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Presentation

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In Pro-Research, Teaching and Training of human resources committed to Science. The content of the articles and reviews that appear in each issue are those of the authors and does not necessarily the opinion of the editor in chief.

In the first issue we present, *In vitro induction of extracellular neutrophil traps (NET) with Trichophyton rubrum*, by PRIETO-CORREA, José Roberto, MAYORGA-RODRÍGUEZ, Jorge and ISLAS-RODRÍGUEZ, Alfonso Enrique, with affiliation at Universidad de Guadalajara, as following article we present, *A Case of Inactivated Commercial Vaccine Functionality against Infectious Coryza Serotypes A, B and C (Avibacterium Paragallinarum) in Birds of Combat*, by CARRILLO-DÍAZ, Fernando, MACÍAS-CORONEL, Humberto, SALGADO-MORENO, Socorro and MARTÍNEZ-GONZÁLEZ, Sergio, with secondment in the Universidad Autónoma de Nayarit, as a next article we present, *NBelyax nanoparticle test as a disinfectant agent, applied in the hands of nursing staff. Hospital de la Beneficencia Española and Hospital Nicolás San Juan, as case studies*, by LEON-GUTIERREZ, Gabriela, ALBARRAN, León, LEON-GUTIERREZ, Sergio and ARTEAGA-LOPEZ, Paola, as last article we present, *Response of stomatal conductance and xylem sap abscisic acid concentration of Sorghum bicolor (L.) Moench cv. Tegemeo under re-watered and drought-stressed conditions*, by NERI-LUNA, Cecilia, VILLARREAL-RUIZ, Luis, HUERTA-MARTÍNEZ, Francisco Martín and ROBLES-MURGUÍA, Celia, with affiliation in Universidad de Guadalajara, as last article we present *Optimization and characterization of the immobilization process of trypsin in calcium alginate beads, by* TOVAR-JIMÉNEZ, Xochitl, MURO-URISTA, Claudia Rosario and ARANA-CUENCA, Ainhoa.

Content

Article **ECORFAN Journal**
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In vitro induction of extracellular neutrophil traps (NET) with Trichophyton rubrum

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Abstract

Tinea is a human dermatophytosis caused by *Trichophyton rubrum,* which infects keratinocytes, generates severe lesions in immunosuppressed individuals, and is a public Health problem. The immune system of mammals has kept his innate component based on the recognition of pathogen-associated molecular patterns through its receptor recognition patterns on the surface of immune cells, including macrophages and neutrophils, which besides its phagocytic function, are capable of destroying extracellular pathogen forming traps made of DNA, histones, and antimicrobial peptides (NET). The aim of this study was whether neutrophils isolated from peripheral blood of normal subjects by bringing them into contact with sonicated conidia and conidia of *T. rubrum,* form NET. by means of In vitro culture of *T. rubrum,* isolation and purification of conidia of *T. rubrum,* and obtaining a homogenate of *T. rubrum* conidia by sonication for the induction of Neutrophil extracellular traps (NET). The results shows that by putting in contact conidia and sonicated conidia of *T. rubrum* positively induced formation of NET. It is concluded that both conidia as the sonicated conidia of *T. rubrum* may have a protective function as a component of the innate immune response in individuals at risk of acquiring infection.

Innate Immune Response, Neutrophils Extracellular Traps (NET), *Trichophyton rubrum*

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1. Introduction

Humans have infectious diseases caused by fungal bacteria and pathogenic viruses that develop adaptation mechanisms to evade the Innate Immune Response (RII) and the Adaptive Immune Response (RIA) of the host, survive in it and generate these diseases that are becoming more frequent among the population, since they are resistant to treatment [2, 9, 22].

The most frequent fungal infections among the population are dermatophytosis or tinea infections caused by Trichophyton rubrum, among others. In Mexico this is a problem that occurs between 70 and 80% of all mycoses. Because it has a very high frequency as an infection in different parts of the body, it is believed that Trichophyton rubrum has developed survival modes to escape the host defense mechanisms, as a result the disease is expressed with bothersome and uncomfortable symptoms for the patient [4 , 24, 31].

1.1 Justification

The NETs are related to many infectious diseases, and possibly autoimmune as mentioned, and have shown affinity for pathogenic microorganisms destroying them. Therefore, this mechanism is essential for the innate immunity against T. rubrum, keeping the host free from Ringworm.

