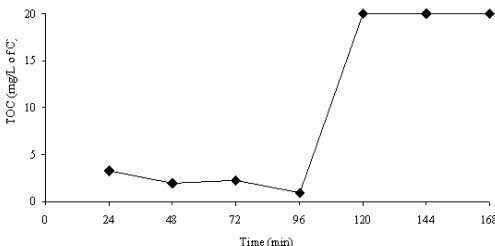
Figure 3. Removal of humic acid by *M. oleifera* lectin immobilized in sepharose.

In this context, immobilized lectin from *M. oleifera* seeds can be used for humic acid removal in water treatment and the immobilization assays with the other preparations (extract and fraction) are being conducted in our laboratory for the production of a filter to be used in a larger scale.

4. Conclusions

With this study, we can conclude that *M. oleifera* lectin preparations showed affinity with humic acid. The immobilization stage can be limiting and new methodologies will be tested. The assays with lectin immobilized in a packed bed column removed humic acid (30.4 mg/g, expressed as mass of humic acid per mass of support). This feature could be explored in the field of water treatment with perspectives to produce a filter for water purification with capacity to remove humic acids.

Acknowledgements

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Characterization of Galactomannan Edible Films to Extend Shelf Life of Fruits

ABSTRACT

Edible films and coatings are of interest since they have potential to improve food shelf life and quality and protect food from deterioration by microorganisms and physical damage. However, lack of edible film or coating properties data has limited their use in food applications. Barrier and mechanical properties of edible films or coatings strongly depend on the nature of the materials, extraction process, manufacture of the films and their final compositions. The objectives of this study were to i) characterise the mechanical and barrier properties of galactomannan based edible films as a function of galactomannan and glycerol concentration, ii) evaluate the influence of temperature and relative humidity on the film properties and iii) evaluate film performance to extend the shelf life of fresh cut packed Royal Gala apples. A response surface methodology was applied to determine the formulation, the mechanical and barrier properties and the environmental conditions that can affect film performance. It was found that films with high tensile, elongation and puncture strengths were obtained at higher galactomannan concentration and lower glycerol concentration. Thickness and tear strength increased with galactomannan and glycerol content. Galactomannan and glycerol concentrations influence significantly Water Vapour Permeability (WVP), Oxygen Permeability (OP), Water Uptake (WU) and Moisture Content (MC). The results indicated that glycerol played a major role on the film's physical-chemicals properties showing a positive effect on the WU, MC and WVP, and negative effect on the OP. The temperature and relative humidity showed a positive highly significant effect on WVP and OP. Apples packed using edible film showed a decrease in weight loss and a delay in the onset of decay as shown by lower colour, pH and moisture content changes during storage of the fruit packed under the conditions tested, confirming the potential application of edible films for extending the shelf life on packaged foods.

TESTIMONIAL

"I went to the largest and better experience in my life, professional, personal, cultural, because I learned innovative techniques used in my research field to whic I had not still had access. Personally I learned how to live independently. I met a new culture and a wonderful country."

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Characterization of Galactomannan Edible Films to Extend Shelf Life of Fruits

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Abstract

Edible films and coatings are of interest since they have potential to improve food shelf life and quality and protect food from deterioration by microorganisms and physical damage. However, lack of edible film or coating properties data has limited their use in food applications. Barrier and mechanical properties of edible films or coatings strongly depend on the nature of the materials, extraction process, manufacture of the films and their final compositions. The objectives of this study were to i) characterise the mechanical and barrier properties of galactomannan based edible films as a function of galactomannan and glycerol concentration, ii) evaluate the influence of temperature and relative humidity on the film properties and iii) evaluate film performance to extend the shelf life of fresh cut packed Royal Gala apples. A response surface methodology was applied to determine the formulation, the mechanical and barrier properties and the environmental conditions that can affect film performance. It was found that films with high tensile, elongation and puncture strengths were obtained at higher galactomannan concentration and lower glycerol concentration. Thickness and tear strength increased with galactomannan and glycerol content. Galactomannan and glycerol concentrations influence significantly Water Vapour Permeability (WVP), Oxygen Permeability (OP), Water Uptake (WU) and Moisture Content (MC). The results indicated that glycerol played a major role on the film's physical-chemicals properties showing a positive effect on the WU, MC and WVP, and negative effect on the OP. The temperature and relative humidity showed a positive highly significant effect on WVP and OP. Apples packed using edible film showed a decrease in weight loss and a delay in the onset of decay as shown by lower colour, pH and moisture content changes during storage of the fruit packed under the conditions tested, confirming the potential application of edible films for extending the shelf life on packaged foods.

Resumo

Filmes comestíveis e revestimentos são de grande interesse comercial, devido ao seu potencial em aumentar o tempo de prateleira dos alimentos sem alterar sua qualidade, protegendo-o da deterioração por microorganismo e danos físico-quimicos. Porém, a ausência de informações referente as propriedades desses materiais tem limitado seu uso. As propriedades mecânicas e de barreira de filmes comestíveis e revestimentos dependem fortemente da natureza dos materiais, processo de extraccao, fabrico dos filmes e suas composições finais. Os objetivos deste estudo eram i) caracterizar as propriedades de barreira e mecânicas de filmes comestíveis de galactomanana em função da concentração de galactomanana e glicerol, ii) avaliar a influência da temperatura e húmidade relativa nas propriedades dos filmes e iii) avaliar o desempenho dos filmes no empacotamento de maçã curtada do tipo Royal Gala com o objetivo de aumentar o tempo de prateleira dessa fruta. A metodologia de resposta de superfície foi aplicada para determinar a formulação, as propriedades de barreira, mecânicas e as condições de armazenamento que

podem afectar o desempenho dos filmes. Foi observado que os filmes com maior elasticidade, alongamento e resistência a perfuração apresentava a maior concentração de galactomanana e menor concentração de glicerol. Os maiores valores de espessura e a resistência ao corte foram obtidos com as maiores concentrações de galactomanana e glicerol. Galactomanana e glicerol influenciam significativamente na permeabilidade ao vapor d'água (WVP), permeabilidade ao oxigênio (OP), absorção de húmide (WU) e conteúdo de húmidade (MC). Os resultados indicaram que o glicerol tem um papel fundamental nas propriedades físico-químicas dos filmes com um efeito positivo no WU, MC e WVP, e efeito negativo no OP. A temperatura e húmidade relativa mostraram um efeito positivo e altamente significante em WVP e OP. Maçã curtada embalada com filme comestível mostrou diminuição na perda de peso e uma maior preservação das propriedades referentes a mudanças de cor, pH e húmidade nas condições testadas, confirmando dessa forma, o potencial dos filmes comestíveis de galactomanana para embalagem de frutas curtadas com vista ao aumento do tempo de prateleira desses alimentos.

1. Introduction

Edible or Biodegradable protein and polysaccharide films with satisfactory mechanical properties and good appearance are potential and ecological alternatives for substituting synthetic packaging in food and pharmaceutical applications [1]. The semi-permeable barrier provided by edible coating/film is aimed to extend shelf life by reducing moisture and solutes migration, gas exchange, respiration and oxidative reaction rates, as well as suppressing physiological disorders of fresh-cut fruits [2]. Edible coating/film acts as a barrier to gases, and generates a sort of modified atmosphere in each coated or packed fruit piece, which when combined with optimal relative humidity and refrigeration temperatures, helps to achieve reasonable shelf-life in fresh and fresh-cut fruits and vegetables. Edible coatings can be used to reduce the deleterious effect brought about by minimal processing, but shelf-life extension may require delay of respiration and physiological process. The main components of the edible coating/film are the plasticizer and film-forming polymer. The addition of a plasticizer agent to edible films is required to overcome film brittleness, caused by high intermolecular forces which react by increasing the mobility of polymer chains, thereby improving the overall flexibility and extensibility of the film. However, plasticizers also generally decrease the gas, water vapour and solute permeability of the film and can decrease elasticity and cohesion [3]. Thus, the edible coating/film have the ability to permeate the gas therefore showing great potential for modifying gas composition in packaging of fresh fruits and vegetables.

The coatings and the polysaccharides films do not have a greasy appearance, have a low caloric content and they can be used to increase the time of shelf of the fruits, vegetables, shellfish or meat products avoiding dehydration, oxidative rancidity and the darkening of the products surface. These coatings became popular in agriculture due to their high permeability to CO_2 and low permeability to O_2 since they allow the creation of modified atmospheres, an advantage over plastic which besides from being costly, is difficult to apply and harmful to the environment [4]

Polysaccharides are nontoxic and widely available. They also are selectively permeable to CO_2 and O_2, and hence, retard the respiration and ripening of many fruits and vegetables by limiting the availability of O_2. Hydrophilic films and coatings, such as polysaccharides, provide a good barrier to CO_2 and O_2 under certain conditions, but a poor barrier to water vapour [5, 6]. Hence, by limiting the availability of O2, they retard the respiration and ripening of many fruits and vegetables. The poor water vapour barrier property allows for the movement of water vapour across the film, thus, preventing water condensation that can be a potential source of microbial spoilage in horticultural commodities [7]. The aim of this study was to i) characterise the mechanical and barrier properties of galactomannan based edible films as a function of galactomannan and glycerol concentration, ii) evaluate the influence of temperature and relative humidi-

ty on the film properties and iii) evaluate film performance to extend the shelf life of fresh cut packed Royal Gala apples.

Galactomannans have been found in the seeds of more than ten plant species. They are present in the endosperm of numerous plants, particularly the Leguminosae, and they have several functions, e.g. as reserve of carbohydrates. Galactomannans are polysaccharides built up of a β-(1-4) mannan backbone with D-galactose branches linked α-(1-6). Their mannose/galactose ratios differ according to the species. They are water soluble hydrocolloids which form highly viscous, stable aqueous solutions. Due to their physicochemical properties, galactomannans are excellent stiffeners and stabilizers of emulsions, and their lack of toxicity allows their use in the textile, pharmaceutical, cosmetics and food industries. Most galactomannans used in pharmaceutical technology and cosmetics are usually unpurified gums.

2. Materials and Methods

2.1. Extraction of Galactomannan: Galactomannan (Gal) was obtained by solubilisation of seed endosperms of *Caesalpinia pulcherrima* in water for a few hours and followed by precipitation with ethanol 96% (v/v).

2.2. Method for the Preparation of Films: The solutions of Galactomannan-Glycerol (GalGly) were prepared following an experimental design with 2 variables (concentration of Gal and Gly) at 3 different levels. Films were dried in an oven at 50 ºC for 12 hours. The analysis of the influence of temperature and relative humidity on the film composition followed an experimental design with 3 variables (temperature, humidity and formulation of GalGly) at 3 different levels.

2.3. Analysis of Galactomannan Films: Films produced were analysed in terms of physical-chemical properties (water uptake, moisture content, water vapour permeability, oxygen permeability) and mechanical properties (thickness, tensile strength, elongation, tear strength and puncture strength). All tests were conducted at least in triplicate.

2.4. Performance analysis of edible film for packaging of fresh cut apples

> **2.4.1. Fruit:** Royal Gala apples (Malus domestica Borkh) were provided by Tesco Ireland and stored at 4 ± 1 ºC until processed.
> **2.4.2 Performance analysis of galactomannan edible film for packaging:** Sliced apples (150 g) were packed in plastic trays with and without galactomannan films. The packages were stored at 15ºC and 55% RH and quality parameters were analysed throughout time.
> **2.4.3. Quality evaluation:** The quality of packed sliced apples such as weight loss, colour, moisture content and pH were evaluated at least in triplicate throughout time.