1.2 Problem

Trichophyton rubrum is a pathogen that mainly affects the keratinocytes, feeding on keratin, which is a necessary nutrient for it to develop, causing one of the most common fungal diseases in humans; ringworm and athlete's foot among others. This parasite shows to be rebellious to the treatments, since it has developed mechanisms of resistance and evasion [25, 19].

1.3 Hypotesis

The complete conidia and the sonicated extract of conidia of the fungus Trichophyton rubrum, induce the formation of extracellular networks of neutrophils of human peripheral blood in in vitro conditions.

1.4 Objectives

1.4.1 General objective

Experiment if the complete conidia and the sonicated extract of conidia of the fungus Trichophyton rubrum, are able to induce the formation of extracellular DNA networks of human peripheral blood neutrophils under in vitro conditions.

1.4.2 Specific objectives

- Isolation and Culture T. rubrum.
- Isolation of conidia from T. rubrum.
- Homogenate conidia of T. rubrum.
- Isolation and culture of polymorphonuclear neutrophils from human peripheral blood.
- Induction of extracellular neutrophil traps (NET).

2. Theoretical framework

Classically, physical barriers such as skin, mucous membranes, hair, body secretions, etc., constitute the first line of defense of the RII. Antimicrobial peptides, phagocytosis and the involvement of the complement system are important mechanisms of this response [30].

The RII is also largely integrated by the action of granulocytes and macrophages, its main function is to phagocytose fungi and destroy bacteria. They are very important cells for immunity [15, 32].

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Neutrophils are a type of granulocyte, whose function is to participate in the initiation of the inflammatory process and respond to the stimulation of fungi, bacteria, and other parasites to eliminate them, trying to make this mechanism effective [3, 23, 26, 27, 39, 41]. Recent research indicates that neutrophils under infection conditions can catapult extracellular networks or fibers at the site of an infection in response to pathogens, called traps.

Extracellular neutrophils, by its acronym in English (NET), which are structures composed mainly of DNA, histones, elastase and antimicrobial peptides, which are present in the nucleus and granules of the cytoplasm of neutrophils [11, 12, 14, 34, 35, 37, 38, 43, 44, 47]. The DNA of the nucleus of the living cell is violently expelled to the outside of the membrane, and as a result the microorganisms are trapped in the network, eliminating them and initiating the process of healing and regeneration of the tissue, all at the cost of the death of the neutrophil. [5, 7, 8, 16, 17, 18, 20, 21, 33, 36, 42, 48].

There is still much to be deciphered regarding the role of NETs in the development or control of infectious diseases; however, the excessive formation of NET or its persistence would play a fundamental role in some infectious and autoimmune diseases. It is not clear if severe lung dysfunction or other diseases in conjunction with viral infections could be due to the formation of NETs as a consequence of the inflammatory response. Finally, it should be mentioned that the formation of NET is preferably induced by pathogenic microorganisms [28, 45, 46, 49].

3. Metodology

Isolation and Culture of T. rubrum.- Trichophyton rubrum was directly isolated from 1 patient diagnosed with dermatophytosis (at the Dermatological Institute of Jalisco, located at North Federalism Street # 3102, Atemajac del Valle, 45190 Guadalajara, Jalisco) according to the technique of Dr. Jorge Mayorga of the Mycology laboratory of the aforementioned institute. It was cultivated in glass tube with Agar Saboraud (J.T. Baker®) solid.

It was incubated at 37 ° C for 24 hrs (after this time it was left to incubate at room temperature between 7 and 15 days). The nichrome handle was introduced with a self-adhesive strip frame on the tube with Trichophyton rubrum, it was scraped and a part of the colonies was captured. The tape was placed in an object holder and two drops of the methylene blue dye (J.T. Baker®) were added, the object cover was placed and the identification of the microorganism was observed in the compound microscope.

The strain of T. rubrum was inoculated into the Petri dish containing the solidified Sabouraud Agar. Petri dishes were labeled with the name of the strain and incubated at 37 ° C for 24 hrs, the Petri dishes were left until their maximum growth at room temperature [19].

Conidial isolation of T. rubrum.- For the isolation of the conidia, 10mL of bidistilled water was used (obtained from the laboratory of Dr. Anne Santerre), sterilized by autoclave and added to a culture box with the microorganism in its maximum growth and scraped the agar surface with a spatula. The liquid was recovered and vortexed for 3 minutes (GENIES II®) three times to detach the conidia. The suspension was purified with sterile Wathman No.1 filters.

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The conidia were quantified with a Neubauer chamber (Loptik Labor®) obtaining 7X105 = 700,000 conidia of T. rubrum in 1 mL of water. The conidial solution was divided into two tubes of 15mL with a volume of 5mL per tube, one tube was cooled to -20 ° C and the second was placed in a sonicator (BRANSON®) to break the conidia [19].

Homogenate of conidia of T. rubrum.- Four sonication cycles of 30 minutes each were used. The solution was filtered through a sterile 0.22 μ membrane (Millipore®). The breaking of the conidia was confirmed by visualization in the microscope. The solution with the conidial homogenate was refrigerated at -20 ° C [19].

Isolation and culture of polymorphonuclear neutrophils from human peripheral blood. - 7mL (per donor) of human peripheral blood was obtained from 6 clinically healthy donors in heparin vacuator tubes (BD®), a tube of 15mL was prepared with 7mL of histopaque (Ficoll-SIGMA®) to perform the first density gradient. The 7mL of blood was slowly poured into the tube with the 7mL of ficoll, taking care not to break the gradient, centrifuged at 2500rpm for 20min, at room temperature.

The different phases were discarded: plasma, ficoll and mononuclear, the granulocyte phase was taken and placed in a new tube. 10mL of PBS pH 7.2 was added to the tube with the granulocytes and mixed by inversion, centrifuged at 1600rpm for 10min, (in a Jouan® centrifuge). At the end of the centrifugation, the supernatant was discarded and 4mL of PBS was added, the cells were resuspended. This solution was slowly passed to the tube containing 10mL of the percoll gradients *. Centrifuged at 2,500rpm for 20min, at room temperature. The polymorphonuclear ring was recovered and added to a new tube, 10mL of PBS was added and mixed by inversion, centrifuged at 1,600rpm for 10min, at room temperature.

The supernatant was removed and 6mL of RPMI medium (SIGMA®) was added, the cells were resuspended. The cells were quantified in a Neubauer chamber, concentration of 90μL of trypan blue (SIGMA®) with 10μL of the sample with the cells. [29, 1].

Formulation of the decreasing gradient of Percoll (SIGMA®) .- The solution for the percoll gradient was prepared: 36mL of percoll were mixed with 4mL of 10% PBS, to have a 100% solution. In a new 15mL tube, 1.5mL of Hank's solution was added with 8.5mL of percoll leaving an 85% solution, 2mL of Hank's solution was added with 8mL of percoll to another new tube leaving the 80% solution , in the next tube, 2.5mL of Hank's solution (SIGMA®) was placed with 7.5mL of percoll leaving the solution at 75%, in the following tube 3mL of Hank's solution was added with 7mL of percoll to remain a 70% solution, and 3.5mL of Hank's solution was added to the last tube with 6.5mL of percoll to be 65%. In a new tube of 15mL 2mL of 85% percoll was added, slowly to the same tube 2mL of 80% percoll, 2mL of 75% percoll, 2mL of 70% percoll and finally 2mL of 65% percoll were added. (the solutions were added as slowly to avoid breaking the gradient).

Induction of neutrophil extracellular traps (NET). - 6 covers were placed round objects 5 min in 70% ethanol, 5 min in 100% ethanol and 5 min in poly-L-lysine (SIGMA®) solution 1:10, subsequently They were placed on the 6-well plate (one covers per well).

It was washed with 1mL of PBS pH 7.2 at the periphery of the well to remove excess poly-L-lysine. 3X106 of neutrophils were added to each well, 200μL of autologous serum, two wells were left as negative control, no stimulus was received (C-), two were established as positive control (C +) with Forbol Miristato Acetate (for its acronym in English PMA SIGMA ®) at a concentration of 1μg / 1mL.

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And the last two wells were the experimental conditions and were stimulated with 70,000 complete conidia of the fungus T. rubrum in 100µL of bidistilled water each.