2.5. Statistic Methods: The full factorial design experiments were carried out, and then all the data collected was analysed using Statistic software. (Version 7.1, Statsoft).
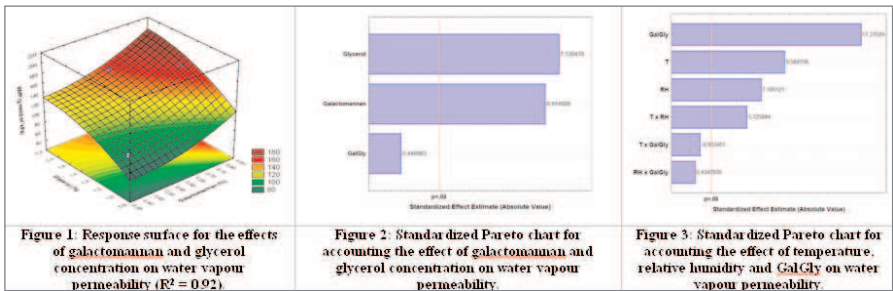
3. Results and Discussion
3.1. Physical-Chemical Properties

> **3.1.1. Water Uptake (WU) and Moisture Content (MC):** The WU and MC of the films varied significantly with different amounts of Gal and Gly. The Gal concentration had a significantly negative influence on WU and MC. The optimal composition (determined using res-
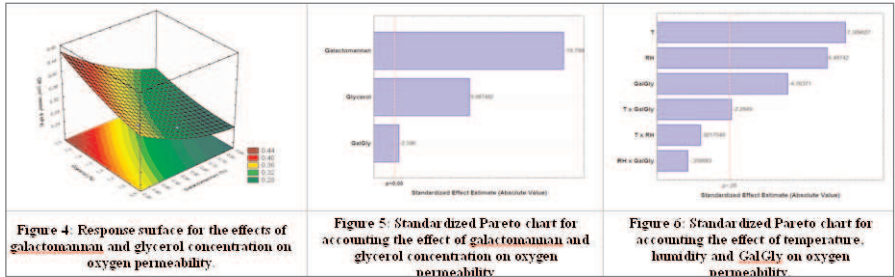
ponse surface methodology) than maximized and minimized WU was (0.4:2.0) and (0.5:1.0) for Gal and Gly, respectively. An increasing Gly concentration increased WU and MC (Fig. 1). This fact is probably due to the greater hygroscopic character of Gly, which has a higher affinity for binding water. The optimal composition that maximized MC at 73% was (0.4:2.0), and which minimized MC at 41% was (0.8:1.0) for Gal and Gly, respectively. Similar results were found by [8] who studied the variation of moisture content in films of β-lactoglobulin plasticized with different quantities of glycerol.

3.1.2. Water Vapour Permeability (WVP): The WVP increased significantly as the Gal and Gly concentration increased (Figure 1). Combining the two variables the lowest level of WVP (83 g mm/d m^2 kPa) was found when the Gal and Gly concentrations were 0.4% and 1.0%., was (0.4:2.0).The concentrations of Gal and Gly had a positive effect on WVP values of the film as can be seen from the Pareto chart (Figure 2). The model described the process adequately (R^2=0.92), showing a good agreement between experimental and predicted WVP. These results are in agreement with previous findings [9] which attributed the permeability increment to the plasticizing effect of glycerol, which reduces polymeric packaging density. On the other hand, the increased interactions between Gal molecules (hydrogen bonding type) increased with Gal concentration, therefore increasing the water vapour transmission rate. The effects of tempera-ture and humidity on WVP were quantified with a Pareto analysis at 95% significant level (Figure 3), showing that in the range of conditions studied, both temperature and humidity were the influential variables. The effect of temperature (T) on WVP was more pronounced than that of relative humidity (RH). The interactive effects between T and RH were also significant, with WVP, increasing with increasing T and RH.

Figure 1: Response surface for the effects of galactomannan and glycerol concentration on water vapour permeability (R^2 = 0.92).

Figure 2: Standardized Pareto chart for accounting the effect of galactomannan and glycerol concentration on water vapour permeability.

Figure 3: Standardized Pareto chart for accounting the effect of temperature, relative humidity and GalGly on water vapour permeability.

3.1.3. Oxygen Permeability (OP): Oxygen is the key factor that can cause oxidation, which initiates several food changes such as odour, colour, flavour and nutrient deterioration, so obtaining films with proper oxygen barriers can help improve food quality and extend food shelf life. OP is one of the most commonly studied transport properties of edible polymer films and the higher the OP, the lower is the OP barrier ability of the films. OP decreased with increase in Gal concentration and increased with increase Gly concentration (Figure 4). The low oxygen permeability at high Gal concentration was most probably due to the increased density of the film solution which resulted in larger film thickness and concomitant reduction in the intersti-tial spacing between the molecules of the cast polymer films. As a consequence, OP is lowered due to obstruction of oxygen molecules passing through the more closely packed polysaccha-ride network. Glycerol may compete with water for the active sites on the polymer, thus pro-

moting water clustering and increased free volume in the polymers at low moisture levels. Both Gal and Gly concentration and GalGly interaction has a significant effect on the OP of the edible films (Figure 5). Temperature (T) played a major role on the OP, having a positive effect as shown by the Pareto chart (Figure. 6). The temperature effect is observed due to thermal expansion of polymer materials [10], and permeating molecules also have an increased energy level with temperature.

Figure 4: Response surface for the effects of galactomannan and glycerol concentration on oxygen permeability.

Figure 5: Standardized Pareto chart for accounting the effect of galactomannan and glycerol concentration on oxygen permeability

Figure 6: Standardized Pareto chart for accounting the effect of temperature, humidity and GalGly on oxygen permeability

Relative humidity (RH) also had an important positive effect on the OP (Figure 6), which increased linearly with RH in the range of 50-95%. Hydrophilic polymers, which contain polar groups with hydrogen binding capacity, generally tend to strongly absorb water from humid environmental air. The effect of RH on OP has been previously observed for whey protein films [11], as well as for other biopolymer film types, including shellac [12], egg white protein [13], and casein [14]. Such effects of RH on OP values of the coated films are important for many practical applications.

3.2. Mechanical Properties

Mechanical properties reflect the ability of films and coatings to maintain a good integrity either as standalone films or applied as coatings to a food product. Polysaccharides (starch and others) are often used in industrial food. They produce films with good mechanical properties and coverings that are efficient barriers against low polarity compounds, however, they do not offer good barriers against humidity.

3.2.1. Thickness: The thickness of the films varied with Gal and Gly concentration and both factors showed a highly significant effect on thickness of the edible films (Figure 7a). The thickness of the film is at its greatest when Gal and Gly concentrations were increased.

3.2.2. Tensile Strength and Elongation: Tensile strength (TS) represents the maximum TS that can be sustained by a film, whereas Elogation (E) shows the ability of a film to stretch before it breaks. TS and E are the most commonly reported responses to describe mechanical properties of edible films and coatings. TS increased with an increase in Gal and decreased with Gly (Figure 7b), and both Gal and Gly have a statistically significant effect on TS. Elongation decreased with an increase in Gal concentration and increase with Gly (Figure 7c). TS decreased and E increased as Gly content increased;. since Gly is a plasticizer it reduces interactions between polymer chains, thereby reducing film strength and increasing flexibility [15].

3.2.3. Puncture Strength and Tear: Puncture strength (PS) is the force required to penetrate the film using a stainless steel point and the optimum variable levels should give the film the

highest PS possible. PS decreased with increasing Gly and increased significantly with increased Gal concentrations (Figure 7d), as Gly and Gal concentrations increased resulted in reduction of intermolecular interactions and increased of mobility of polymer chains. Tear increase significantly with Gal and Gly concentrations (Figure 7e). The decrease of PS and increase of T with increasing Gly content in hydrophilic films have been reported previously [16] and this behaviour could be related to the structural modifications of starch network when Gly was incorporated, as the film matrix became less dense and under stress, movements of polymer chains were facilitated.

Figure 7: Response surface for the effects of galactomannan and glycerol on the a) thickness (R2 = 0.95), b) tensile strength (R2 = 0.97), c) elongation (R2 = 0.95), d) puncture (R2 = 0.99) and e) tear (R2 = 0.93)
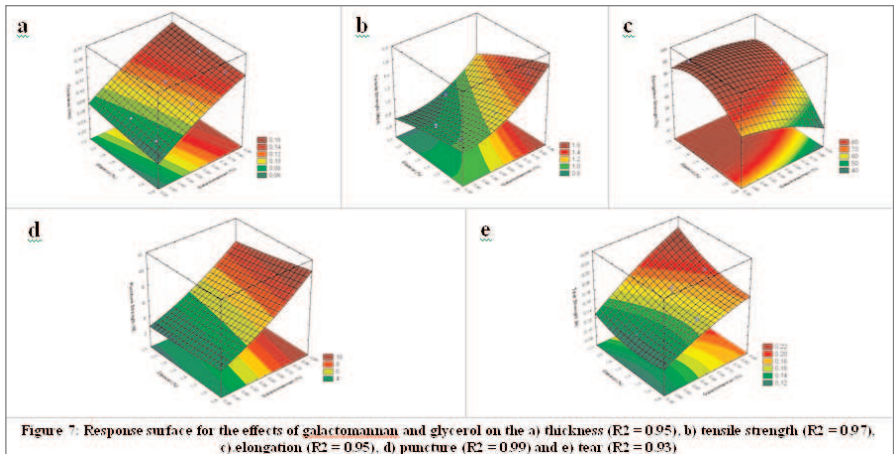
3.3. Performance analysis of galactomannan edible film for packaging

3.3.1. Colour changes: Colour is an important parameter as it determines consumer acceptance. The galactomannan films did not change the colour of the fruit, but it delayed browning. A significant difference was observed in the total colour change of the sliced apples with and without film, showing that the fruits packed with film presented a reduction in the variation of the colour (Figure 8a).

3.3.2. Weight loss: A large reduction in weight loss was observed in the apples packed with edible film. After 33 hours there was a loss of 2.32% in the apples with films and 12.52% in the apples without the film (Figure 8b). The reduction in weight loss can be attributed to the film used for sample storage, which created a saturated or nearly saturated atmosphere with regard to water vapour, and minimized water loss in spite of the increased transpiration rate through the peeled surface.

3.3.3. pH: The pH of the apple decreased with storage time, as shown in Figure 8c. However, pH did not change significantly during storage for both samples, probably due to the effect of the buffering capacity of the apple tissue. This stability of pH may have several positive implications: low activity of polyphenoloxidases in this range of pH, 3.5-3.7, and reduced microbiological development which would contribute to the preservation of the apple. It is also desirable from the sensorial point of view, since a variation in pH value would most certainly imply a negative change in flavour.

3.3.4. Moisture content: The moisture content decreased significantly in both samples, with the reduction being more significant in the sample without edible film (Figure 8d), probably due the high transpiration rate of the fruits.
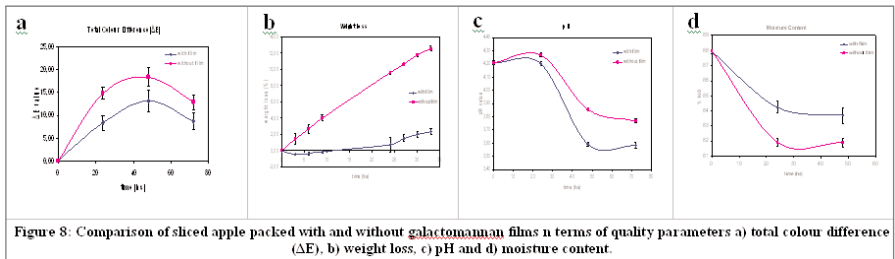
Figure 8: Comparison of sliced apple packed with and without galactomannan films n terms of quality parameters a) total colour difference (ΔE), b) weight loss, c) pH and d) moisture content.

Conclusions

A response surface methodology was applied to the results obtained in this work to understand the behaviour of edible films of Galactomannan and Glycerol in terms of better mechanical properties and lower water vapour and oxygen permeability. Predictive models indicated that increased glycerol exhibited good elongation and tear strength. This behaviour is adequate for films that require flexibility and resistance. However, high levels of glycerol increased oxygen permeability. Increased galactomannan concentration decreased oxygen permeability and increased water vapour permeability and puncture strength. Relative humidity and temperature also have significant influence on the barrier properties. In this way, the films consistency and its barrier properties can be controlled using the concentration of galactomannan and glycerol used in the formulation of the edible film, and combined with adequate conditions of temperature and relative humidity it is possible to obtain a film with good properties for application in fruits. Application of an edible film in apples proved to extend the shelf-life of cut apple by decreasing weight loss, and delaying the decay incidence changes in color, pH and moisture content during storage in the conditions tested, confirming the potential of edible packaging to become an integral part of food processing.