The plate was incubated for 3hrs at 37 ° C with 5% CO2 (in a Thermo® incubator) After the incubation time, each well was washed with 1mL of PBS to remove excess PMA and conidia. 500µL of 4% paraformaldehyde was added per well, incubated for 20min at room temperature. It was then washed with 1mL of PBS each well to remove the excess. 100µL of DAPI in concentration 1mg / 1mL was added to each well and incubated for 1h at room temperature. Was washed with 1mL of PBS each well to remove excess DAPI (SIGMA®), incubated for 24h at 4 ° C. The next day two drops of PBS pH 7.2 were placed in the holder and covers were mounted, samples were observed under the Karl Zeiss® fluorescence microscope) and the images were captured [10, 13].

4. Results

Isolation and Culture of T. rubrum.

As can be seen in Figure 1, it was possible to maintain the viability and maximum growth of the Trichophyton rubrum strain that was isolated directly from a patient.

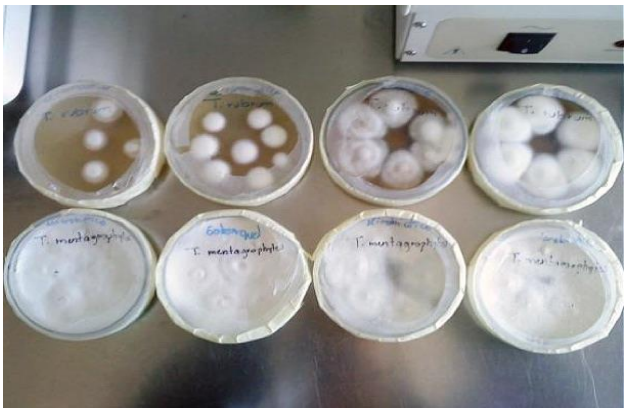

Figure 1 Maximum growth at room temperature of T. rubrum inside the Petri dish containing the solidified Sabouraud Agar. [19]

Induction of extracellular neutrophil traps (NET)

After isolating, culturing and stimulating the neutrophils of the seidonants with the different controls and experimental conditions, it was possible to observe, that the neutrophils of the first duplicate, of each of the 6 experiments (one from each donor), corresponding to the negative control (C-) remain intact when no stimulus is received, while the duplicates of each experiment with cells that were stimulated with PMA, corresponding to the positive control (C +), showed the DNA released and defragmented.

It can be seen that the cell loses all its structural properties, releasing their genetic and cytoplasmic material to the extracellular space in the form of a network (NET). The following duplicates correspond to the stimulation of complete conidia (3 first donors) and finally with conidial homogenate (donors 4, 5 and 6), the induction and release of the genetic material was also observed, with the formation of NETs. (See Figures 2-7).

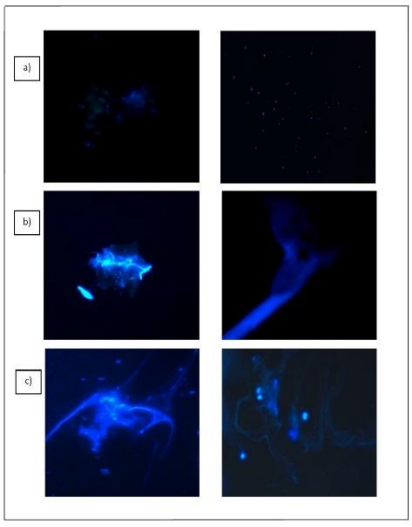

Figure 2. Induction of NET in vitro with conidia of Trichophyton rubrum, images obtained by fluorescence microscopy (40X) (a). Neutrophils without stimulus (C-). (b) Neutrophils stimulated with PMA (C +). (c) Neutrophils stimulated with complete conidia of Trichophyton rubrum.