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CARLOS EDUARDO ALVES SOARES

Characterisation of corn starch-galactomannan films by adsorption isotherms

ABSTRACT

The aim of this work was to characterise different samples of edible films made of blend of galactomannan-corn starch through the behaviour of their water activity. A dispersion of corn starch was prepared and this dispersion was mixed with different parts of dispersion 1.0% of galactomannan from *Adenanthera pavonina* according to a designed experimental matrix. Glycerol (30% w/w) was used like a plasticizer. Different films encoded by different parts of polysaccharide dispersions were prepared. An equipment LabMaster-aw (Novasina, Switzerland) was used to perform all measurements of water activity. All data acquired were done at constant temperature of 25 °C. Adsorption curves were built with Microsoft Excel software and three sorption models were tested B.E.T., G.A.B. and Langmuir. All adsorption curves showed distinct plots. The water absorption in lowest values of aw was influenced by corn starch content. With the increasing of the parts of corn starch dispersion into galactomannan dispersion, it was possible to see the films had absorbed water in the lower values of aw (0.11, 0.33 and 0.53). An opposite behaviour was seen with decreasing of parts of corn starch dispersion. The best fitted model was Langmuir ($0.9704 < R^2 < 0.9992$) but the films fitted well with B.E.T. and G.A.B. models too ($0.9585 < R^2 < 0.9999$ and $0.9566 < R^2 < 0.9965$, respectively). The behaviour of water activity in the different samples of films made of blend of galactomannan-corn starch is an accurate parameter to evaluate the stability and characterize these films.

TESTIMONIAL

"I have got an excellent experience."
"My research many times was funny and constructive for me."
"The friends that I did in Spain changed the sad moments and make me strong to stay for a full year."

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Characterisation of corn starch-galactomannan films by adsorption isotherms

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Abstract

The aim of this work was to characterise different samples of edible films made of blend of galactomannan-corn starch through the behaviour of their water activity. A dispersion of corn starch was prepared and this dispersion was mixed with different parts of dispersion 1.0% of galactomannan from *Adenanthera pavonina* according to a designed experimental matrix. Glycerol (30% w/w) was used like a plasticizer. Different films encoded by different parts of polysaccharide dispersions were prepared. An equipment LabMaster-aw (Novasina, Switzerland) was used to perform all measurements of water activity. All data acquired were done at constant temperature of 25 ºC. Adsorption curves were built with Microsoft Excel software and three sorption models were tested B.E.T., G.A.B. and Langmuir. All adsorption curves showed distinct plots. The water absorption in lowest values of aw was influenced by corn starch content. With the increasing of the parts of corn starch dispersion into galactomannan dispersion, it was possible to see the films had absorbed water in the lower values of aw (0.11, 0.33 and 0.53). An opposite behaviour was seen with decreasing of parts of corn starch dispersion. The best fitted model was Langmuir (0.9704 $< R^2 <$ 0.9992) but the films fitted well with B.E.T. and G.A.B. models too (0.9585 $< R^2 <$ 0.9999 and 0.9566 $< R^2 <$ 0.9965, respectively). The behaviour of water activity in the different samples of films made of blend of galactomannan-corn starch is an accurate parameter to evaluate the stability and characterize these films.

Keywords: adsorption isotherms, galactomannan, water activity, edible films

Resumen

El objetivo de este trabajo fue caracterizar diferentes películas comestibles hechas de mezcla de galactomanano y almidón de maíz a través de su comportamiento de actividad de agua. A la dispersión de almidón de maíz, diferentes partes de una dispersión 1,0% de galactomanano de *Adenanthera pavonina* fueron mezcladas de acuerdo con una matriz experimental diseñada. Glicerol (30% w/w) se utilizó como plastificante. Distintas películas codificadas por diferentes partes de las dispersiones fueron hechas. Un equipo LabMaster-aw (Novasina, Suiza) fue utilizado para realizar todas las mediciones de actividad de agua a la temperatura constante de 25º C. Curvas de adsorción se construyeron con el software Microsoft Excel y tres modelos de sorción se probaron BET, GAB y Langmuir. Todas las curvas de adsorción se mostraron distintas. Con el aumento de las partes del almidón de maíz en la dispersión de galactomanano fue posible ver que las películas habían absorbido el agua en la parte baja de los valores de aw (0,11, 0,33 y 0,53). Un comportamiento contrario se observó con la disminución de partes de la dispersión de almidón de maíz. Lo mejor modelo fue instalado Langmuir (0.9704 $< R^2 <$ 0.9992) pero las películas encajaban

bien en los modelos BET y G.A.B. ($0.9585 < R^2 < 0.9999$ and $0.9566 < R^2 < 0.9965$, respectivamente). El comportamiento de la actividad de agua en las diferentes muestras de películas hechas de mezcla de galactomanano y almidón de maíz es un parámetro exacto para evaluar y caracterizar la estabilidad de estas películas.

Palavras claves: isotermas de adsorción, galactomanano, actividad de agua, envases comestibles

1. Introduction

Packaging is an important sector of the world industry. This sector represents about 2% of the Gross National Product of the developed countries. Nowadays packaging industry also presents continuous growth in importance and size. Preservation processes of foods still depend on effective packaging. So thanks to modified-atmosphere packaging a lot of products can be sold fresh or only chilled. In fact, food packaging has developed a lot due to increased demand on self-life extension, product safety, cost-efficiency and consumers convenience [1].

Edible films and coatings are an interesting alternative to increase the shelf-life of food in spite of packages made of petroleum derivates. Edible films and coatings are capable of providing functional advantages like modified and controlled-atmosphere, being carriers of antimicrobials, antioxidants or other preservatives, retaining the volatile flavour and they can also decrease packaging wastage associated with processed foods [1,2].

The concept about water activity was used for the first time to show that microorganisms have a limiting level of water activity for growth and development. Nowadays this concept gains high importance and it generally relates closely with microbial, chemical and physical properties of food and foodstuff or others products that water is in their moisture content [3].The role of water activity in foods relates with food stability and it can be described to a significant because the water contributes physical and chemical in deteriorative reactions. This concept is important, useful and commonly used to relate food safety and quality. One the methods more effective to preservation of food is control the aw against microbial growth and chemical deterioration [4].

The relation between moisture content of food/foodstuff and water activity relates with atmosphere equilibrium between sample and salt saturated solution. Thus, it is possible the representation of this equilibrium relationship by the moisture sorption isotherms. To understand the water relationships in a food system, it is important determine aw levels corresponding to the range of water content to which the food product may be subject [1]. The range from 0,2 to 0,3 of aw in which the monolayer moisture (m_o) represents the optimal moisture content region where dehydrated foods have a maximum shelf-life is many critical for physical and chemical reactions. Above the monolayer, chemical reactions that require a water phase begin, while above and below the region, the rate of oxidation of lipids increases compromising shelf-life. Another range where physical state changes begin, such as loss of crispiness, stickiness of powders and hard candies, followed by the recrystallization of amorphous state sugars causing irreversible caking is from 0,35 to 0,45. These physical state changes are also controlled by the Tg which can more accurately define the critical moisture content where such changes begin [2,5].

The polysaccharides consist of long chains containing hundreds or thousands of units' monosaccharides. The polysaccharides are most abundant starch and cellulose, synthesized by plants, consisting of recurring units of D-glucose, but which differ in the type of connection glucosidic. They are also very important as hemicelluloses, components of the cell wall of plants that besides structural function may also play a role in protection against water stress [6]. The most cited polysaccharides in the literature to do edible films are starch, particularly amylose, chitosan and galactomannans [4,6].

The aim of this work was characterize edible films galactomannan (GLM) and corn starch amylose (HACS) based by the technique of water activity measurement. Plots of sorption isotherms were made and the data were adjusted to three mathematical models.

2. Development

2.1. Extraction of the galactomannan polysaccharide

Seeds from *Adenanthera pavonina* L. were collected in Fortaleza-Ceará, Brazil. One hundred grams of seeds were boiling for 20 minutes. After that, the seeds stayed 24 hours in distilled water for imbibition. Testa and cotyledons were removed manually and only the endosperms were used. About 500 mL of distilled water were mixed with endosperms to obtain a viscous liquid. This liquid was added to ethanol 95º (1:2, v/v, respectively) to precipitate the soluble polysaccharides. The precipitate was washed with acetone and dry with vacuum desiccators.

2.2. Preparation of formed film dispersion and edible films

Corn starch-galactomannan blends were prepared**.** A dispersion of corn starch (HACS) was prepared according to Bertuzzi *et al.* [7]. This dispersion was mixed with solution of galactomannan (GLM) 1.0% (w/w) in different proportions according to an experimental matrix presented below (TABLE 1). A proportion of 3 parts of HACS dispersion and 7 parts of GLM dispersion was chose such as matrix centre because presented more time resistance in agar plates according to gravimetric test presented in the first report. This film was coded like film 7.0-3.0. Glycerol was added as a plasticizer (30%, w/w) for both dispersions, HACS and GLM.

2.3. Water activity (aw)

2.3.1. Conditions of drying of sample films and aw measurement

An equipment LabMaster-aw (Novasina, Switzerland) was used to perform all measurements of water activity according to the manufacture instructions. The standard salts used were 0.11, 0.33, 0.53, 0.75, 0.90 and 0.97 aw [8]. All data acquired were done at constant temperature of 25 ºC. The weight of samples before and after equilibrium in the measurement chamber of the equipment was registered with a precision balance Sartorius model 2462 (±0.0001 g).

2.3.2. Moisture sorption isotherms

2.3.2.1. Adsorption and desorption isotherms

Only for the film 7.0-3.0, both adsorption and desorption isotherms were plotted. Six points of aw were measured by equilibrium of salt saturated solutions with the film samples. Adsorption isotherms were plotted for defining the best treatment of drying to all formed films and the film 7.0-3.0 was used as experimental model. Three replicates were done by film.

2.3.2.2. Modelling of moisture sorption isotherms

Adsorption and desorption curves for all samples were plotted with Microsoft Excel software. Three mathematical models of sorption were used with the experimental matrix films (B.E.T., G.A.B. and Langmuir).

Table 1 – Experimental matrix of different contributions of formed film dispersions of corn starch and galactomannan. The GLM 0,000 and HACS 0,000 coded values represent the centre of matrix.

| Coded values | | Natural values | | |
GLM*	HACS**	GLM	HACS	FV***
1,000	1,000	6,667	3,333	10,000
1,000	-1,000	9,091	0,909	10,000
-1,000	1,000	4,444	5,556	10,000
-1,000	-1,000	8,000	2,000	10,000
-1,267	0,000	8,726	1,274	10,000
1,267	0,000	6,612	3,388	10,000
0,000	-1,267	7,000	3,000	10,000
0,000	1,267	7,000	3,000	10,000
0,000	**0,000**	**7,000**	**3,000**	**10,000**
0,000	**0,000**	**7,000**	**3,000**	**10,000**
0,000	**0,000**	**7,000**	**3,000**	**10,000**
0,000	**0,000**	**7,000**	**3,000**	**10,000**

*Galactomannan. ** High amylose corn starch. *** Final volume of the blend.

2.4. Results and discussion

Films made of blend galactomannan and amylose were found to be easy to handle. Glycerol was used like a plasticizer and these films showed no stickiness. Talja et al. [9] worked with potato starch films plasticized with binary mixtures of polyols. The concentration of plasticizers was of 20, 30, 40 and 50%. Concentrations up to 40% of binary mixtures of polyols produced films handle.

All adsorption curves showed distinct plots (Figure 1 A). The water absorption in lowest values of aw was influenced by corn starch content. With the increasing of the parts of corn starch dispersion into galactomannan dispersion, it was possible to see the films had absorbed water in the lower values of aw (0.11, 0.33 and 0.53). An opposite behaviour was seen with decreasing of parts of corn starch dispersion. According to table 2, the best fitted model was Langmuir ($0.9704 < R^2 < 0.9992$) but the films fitted well with B.E.T. and G.A.B. models too ($0.9585 < R^2 < 0.9999$ and $0.9566 < R^2 < 0.9965$, respectively). The behaviour of water activity in the different samples of films made of blend of galactomannan-corn starch is an accurate parameter to evaluate the stability and characterize these films.

The figure 1 B shows adsorption and desorption isotherms plot from the film 7.0-3.0. The hysteresis phenomenon is noticed because the curves did not overlay. In general, the moisture content of any sample will be greater for desorption than adsorption at any aw given [4]. The best fit was Langmuir model and the range of R^2 was between 0.97 and 0.99, such as observed for the films of experimental matrix.
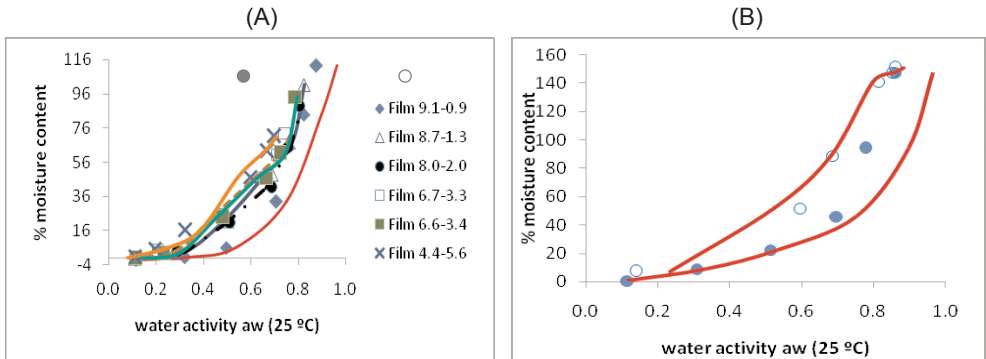
Figure 1 – Adsorption isotherm plots for all edible films from experimental matrix (A). The continuous lines represent Langmuir model. With the increasing of HACS content it is possible to notice increase in the aw and water gain values for those films. Adsorption ( ) and desorption ( ) isotherms plot from the film 7.0-3.0 (B). The red continuous line represents Langmuir model. Each plot represent one experiment at least three replicates.

Sample coded film	Models									
	B.E.T.*			G.A.B.				Langmuir		
	C	Xm	R^2	K	Xm	C	R^2	K	C	R^2
7.0-3.0	4.85	0.287	0.9991	0.105	2.869	3.25	0.9965	32.60	0.031	0.9956
	3.72	0.298	0.9975	0.104	2.851	3.23	0.9890	5.77	0.176	0.9900
	4.32	0.292	0.9997	0.104	2.906	3.17	0.9925	5.55	0.185	0.9938
	3.83	0.298	0.9994	0.104	2.830	3.25	0.9931	4.40	0.243	0.9953
9.1-0.9	5.86	0.273	0.9926	0.524	1.771	1.58	0.9940	35.49	0.028	0.9992
8.7-1.3	4.06	0.285	0.9989	0.102	2.884	3.19	0.9928	0.18	5.540	0.9938
8.0-2.0	4.21	0.291	0.9999	0.103	2.714	3.38	0.9916	0.28	3.749	0.9946
6.7-3.3	2.38	0.300	0.9585	0.469	2.220	1.74	0.9566	6.79	0.131	0.9716
6.6-3.4	3.07	0.286	0.9812	0.096	2.922	3.17	0.9834	0.11	7.981	0.9838
4.4-5.6	11.23	0.162	0.9931	0.365	25.849	39.68	0.9723	160.53	0.004	0.9704

Table 2 – Values of constants (K, Xm and C) and R^2 of water activity measurement for edible films fitted with three mathematical models in the range between 0, 11 and 0, 97.

3. Conclusions

The HACS content contributes to increase the values of aw and the moisture content of films blend corn starch GLM-based. It was possible noticed that the water activity was a useful parameter to characterize the edible films produced.

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MICHELE SOLAROLI

Production and Purification of Protease Produced by *Penicillium aurantiogriseum*

ABSTRACT

Submerged culture (SmC) is the process employed to produce commercially the tannase enzyme, however in this culture system, tannase is mainly expressed in intracellular manner representing high costs of production. Solid-state culture (SSC) is a process reported as good alternative to produce extracellularly the enzyme. To demonstrate that fungal tannase excretion is strongly influenced by the culture conditions, in this PhD study the tannase production by SmC and SSC using different bioreactors was evaluated. In this report, we present the obtained results for the tannase production by SmC. *Aspergillus niger* GH1 was firstly re-purified and conserved before the initial fermentations in order to assure the pure culture. Two sets of experiments were conducted using in a fist step a 1.5 L bioreactor and in a second step 100 mL Erlenmeyer flasks. Modifications to culture conditions previously reported, allowed to obtain positive results for the tannase production in this SmC, the culture conditions were: initial pH 5, Temperature 35°C, Substrate concentration 25g/L and agitation 200 rpm.

TESTIMONIAL

"I think it has been a life experience. I learnt, I meet people and I saw thing that I will never forget. Saudade do Brasil."

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Production and Purification of Protease Produced by *Penicillium aurantiogriseum*

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Abstract

In this study, a protease from *Penicillium aurantiogriseum* has been produced in a soy medium MS during 86 h at 28 °C in Erlenmeyer flasks. The supernatant (crude extract) obtained, that presented a protein concentration of 0.04 mg/ml and an activity of 4.07 U/ml, was subjected to further manipulations. At first, the ATPS technique with a polyethylene glycol (PEG) and cytrate system was applied in order to pre-purify the protease. A 2(4)-experimental design was used to evaluate the influence of PEG molar mass and concentration, citrate concentration and pH on protease partition coefficient, purification factor and protease purification yield. It has been found that the use of lowest concentration of M_{PEG}, C_C and C_{PEG} allowed the best value of purification (1.62). In a second time, since a good degree of purity is required for characterization assays, proteins produced by *P. aurantiogriseum* and recovered from cultures media have been purified through different chromatographic techniques (hydrophobic chromatography, gel filtration) and various treatments (ammonium sulfate fractionation). The results on the whole shows that the used techniques are not sufficiently selective to provide total enzyme purification. In conclusion, in order to reach our goal, the purification and the characterization of the protease produced by *P. aurantiogriseum*, will be necessary planning new purifications strategies.

Resumen

En este trabajo, una proteasa del *Penicillium aurantiogriseum* ha sido producida en un medio de soya MS durante 86 h a 28 °C en Erlenmeyers. El sobrenadante (extracto crudo) obtenido, que presentaba una concentración proteica de 0.04 mg/ml y una actividad de 4.07 U/ml, fue manipulado con varias técnicas. En principio, se usó la técnica del ATPS con un sistema de polyethylene glycol (PEG) e de citrato para una pre-purificación de la enzima. Se utilizó un diseño experimental para evaluar la influencia de la masa molar y concentración del PEG, la concentración del citrato y del pH sobre el coeficiente de partición de la proteasa, el factor de purificación y el rendimiento de la purificación de las proteinas. Se ha encontrado que el uso de concentración más baja de M_{PEG}, C_C y C_{PEG} permitio el mejor valor de purificación (1,62). A continuación, ya que un buen grado de pureza es necesario para los ensayos de caracterización, las proteínas producidas por *P. aurantiogriseum* y recuperadas desde el medio de cultivo se han purificado a través de diferentes técnicas cromatográficas (cromatografía hidrofóbica, gel de filtración) y diversos tratamientos (fraccionamiento con sulfato de amonio). En el conjunto, los resultados muestran que las técnicas utilizadas no son lo suficientemente selectivas para proporcionar la total purificación de la enzima. En conclusión, con el fin de alcanzar nuestro objetivo, o sea la purificación y la caracterización de la proteasa producido por *P. aurantiogriseum*, será necesario planificar nuevas estrategias de purificación.

Introduction

Proteases represent the class of enzymes which occupy a pivotal position with respect to their physiological roles as well as their commercial applications. They perform both degradative and synthetic functions. Since they are physiologically necessary for living organisms, proteases occur ubiquitously in a wide diversity of sources such as plants, animals, and microorganisms. Despite the diversity of microorganisms' proteases producers, only some are deemed suitable for commercial producing. A large number of microorganisms such as bacteria, fungi and yeasts are producers of alkaline proteases [9]. Microbes are an attractive source of proteases owing to the limited space required for their cultivation and their ready susceptibility to genetic manipulation [1].

Moreover, the microbial proteases have a longer life and can be stored under ideal conditions for weeks without significant loss of their activity. In general the microbial proteases are extracellular ones, secreted directly in the culture medium by the producer, thereby simplifying the process of "dowstream" of the enzyme compared to the proteases obtained from plants and animals. Traditional methods to isolate and purify proteins involve several steps such as ammonium sulfate precipitation, ionic and affinity chromatography, dialysis and final concentration of the product, which are time and cost consuming, induce loss of their biological activity and reduce the yield of the whole process.

A possible way to extract proteases from fermented broths is to use aqueous two-phase system (ATPS), which are made up of two aqueous solutions of two water-soluble polymers or a polymer and a salt [10, 11] They have recently been used to separate biomolecules such as enzymes, other proteins and antibiotics, mainly because of the possibility of being tailored to a specific separation by varying their features and/or those of the extraction systems [15, 12, 8, 13, 14]. Their technical simplicity, easiness of scaling-up and suitability for continuous operation make this process a promising alternative for large-scale operation [2]. Proteases find extensive applications in the food and dairy industries. Microbial alkaline proteases dominate the worldwide enzyme market, accounting for a two-thirds share of the detergent industry. Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially [1]. The current estimated value of the worldwide sales of industrial enzymes is \$1 billion. Among industrial enzymes, 75% are hydrolytic. The proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sales of enzymes [3].

Results and Discussion

The fermentation of *P. aurantiogriseum* (URM- 4622) was effectuated in 3 different Erlenmeyer flasks (500ml) containing 100 ml of soy medium MS [4], during 86 h at 28 °C in Erlenmeyer flasks. The supernatant (crude extract) obtained presented a protein concentration of 0.04 mg/ml and an activity of 4.07 U/ml and was subjected to the further manipulations by ATPS. The results of protease partition coefficient as function of PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), citrate concentration (C_C) revealed them all to be significant, excepting pH results (Table 1 and Fig.1). A negative effect of M_{PEG} and a positive effect of C_C and C_{PEG} on the partition coefficient (K) were observed. About the yield (Y), concerning the top part of the system, it shows that C_{PEG} and C_C had a significant positive effect, while M_{PEG} had a negative effect. The use of highest C_C and C_{PEG} allowed excellent recovery, as demonstrated by Y values always higher than 100%. Yields above 100% are frequently reported for liquid-liquid extraction of enzymes. According to [8], this could be the result of removal of inhibitors from PEG phase during extraction. The yield concerning the bottom phase shows the importance of the M_{PEG} and C_C that present a positive algebrical sign and of C_{PEG} and the interaction between M_{PEG} and C_C that have a negative sign. The Purification Factor (PF) of the top part of the system (PF_t), indicates that a reduction of M_{PEG} is likely to
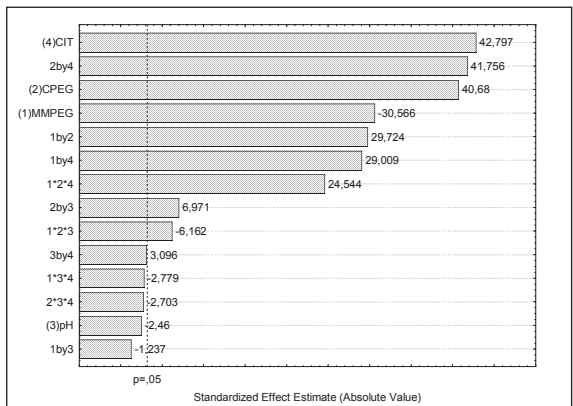
improve the purification (Fig.2). The use of lowest concentration of M_{PEG}, C_C and C_{PEG} allowed the best value of purification (1.62). Concerning the PF of the bottom phase (PF_b) the statistical analysis reveal that only the C_{PEG} had a significant effect.
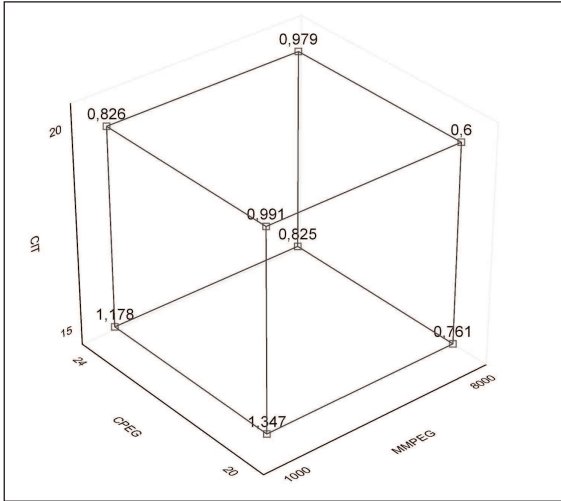
Table 1 Experimental schedule and results of protease removal from *P. aurantiogriseum* fermentation broth by ATPS

Run	M_{PEG}	C_{PEG}	pH	CIT	K_p	K_a	Y_t	Y_b	PF_t	PF_b
1	1000	20	6	15	0.838	1.012	224.5	164.1	**1.62**	1.34
2	8000	20	6	15	0.879	0.533	118.7	255.5	0.94	1.56
3	1000	24	6	15	0.791	0.867	208.9	156.2	1.34	1.22
4	8000	24	6	15	0.792	0.535	122.3	208.4	0.90	1.33
5	1000	20	8	15	0.786	0.862	181.1	175.2	1.35	1.23
6	8000	20	8	15	0.915	0.481	110.7	251.6	0.86	**1.63**
7	1000	24	8	15	0.796	0.924	225.2	132.6	1.30	1.12
8	8000	24	8	15	0.703	0.533	136.0	238.4	1.03	1.36
9	1000	20	6	20	0.879	0.934	195.9	186.4	1.40	1.31
10	8000	20	6	20	0.831	0.547	101.8	265.7	0.95	1.44
11	1000	24	6	20	1.261	1.050	220.3	178.5	0.92	1.11
12	8000	24	6	20	1.536	1.470	208.2	210.3	1.09	1.14
13	1000	20	8	20	1.262	0.894	166.1	251.0	0.86	1.22
14	8000	20	8	20	1.362	0.539	95.9	277.9	0.53	1.34
15	1000	24	8	20	1.196	1.152	226.1	187.1	1.01	1.04
16	8000	24	8	20	1.452	1.433	244.1	201.7	1.14	1.16
17 (C)	3350	22	7	17.5	1.490	0.476	97.8	166.8	0.38	1.18
18 (C)	3350	22	7	17.5	1.384	0.496	92.6	186.7	0.38	1.05
19 (C)	3350	22	7	17.5	1.623	0.493	91.7	181.6	0.40	1.32
20 (C)	3350	22	7	17.5	1.386	0.469	87.3	190.6	0.37	1.08

M_{PEG}: PEG molar mass; C_{PEG}: PEG concentration; C_C: Citrate concentration; Kp: Protein partition coefficient; Ka: Activity partition coefficient; Yt: Protease purification yield referred to the top phase; Yb: Protease purification yield referred to the bottom phase; PFt: Purification factor referred to the top phase; PFb: Purification factor referred to the bottom phase; (C): Central point.

Fig.1: Contrast values calculated for the Ka. Pure Error = 0.0001765, (M_{PEG}: PEG molar mass, C_{PEG}: PEG concentration, CIT: Citrate concentration) Ka: Activity partition coefficient

(4)CIT — 42,797
2by4 — 41,756
(2)CPEG — 40,68
(1)MMPEG — -30,566
1by2 — 29,724
1by4 — 29,009
1*2*4 — 24,544
2by3 — 6,971
1*2*3 — -6,162
3by4 — 3,096
1*3*4 — -2,779
2*3*4 — -2,703
(3)pH — 2,46
1by3 — -1,237

p=,05

Standardized Effect Estimate (Absolute Value)

Fig.2: Cubic plot of the PFt coefficient values. Pure Error = 0.0002474(MM$_{PEG}$:PEGmolar mass, C$_{PEG}$: PEG concentration, CIT: Citrate concentration). PFt: Purification factor referred to the top phase.

Our goal is the purification and the characterization of the protease produced by *P. aurantiogriseum*, with a view to its biotechnological application.

Since a good degree of purity is required for characterization assays, proteins produced by *P. aurantiogriseum* and recovered from cultures media have been purified through different chromatographic techniques (hydrophobic chromatography, gel filtration, ATPS) and various treatments (ammonium sulfate fractionation). Before passing the sample through the purifier system (FPLC), the crude extract was fractionated by the addition of ammonium sulfate in order to obtain the 40-80% fraction, that in the precedents experiments results the best in terms of purification factor (4,57) and specific activity (465 U ml^{-1}).

This fraction was dialyzed with equilibrium column buffer to wash away the high concentration salt and successively lyophilized. The lyophilized sample was resuspended in the desired loading volume (0,5 ml). At first, the sample was loaded into hydrophobic column (Fig. 3).
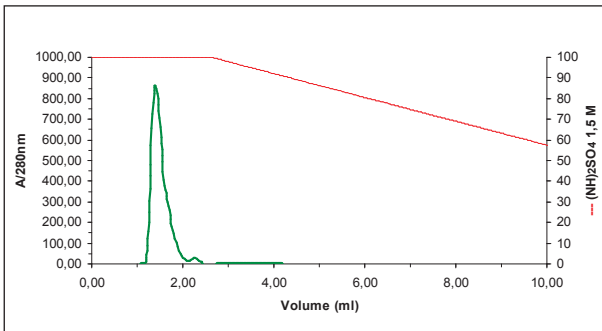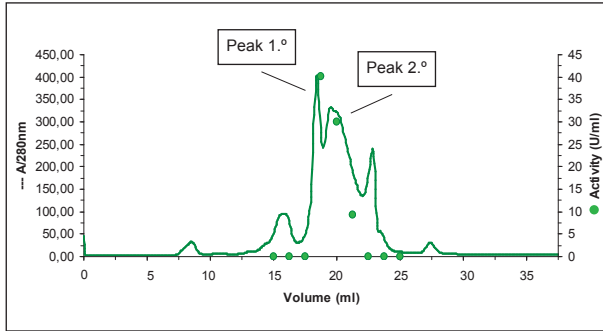

Sample	Total Actvity (U)	Yield U(%)
Crude Extract	360,0	100,0
RESOURCE™ 15ISO		
N.R.	234,4	65,0

Table 2: Yield obtained in relation of the enzymatic activity of the different fraction of the hydrophobic chromatography.

Fig.3: Elution profile by the hydrophobic column. Column type: RESOURCE™ 15ISO (isopropil) equilibrated in 50 mM phosphate buffer, containing 1,5 M $(NH_4)_2SO_4$ pH 7 (Buffer B). Sample: 40-80% $(NH_4)_2SO_4$ fraction of the crude extract. Gradient: 100-0% of 1,5 M $(NH_4)_2SO_4$ in 20 beds column volume. Flow rate: 1 ml/min. Fraction volume: 1 ml.

It's possible to observe that the whole peak protein is directly eluted in the equilibrium buffer. It's means that the protease is not able to stick to the hydrophobic matrix, making difficult to purify the protease by different elution buffers. It was possible to recover part of the activity loaded (65%), but we were not able to separate the protease from other possible contaminants.

In order to get more information about the state of the proteins presents in the crude extract, we used a gel filtration chromatography. The profile is shown in the Fig.4.
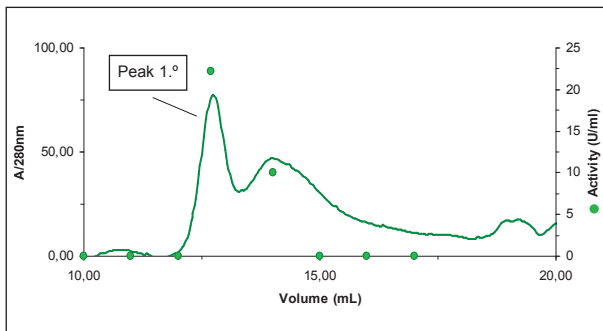

Sample	Total Actvity (U)	Yield U(%)
Crude Extract	360,0	100,0
SUPERDEX 200 10/300GL		
Peak 1°	40,1	11
Peak 2°	30,5	8
Peak 3°	9,8	2,5
TOTAL	**80,4**	**21,5**

Table 3: Yield obtained in relation of the enzymatic activity of the different fraction of the gel filtration chromatography.

Fig.4: Eluition profile by the gel filtration column. Column type: Superdex 200 10/300GL equilibrated in 50 mM Tris-HCl, containing 0,15 M NaCl pH 7,2. Sample: 40-80% $(NH_4)_2SO_4$ fraction of the crude extract. Flow rate: 0,25 ml/min. Fraction volume: 1 ml.

Are detected three peaks of enzymatic activity corresponding to the three mayors absorbance peaks, but their grade of separation is not satisfactory. Trying to find a better peak resolution, we collected the fractions corresponding to the two peaks with mayor enzymatic activity (peak 1° and 2°) and load into a smaller gel filtration column (Fig.5). The loaded sample is previous lyophilized and resuspended in the desired volume (0,5 ml).

Sample	Total Actvity (U)	Yield U(%)
Crude Extract	60,0	100,0
SUPERDEX 75 10/300GL		
Peak 1°	22,1	37
Peak 2°	10	16
TOTAL	**80,4**	**21,5**

Table 4: Yield obtained in relation of the enzymatic activity of the different fraction of the gel filtration chromatography.

Fig.5: Eluition profile by the gel filtration column. Column type: Superdex 75 10/300 GL equilibrated in 50 mM Tris-HCl, containing 0,15 M NaCl pH 7,2. Sample: 40-80% $(NH_4)_2SO_4$ fraction of the crude extract. Flow rate: 0,5 ml/min. Fraction volume: 1 ml.

Unfortunately, the peaks resolution is not good and also the protein and enzymatic recover is poor. In order to check the state of the peaks of the gel filtration chromatography, the samples are analyzed by a gel electrophoresis analysis (*SDS-PAGE*) (Fig.6).

Conclusion

Concerning the ATPS, the main effects of these independent variables (M_{PEG}, C_{PEG}, C_C, pH) on the partition coefficient (K), the protease removal yield (Y) and the purification factor (PF) can be summarized as follows: both K and Y_t was improved by an increase in C_C, C_{PEG} and a decrease in M_{PEG}; Y_b was improved by an increase in M_{PEG} and C_C; PF_t was effectively improved by a decrease in M_{PEG} and C_C. These results on the whole prove that ATPS is not sufficiently selective to provide total enzyme purification; therefore, was tried to purify by chromatography techniques but without reaching the expected degree of purity. In conclusion, in order to reach our goal, the purification and the characterization of the protease produced by *P. aurantiogriseum*, will be necessary planning new purifications strategies. About the ATPS, it should be recognized as a potential and powerful primary concentration/decontamination step in the overall enzyme purification protocol, which could be used for the purification of bioproducts of industrial concern. In particular, it could be successfully exploited in the pharmaceutical industry as the first step of the purification of biomolecules as antibiotics, immunoglobulins, pigments and enzymes.
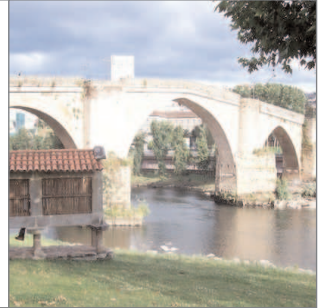
Acknowledgements

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DARÍO SPELZINI

Production of fungal peptidase aspartic from alternative culture media

ABSTRACT

During the canned tuna processing, boiling is an essential process. A medium size processing plant yields more than 15 tons/day of tuna cooked waste water (TCWW), which contains approximately 4% of valuable proteins. However, the cooked waste water that contains a high biological oxygen demand value is rejected. This results in water pollution as well as loss of a valuable protein source. Therefore a possible way to reuse this waste is their utilization as microbiological culture medium.

In this work, the production of extracellular peptidase aspartic of the fungi Aspergillus awamori NRRL 3112 was studied in submerged fermentation using tuna cooked waste water as culture medium.

Additionally the partitioning of extracellular peptidases aspartic from filtrate A awamori broth culture produced in stirred tank reactor was carried out in aqueous-two phase systems (ATPS) formed by polyethyleneglycol (PEG) and potassium phosphate. Previous results showed that aspartic peptidase have high affinity for the PEG rich phase [2]. The method appears to be suitable as a first step for the purification of these proteins from their natural sources.

TESTIMONIAL

"Estoy muy agradecido por la calidez de mis compañeros todos los momentos que pase con ellos, desde salir de pinchos a pasar navidad a orillas de la ría de Vigo, y hasta fui honrado con la oportunidad de codirigir un trabajo final de carrera… Muchas gracias y hasta pronto."

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Production of fungal peptidase aspartic from alternative culture media

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Abstract

During the canned tuna processing, boiling is an essential process. A medium size processing plant yields more than 15 tons/day of tuna cooked waste water (TCWW), which contains approximately 4% of valuable proteins. However, the cooked waste water that contains a high biological oxygen demand value is rejected. This results in water pollution as well as loss of a valuable protein source. Therefore a possible way to reuse this waste is their utilization as microbiological culture medium

In this work, the production of extracellular peptidase aspartic of the fungi *Aspergillus awamori* NRRL 3112 was studied in submerged fermentation using tuna cooked waste water as culture medium

Additionally the partitioning of extracellular peptidases aspartic from filtrate *A awamori* broth culture produced in stirred tank reactor was carried out in aqueous-two phase systems (ATPS) formed by polyethyleneglycol (PEG) and potassium phosphate. Previous results showed that aspartic peptidase have high affinity for the PEG rich phase [2]. The method appears to be suitable as a first step for the purification of these proteins from their natural sources.

Resumen

En el procesamiento del enlatado de atún, la cocción es un proceso esencial. Una planta de producción de tamaño medio genera más de 15 ton/día de caldo de cocción de atún, el cual contiene aproximadamente 4% de proteínas de valor. Sin embargo, el caldo de cocción, que tiene una alta demanda de oxígeno biológico, es desechado. Esto resulta en el desperdicio de una fuente de proteína importante como así también en la contaminación del agua. Por lo tanto una vía para recuperar el caldo de cocción de atún (CCA) es su uso como medio de cultivo de microhongos.

En este trabajo se estudió la producción de peptidasas aspárticas extracelulares a partir del hongo *Aspergillus awamori* NRRL 3112 en cultivos sumergidos utilizando CCA como base para la formulación de un medio de cultivo.

Adicionalmente se estudió la extracción de las peptidasas aspárticas extracelulares de caldos de cultivo filtrados de *A awamori* con sistemas bifásicos acuosos formados por polietilenglicol y fosfato de sodio. Las proteasas se reparten hacia la fase rica en polietilenglicol, como muestran resultados previos [2]. Este método de extracción mostró ser adecuado para las primeras etapas de purificación de estas enzimas desde fuentes naturales.

Introduction

Agro-industrial residues are generally considered a good source of substrates for the production of enzymes by fermentation processes. The selection of a substrate for enzyme production in a fermentation process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. Usually, a substrate based on agro-industrial wastes no provides all the needed nutrients to the microorganisms for growing and metabolite production being necessary to supplement them with other nutrients.

Proteolytic enzymes account for nearly 60% of the industrial market in the world. They find application in a number of biotechnological processes, viz. in food processing and pharmaceuticals, leather industry, detergent industry, etc

ATPS have been widely used in biochemical research for the separation and purification of macromolecules, cells and cell particles. ATPS have also been employed in several fields of the biotechnological industry for full-scale enzyme purification. This technique is considered potentially attractive for obtaining industrial enzymes, due its ease of scale-up, low-material cost, and minimization of denaturation of the proteins, among other advantages. In order to achieve a high yield, recovery, and also a good purification factor for a target protein, a composition for the ATPS has to be selected to quantitatively extract the desirable protein from one of the phases with minimal concentration of contaminant molecules.

In this work the production of aspartic peptidase from *Aspergillus awamori* using tuna canned cook wastes as substrate is presented, and a procedure to recovery the enzyme from the culture broth by aqueous-two phase systems purposed.

Materials and Methods

Fungi strains

A. awamori NRRL 3112 (also referred to as *A. niger* var. *awamori*) a strain producing bovine chymosin.

Culture media from TCWW

The TCWW from industrial installations was filtered through two layers of gauze to remove the floating fat and solids and then ultrafiltred following of diafiltration with membranes with a molecular weight cut-off at 10 000 Da in order to remove the saline contain and the low weigh molecular components. The process stopped when a dilution factor for salt of 2,5 was reached keeping the constant the proteins.

The culture medium contained 25% v/v diafiltrated concentrated TCWW , 20 g glucose, 1 g yeast extract and 1L water. The medium was autoclaved at 121 ºC for 45 min

Culture Conditions

Fermented operation was performed with 3 litres of medium in a 5-liter jar fermenter without pH control at 30ºC and 150 r.p.m. An inoculum suspension with $0,5 \times 10^6$ conidia/mL was used.

Partitioning in aqueous biphasic system

The free cells postincubated from *Aspergillus spp* culture was filtrated through nylon mesh and was used to assemble ATPS formed by PEG-potassium phosphate. The aspartic peptidase activity and protein content were determined with Anson method [3] and bicinchoninic acid method respectively.

The ATPS were obtained at pH 7 at longer tie line from Xia et al [4], the water content was partially replaced for filtrate broth as shown in table 1

Table 1

PEG MW (g)	Pi 28 % w/w (g)	PEG (g)	Water (g)	Culture broth filtrate (g)
1500	4.9	1.86	1.40	2.00
1500	4.9	1.86	2.40	3.00
8000	4.1	1.55	2.35	2.00
8000	4.1	1.55	3.35	3.00

The partition of culture broth between both phases was analyzed at 8 ºC. After mixing by inversion for 1 min, and leaving it to settle for at least 30 min, the system was centrifuged at low speed for the two-phase separation. Samples were withdrawn from separated phases and aspartic peptidase activity and total protein content in top phase were determinated and partition coefficient (Kp), purification factor and yield percentage were calculated in top phase.

Results and Conclusions

Composition TCJ ultra filtrate
The composition of both retentate and permeate in each stage of ultrafiltration process are shown in the table 2:

Table 2

	Protein g/L	Carbohydrate g/L	NaCl g/L
TCWW raw	10.00	16.55	3.46
Permeate 1	3.00	6.00	2.83
TCWW retentate 1	14.84	14.32	2.56
Permeate 2	2.43	0.46	1.48
TCWW retentate 2	11.44	8.69	1.4

step 1, concentration; step 2, diafiltration.

It was observed an asymmetric distribution on protein and carbohydrate contents between both permeate and retentate fractions.
Concentration stage showed achieve protein concentrations adequate for support the fungi culture, although the carbohydrate level is not sufficient.
The diafiltration stage showed to be useful for desalting of the TCWW, since the initial NaCl concentration in these juices usually is high.

Bioreactor cultures
Typical culture profiles are shown in the figure 1. Biosynthesis conditions were fixed taking into account the previous results obtained in agitated flask culture (data not shown), which was observed higher enzymatic activity production.
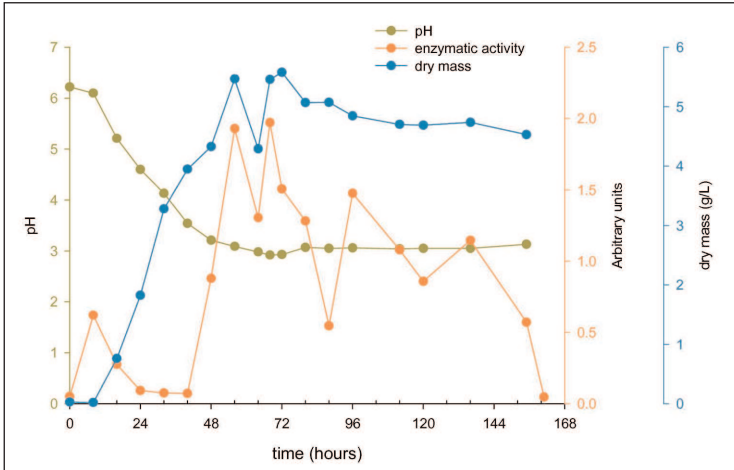
Figure 1: Time course of pH, enzymatic activity, and biomass production for *A awamori* NRRL 3112.

The culture profiles observed show the maxima production of biomass and enzymatic activity on the 2nd day when pH value was below 3.5; the biomass staying constant until 7th day; the extracellular aspartic peptidase production decays from the 4th day. This decay of activity enzymatic occurred when about the 60% of carbohydrates was consumed. The total protein content was constant as can be seen in figure 2.
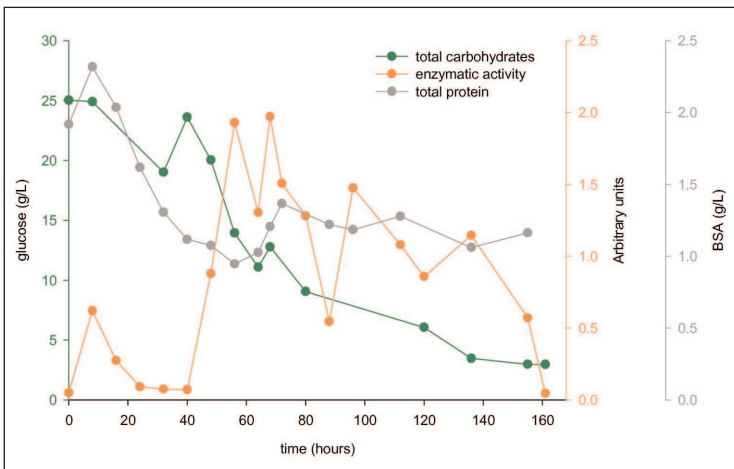

Figure 2: Carbohydrate and protein content in medium B supernatant. Culture A awamori NRRL 3112.

The non-conventional media derivates from ultrafiltrate TCWW, supplemented with glucose and vitamin was adequate to sustain the *A awamori* growth and production of extracellular aspartic peptidase activity in submerged culture .This media uses a waste product and could be applied on other fungi culture to obtain high valuable compounds under other growing conditions.

Filtrate Culture Broth Partitioning in ATPS:

After thermodynamic equilibrium was reached an amorphous precipitate was observed in interface in all cases assayed. The figure 3 show the Kp for aspartic peptidase activity and total protein value for partition of filtrate culture broth in ATPS formed by PEG-potassium phosphate.
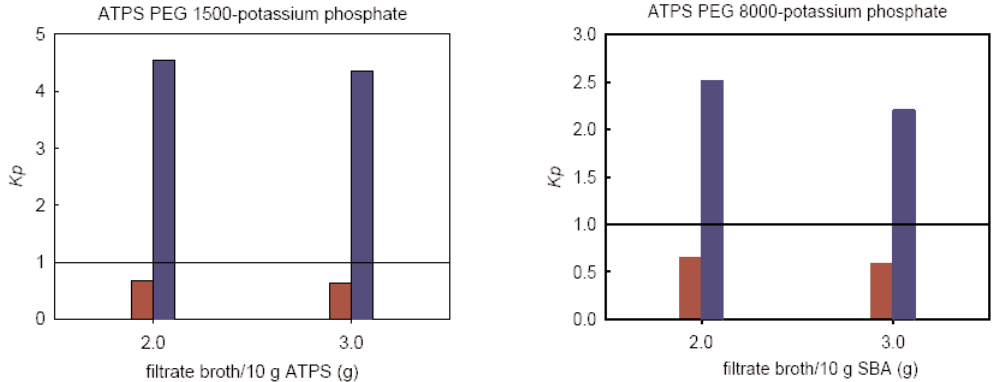
Figure 3: Kp for total protein (brown bars) and enzymatic activity (blue bars) of filtrate broth in ATPS

In all cases the other proteins were spread to interface or bottom phase (would its form part of precipitate), so Kp resulted lower than 1, while the extracellular aspartic peptidase was in top phase, so Kp was higher than 1. The Kp values for enzymatic activity were higher for ATPS formed with PEG 1500.

The tables 3 and 4 shows purification factor and yield percentage for extracellular aspartic peptidase in ATPS formed with PEG 1500 and PEG 8000 respectively; and potassium phosphate

Table 3: Purification table of partition of filtrate broth in ATPS PEG 1500-potassium phosphate pH 7, 8ºC

Culture broth (g)*	Total protein (mg/mL)	Total activity (AU/mL)	Specific activity (AU/mg)	Purification factor	y (%)
Filtrate	5,29	2,91	0,55	1,0	100,0
3,0	1,54	1,98	1,28	2,3	68,0
2,0	1,05	1,28	1,22	2,2	44,0

*g culture broth filtrate added for each 10 g ATPS. y: yield

Table 4: Purification table of partition of filtrate broth in ATPS PEG 8000-potassium phosphate pH 7, 8ºC

Culture broth (g)*	Total protein (mg/mL)	Total activity (AU/mL)	Specific activity (AU/mg)	Purification factor	y (%)
Filtrate	1,89	0,87	0,46	1,0	100,0
3,0	0,47	0,47	0,95	2,1	57,7
2,0	0,33	0,31	0,93	2,0	35,0

*g culture broth filtrate added for each 10 g ATPS. y: yield

The y% was higher for PEG 1500-containing ATPS, while purification factors were independent of molecular weight of PEG used. There were no substantial differences when different amounts of culture broth were added to ATPS. The percentage yield increase when the amount of broth added was greater.

The ATPS used extracted the target protein of a complex mixture, since this molecule is distributed mainly to the upper phase rich in PEG and its impurities are driven towards the interface or the bottom phase. Senthilkumar et al [5] reported purification factors higher than those presented in this work but with similar yields even after a precipitation with ethanol followed by an ion exchange chromatography and size-exclusion chromatography for the isolation of a aspartic peptidase from *Streblus asper*.

Despite this, the extraction of aspartic peptidases from broth culture could be a method to apply a first phase extraction with adequate yield.

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GISELA TUBÍO

Liquid-Liquid Equilibrium and Partitioning Features of Bovine Trypsin in Ucon 50 HB5100 /Sodium Citrate Aqueous Two Phase Systems

ABSTRACT

The phase diagrams of Ucon 50-HB-5100, a non-ionic random copolymer of ethylene oxide and propylene oxide (EOPO) and sodium citrate aqueous two-phase systems were determined at different pHs (5.20 and 8.20) and temperatures (5, 20 and 40º C). The binodal curves were determined by refractive index and enzymatic assay of the solution and described using a four-parameter sigmoidal equation, the reliability of the measured tie line compositions was ascertained by correlation equations given by Othmer Tobias and Bancroft. The two-phase area was expanded by increasing both pH and temperature. The partitioning of bovine trypsin and α-chymotrypsin, proteases of similar physico-chemical properties was investigated in order to evaluate the applicability of partitioning as a putative method to isolate from pancreas and to obtain any information about their partitioning mechanism. The effect of different factors such as pH, tie line length and the presence of an inorganic salt on the protein partition coefficient were analyzed.

TESTIMONIAL

"Mi instancia en Braga fue una experiencia verdaderamente inolvidable y enriquecedora"
"logre forjar intensos lazos de amistad que aun hoy perduran."

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Liquid-Liquid Equilibrium and Partitioning Features of Bovine Trypsin in Ucon 50 HB5100 /Sodium Citrate Aqueous Two Phase Systems

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Abstract

The phase diagrams of Ucon 50-HB-5100, a non-ionic random copolymer of ethylene oxide and propylene oxide (EOPO) and sodium citrate aqueous two-phase systems were determined at different pHs (5.20 and 8.20) and temperatures (5, 20 and 40º C). The binodal curves were determined by refractive index and enzymatic assay of the solution and described using a four-parameter sigmoidal equation, the reliability of the measured tie line compositions was ascertained by correlation equations given by Othmer Tobias and Bancroft. The two-phase area was expanded by increasing both pH and temperature. The partitioning of bovine trypsin and α-chymotrypsin, proteases of similar physico-chemical properties was investigated in order to evaluate the applicability of partitioning as a putative method to isolate from pancreas and to obtain any information about their partitioning mechanism. The effect of different factors such as pH, tie line length and the presence of an inorganic salt on the protein partition coefficient were analyzed.

Resumen

Se caracterizaron las curvas binomiales correspondientes a los sistemas bifásicos acuosos formados por Ucon 50-HB-5100, un copolímero al azar de óxido de etileno y óxido de propileno (EOPO) y citrato de sodio a diferentes pHs (5,20 y 8,20) y temperaturas (5, 20 y 40º C). Las curvas binomiales se obtuvieron por determinación del índice de refracción y ensayos enzimáticos de las soluciones correspondientes; las composiciones de las líneas de unión se corroboraron por las ecuaciones propuestas por Othmer Tobias y Bancroft. El aumento del pH y la temperatura condujeron a un aumento del área bifásica. También se ensayó el comportamiento de reparto de dos proteasas tripsina y α-quimotripsina con el objetivo de emplear los principios de partición como método de aislamiento y purificación de Tripsina a partir de páncreas bovino y de obtener información acerca del mecanismo de partición de la misma. Se analizó el efecto del pH, longitud de la línea de unión y presencia de sales inorgánicas sobre el coeficiente de partición de las enzimas.

1. Introducion

Bovine trypsin is an enzyme that is widely used for commercial purposes to digest or process other proteins, including some therapeutic proteins. Besides, with a high purity grade is employed with researching purposes in protein sequenciation. Another well-known pancreatic serine protease, ?-chymotrypsin, represents the principal contaminant since both proteases exhibit chemical similarities. Their separation is achieved after different chromatographic steps which are long and require expensive materials [1].

The liquid-liquid extraction principle applied to aqueous two-phase systems (ATPSs) offers a method for purification of biologically active materials since they allow the separation of these substances in biocompatible surroundings [2]. In laboratory scale separations the most commonly used systems are composed by the polymers polyethyleneglycol (PEG) and dextran while for large scale enzyme extraction, PEG/salt systems are used. These systems are attractive because of their low cost and rapid phase disengagement. Previous studies [3] have demonstrated that replacing the inorganic salts by other biodegradable and non-toxic ones such as citrates could be considered a good alternative, since citrates can be discharged into biological wastewater treatment plants. Recently, the use of thermo-separating polymers in ATPSs has been introduced [4]. When such polymers are heated above a lower critical solution temperature (LCST), the solubility of the polymer will decrease and a system composed of water and a polymer phase is formed. This makes it possible to perform temperature induced phase separation whereby a target protein can be separated from the polymer and recovered in the water phase.

The general mechanism governing the partition of biological material in aqueous two-phase systems is still not well understood; therefore the ATPS which provides the optimal separation conditions can only be selected after an experimental work. In this work, Ucon 50 HB-5100, an EOPO random copolymer of 50 % ethylene oxide and 50 % propylene oxide (mass) with an average molecular mass of 3900 was selected to form ATPSs with NaCit at pH 5.20 and 8.20. The corresponding phase diagrams were determined and the effect of temperature on the binodal curves was also studied. Then we describe the partitioning features of bovine trypsin and ?-chymotrypsin in the characterize Ucon/NaCit ATPSs in order to evaluate the ability of these systems of separating both proteins.

2. Experimental

2.1. Chemicals: Trypsin (TRP), α-chymotrypsin (ChTRP) from bovine pancreas, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-Benzoyl-L-tyrosine ethyl ester (BTEE) and Citric acid were purchased from Sigma Chem. Co. and used without further purification. Enzymes for citrate determination were obtained from Boehringer Ingelheim, Germany. Ucon 50 HB-5100 of average molecular mass of 3900 was obtained from Union Carbide (NY). All the other reagents were of analytical quality. Stock solutions of UCON of (40-50 % w/w) and NaCit (25 % w/w), of a given pH, were prepared by weighing known quantities of the polymer and citric acid respectively.

2.2. Phase diagram determination: A phase diagram is constituted of a binodal curve and tie lines. The determination of the binodal curve was carried out by a turbidimetric titration method [5]. The system temperature was maintained constant and controlled by immersing the glass tube and the stock solutions in a thermostatic bath.

For the determination of the tie lines, a series of ATPSs of at least three different known total compositions were prepared. When phases were separated, citrate and Ucon equilibrium concentrations were determined by an enzymatic assay described by Mollering and Gruber [6] and refractive index measurements [7] using a refractometer ABBE, NAR 3T (Atago Japan).

2.3. TRP and ChTRP enzymatic activity determination: Trypsin activity was determined with the substrate BAPNA using a method modified from Gildberg and Overbo [8]. The α-chymotrypsin assay is based on the hydrolysis of BTEE [9]. Both enzyme assays were performed at constant temperature of 22 °C. The activities were calculated from the initial linear portion of the absorbance vs. time curve.

2.4. Preparation of the aqueous biphasic system: To prepare the biphasic aqueous systems, stock solutions of the phase components of a given pH were mixed according to the binodal diagram previously obtained. Low-speed centrifugation was used after a thorough gentle mixing of the system components to speed up phase separation, and then each phase was mixed to reconstitute several two-phase systems in which the protein partition was assayed. Three different tie lines were assayed numbered (from 1 to 4) according to their increasing TLL.

2.5. Determination of the partition coefficient (Kp): Partitioning behaviour of TRP and ChTRP was analysed by the method given in ref 5. The partition coefficient was defined as:

$$Kp = \frac{[P]_T}{[P]_B} \quad (1)$$

where $[P]_T$ and $[P]_B$ are equilibrium concentrations of the partitioned protein in the Ucon and NaCit-rich phases, respectively. In our case, the Kp for TRP and ChTRP was calculated by the ratio of the enzyme activities in each phase. A correction factor was calculated as the ratio between the activities of reference solutions (of known concentration) of the enzyme in ach phase. All the measurements were developed by triplicate and constant temperature.

2.6 Selection of ATPS with the best separating capability: To select the ATPS with the best separating capability, the theoretical recovery and purity percentages of TRP in the top phase ($R_{TRP,T}$ and $P_{TRP,T}$) after a first extraction step, were calculated according to:

$$P_{TRP,T}(\%) = \frac{R_{TRP,T}}{R_{TRP,T} + R_{ChTRP,T}} 100 \qquad R_{TRP,T}(\%) = \frac{Kp_{TRP}\frac{V_T}{V_B}}{1 + Kp_{TRP}\frac{V_T}{V_B}} 100 \quad (2)$$

where V_B and V_T are the bottom and top phase volumes. The following assumptions were made: - similar concentrations of TRP and ChTRP in the starting sample, - the V_T/V_B ratio equal to be one, -similar Kp values for each protein in the mixture and alone.

3. Results and Discussion

3.1. Binodal curve: The total system compositions and tie line length determined for the studied systems are shown in Table 1. From visual inspection of the parameters and the corresponding determination coefficients (R^2), it is possible to conclude that of a sigmoidal equation is the most suitable to fit the binodal data since their R^2 values are closer to one than those obtained from literature expressions. Fig.1 summarizes the binodal data corresponding to ATPSs of Ucon/NaCit at different pHs. Binodal curves show similar shapes for the several pH values and the two-phase area is found to be expanded when pH is increased. At 20ºC, binodal curves corresponding to the different pHs tend to superimpose at high concentrations of Ucon or NaCit, thus indicating that either the exclusion or the salting out effect respectively prevails in phase-separation processes. When
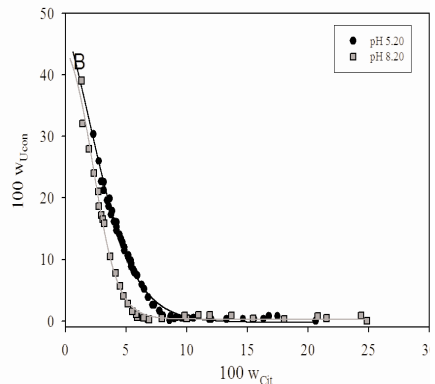
Figure 1. Effect of pH on binodal curve of Ucon/NaCit aqueous two-phase systems at 5 °C.

Ucon and NaCit concentrations adopt intermediate values, a smaller concentration of NaCit is needed for two-phase formation at basic pHs (with higher ratios between trivalent and divalent citrate ions). This effect is also observed in binodal curves at 5°C, even at high Ucon concentrations (low NaCit concentrations) at which no superimposition of binodal curves is observed. At low temperatures, the EO and PO units in the copolymer, are known to be strongly hydrated with two or three water molecules [10]. Similarly, ionic species in solution are known to be hydrated and the extent of hydration depends upon the ion valency.

Table 1. Phase Compositions for Ucon/NaCit ATPSs.

pH	Temp	total compositions		top phase		bottom phase		STL*	σ**	TL	100TLL	Kp***
		$100w_{NaCit}$	$100w_{Ucon}$	$100w_{NaCit}$	$100w_{Ucon}$	$100w_{NaCit}$	$100w_{Ucon}$					
5.20	5° C	7.97	8.96	3.22	22.31	10.97	0.51	-2.81	0.11	1	23.14	3.4
		8.99	8.98	2.81	25.88	12.12	0.39	-2.74	0.08	2	27.14	4.3
		10.06	9.00	2.48	28.82	13.37	0.34	-2.61	0.13	3	30.49	5.4
		10.99	8.98	2.22	30.96	14.44	0.32	-2.51	0.08	4	32.99	6.5
	20° C	5.70	10.65	3.32	21.40	7.28	3.48	-4.53	0.14	1	18.35	2.2
		5.99	11.02	2.84	25.21	7.87	2.52	-4.51	0.09	2	23.24	2.8
		6.22	17.08	1.89	33.40	10.64	0.42	-3.77	0.08	3	34.12	5.6
		6.54	17.87	1.64	35.53	11.38	0.21	-3.63	0.11	4	36.64	6.9
	40° C	6.23	17.15	1.19	43.75	9.32	0.78	-5.28	0.21	1	43.73	7.8
		6.48	17.81	0.97	46.43	9.87	0.19	-5.20	0.16	2	47.09	10.1
		7.09	19.49	0.49	52.51	11.15	0.09	-4.94	0.15	3	53.49	22.6
8.20	5° C	6.45	9.44	1.95	22.73	9.62	0.09	-2.95	0.09	1	23.90	4.9
		7.48	9.37	1.50	26.78	10.71	0.19	-2.89	0.12	2	28.14	7.1
		8.47	9.40	1.12	30.39	11.80	0.19	-2.83	0.11	3	32.03	10.5
		9.41	9.37	0.74	33.90	12.78	0.18	-2.81	0.14	4	35.81	17.2
	20° C	4.32	14.88	2.16	26.45	6.93	0.93	-5.35	0.21	1	25.96	3.2
		4.49	16.33	1.93	29.37	7.58	0.61	-5.09	0.15	2	29.31	3.9
		5.00	17.70	1.63	33.33	8.74	0.33	-4.64	0.14	3	33.76	5.4
		5.49	19.18	1.41	36.24	10.02	0.22	-4.18	0.17	4	37.03	7.1
	40° C	1.99	15.85	0.87	26.95	3.35	2.31	-9.94	0.40	1	24.76	3.8
		2.20	16.39	0.82	28.80	3.83	1.75	-8.97	0.18	2	27.22	4.7
		2.64	20.21	0.42	41.69	5.03	0.04	-9.03	0.27	3	41.90	12
		3.13	23.36	0.41	46.06	5.79	1.12	-8.35	0.25	4	45.26	14.1

*STL tie line slope, **σ Standard deviation, ***Kp Fraction of NaCit retained in the bottom phase divided by the NaCit in the top phase

Thus, triply charged citrate can be expected to be more effective than doubly charged citrate in salting out the copolymer because of competition for water. Therefore, trivalent ions are more efficient than divalent ions in promoting the phase separation [11]. However, this effect does not seem to be significant at low NaCit concentration at 20°C, since binodal curves corresponding to the different pHs overlap. In this case, the breakdown of structured water molecules around the EO/PO-chains associated with an increase in temperature is probably the predominant cause of phase separation.

The effect of temperature on phase-separation processes is given in Fig. 2. An increase in temperature increase from 5ºC to 20ºC induced a slight increase in the biphasic area, while a significant expansion of the two-phase region was observed when the temperature was raised up to 40º C. This trend was also observed for other polymer/salt systems such as PEG/NaCit [12]. According to the model proposed by Kjellander and Florin [2], the entropically unfavourable structuring of water produced by Ucon at low temperatures is overcome owing to the large decrease in enthalpy (due to the energetically favourable and highly directional interactions, such as hydrogen-bonding, between unlike molecules). At higher temperatures, provided that the structure of water in the Ucon hydration shell does not break down too rapidly with increasing temperature [11], the unfavourable entropy contribution becomes prominent and

Figure 2. Effect of temperature on binodal curve of Ucon/Cit pH 8.20 aqueous two-phase system.

the system phase separates itself. In addition by increasing the temperature the magnitude of the tie line slopes increases (see Table 1), indicating the increase of the asymmetry of the diagrams. Similar results were obtained for other polymer/salt systems from literature [13].
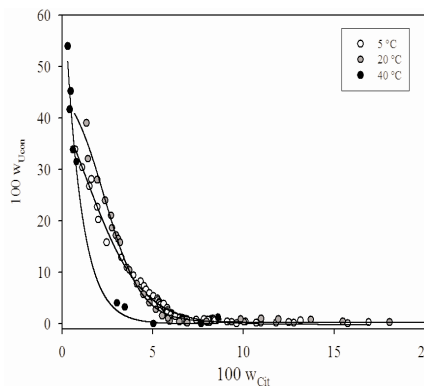
3.2. Tie lines: In Table 1 it also lists the value of the partition coefficient (Kp) of the salt, defined as the fraction of salt retained in the bottom phase divided by the salt in the top phase. High values of Kp were obtained (from 2.2 to 22.6) which is an indication of the separation obtained by adding an amount of Ucon to a given brine solution.

For most of the assayed systems, the tie lines became steeper for total compositions in the vicinity of the critical point. An increase in the STL magnitude indicates an increase in the difference between the polymer concentrations at a given difference in the salt concentrations in the same phase. This implies a decrease in the mutual solubility of the aqueous polymer- and salt-containing media. Empirical equations have been proposed to ascertain the reliability of calculated tie line data in traditional liquid-liquid extraction, being the most widely used those of Othmer-Tobias and Bancroft [14]. Linearization of both equations produced acceptable consistency in the results.

3.3. Partition behaviour of TRP and ChTRP in Ucon/sodium citrate ATPSs: The effect of medium pH on the TRP and ChTRP partition coefficients (Kps) was analyzed. The increase in pH from 5.2 to 8.2 produced an increase of the Kp value for ATPSs formed by Ucon. This behaviour could be satisfactorily explained on the basis of the Albertsson equation [2], which takes into account an electrostatical and a non-electrostatical term. In these systems, the interfacial potencial assumes positive values (since the bottom phase is enriched in the citrate anion); therefore, the electrostatic term will assume the opposite sign to the net protein charge. At pH.5.2 and 8.2 media, TRP and ChTRP are positively charged since their isoelectrical points are 9.1 and 10.5 respectively. When pH raises from 5.2 to 8.2, the net protein charge

decreases, thus increasing both protein Kp values. Protein partitioning behaviour showed to be sensitive the surface. Protein partitioning behaviour showed to be sensitive the surface hydrophobicity [15]. Partition equilibrium for TRP showed to be more displaced to the top phase than ChTRP for assayed ATPSs.

3.4. Influence tie line length and pH on the TRP and ChTRP partitioning: The effect of TLL and pH medium on these proteins partitioning are describes in Fig. 3. The increase in the TLL is accompanied by a decrease in the Kp value. Previous reported data [16] showed that the partition coefficient became more one sided when the tie line length was increased due to the increase in the difference between both the polymer and salt concentrations in the top and bottom phases. For those systems where proteins exhibit a great affinity for the bottom phase, (Kp <1) the partition equilibrium displaced to the bottom phase as the TLL increases while in ATPSs where proteins prefer the polymer rich-phase (Kp >1) the opposite behaviour was observed. Another system variable such as the pH medium was studied. For most of the assayed systems an increase in the Kp values was observed when pH increased from 5.20 to 8.20 in accordance with the Albertsson equation [2]. These findings showed that both TLL and pH would be able to be manipulated in order to separate TRP from ChTRP.

Figure 3: Effect of tie line and pH on the Kp for TRP and ChTRP Ucon/NaCit aqueous two phase system.

Addition of certain salts, such as NaCl, has been reported [4] to displace the partitioning equilibrium to the top phase thus enhancing the protein yield in this phase. Fig. 4 shows that the NaCl presence induces a transfer of trypsin to the top phase. Besides, TRP partitioning showed to be sensitive to salt presence (Kp increases) thus leading to an enhancement of the ATPS selectivity. Our results demonstrate that NaCl addition is a successful tool to improve protein yield and lead to a significant purification factor enhancement but not enough to obtain a satisfactory result.

Figure 4: Effect of tie line and NaCl on the Kp for TRP and ChTRP. Ucon/NaCit aqueous two phase system.

3.5 Selection of optimal separating conditions: According to literature [17], the ratio between the target and contaminant protein partition coefficients ($Kp_{target}/Kp_{contaminant}$) can be considered as a measure of system selectivity, those systems with higher ratio being the most selective ones. In this study we included additional calculations in order to make the decision more appropriate. The theoretical recovery (R) and purity (P) percentages of TRP in the top phase after one extraction step were calculated according to equation (2) in order to select the ATPS with the best capability of separating TRP from ChTRP. From a visual inspection of Fig. 5, it is evident that Ucon/NaCit ATPSs would lead to high TRP purity values (R>70%). This fact is in agreement with the predicted effect of Kp on the R value according to equation (2). In contrast, conduce to lower TRP yields values. At this point, a non-trivial topic to be decided is if either the recovery or the purity should be optimised. The answer depends on the source availability and final application of the target protein.
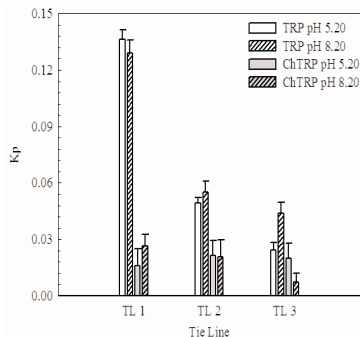
4. Conclusions

The phase diagrams of Ucon/NaCit ATPSs were determined. Reliable and complete data on the composition and properties of these systems were not available at present, being this information necessary for the design of an extraction process. The phase formation proved to be both temperature and pH-dependent. Much lower amounts of polymer and salt than conventional systems are required to form the two phases, thus reducing the environmental impact. In addition, thermo-separating properties of Ucon can be used to recycle this polymer from the polymer rich-phase. These characteristics and several additional advantages such as biodegradability of citrate anion, low cost and rapid phase separation make

Figure 5: Effect of polymer molecular mass, TLL and pH on the theoretical recovery (R) and purity (P) percentage of TRP at the top phase after one extraction step and employing equal top/bottom phase volumes.

Ucon/NaCit ATPSs a promising, versatile and attractive system in the field of bio-separation. TRP and ChTRP partitioning behaviour in Ucon/NaCit ATPSs showed to be sensitive to medium pH and tie line length. In spite of their similar physicochemical properties such as molecular weight and isoelectrical point, both proteins showed different partitioning behaviour. ChTRP is more partitioned to the bottom phase (citrate-riched) than TRP for most assayed systems. The presence of NaCl 3 % enhances the separation capability of Ucon/NaCit ATPSs for the separation of both proteins. Although further work needs to be done to choose the most adequate ATPS, these findings suggest that Ucon/NaCit ATPSs could be employed as a viable and potentially useful first step procedure for the separation of TRP and ChTRP.
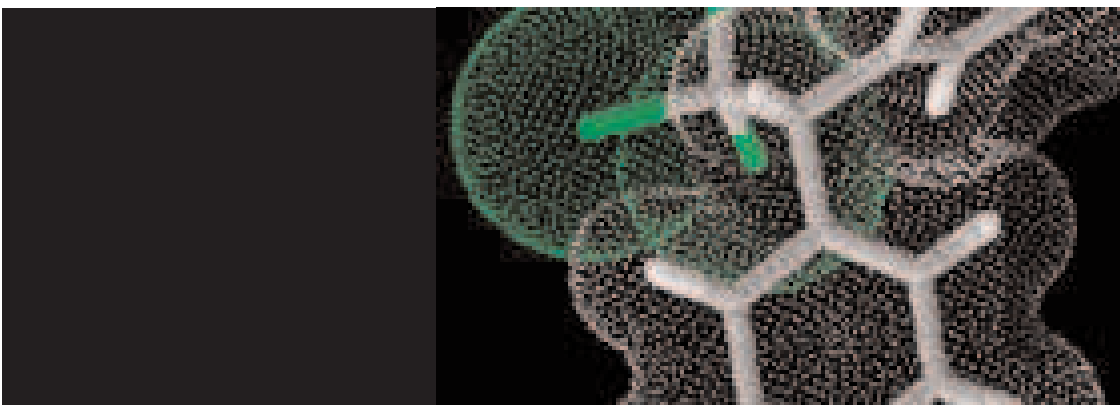
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