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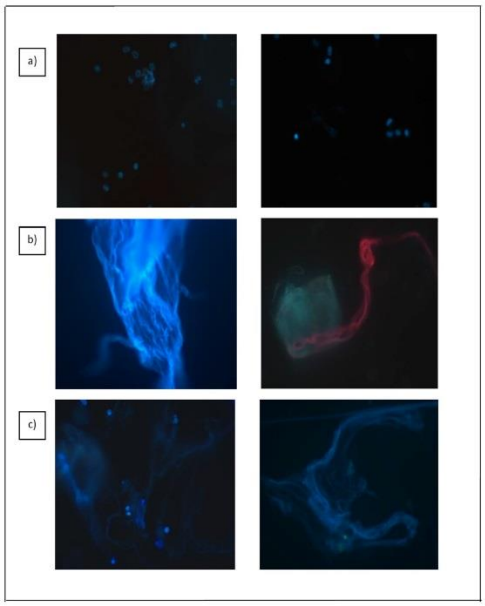

Figure 3 Induction of NET in vitro with conidia of Trichophyton rubrum, images obtained by fluorescence microscopy (40X) (a). Neutrophils without stimulus (C-). (b) Neutrophils stimulated with PMA (C +). (c) Neutrophils stimulated with complete conidia of Trichophyton rubrum

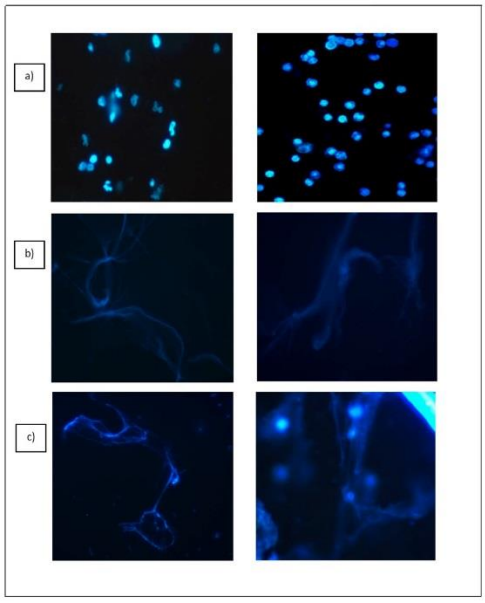

Figure 4 Induction of NET in vitro with conidia of Trichophyton rubrum, images obtained by fluorescence microscopy (40X) (a). Neutrophils without stimulus (C-). (b) Neutrophils stimulated with PMA (C +). (c) Neutrophils stimulated with complete conidia of Trichophyton rubrum

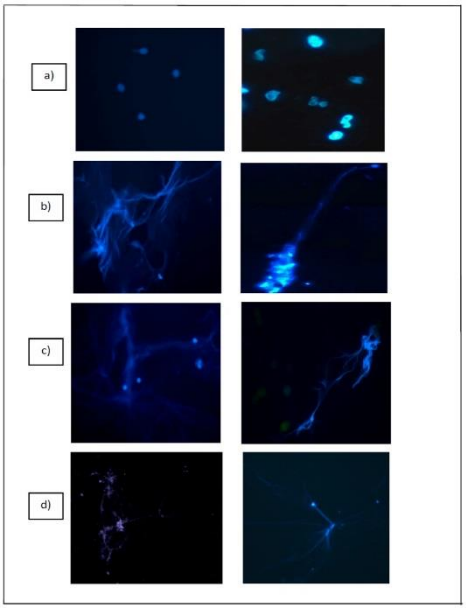

Figure 5 In vitro induction of NET with sonic extract of conidia of T. rubrum: images obtained by fluorescence microscopy (40X). (to). Neutrophils without stimulus (C-). (b) Neutrophils stimulated with PMA (C +). (c). Neutrophils stimulated with complete conidia of Trichophyton rubrum. (d) .Neutrophils stimulated with sonic extract of conidia

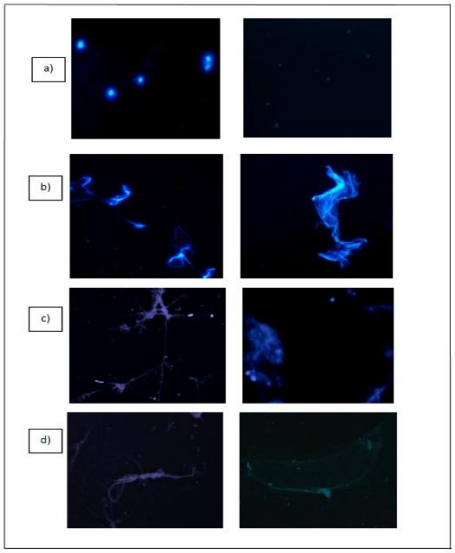

Figura 6 Inducción in vitro de NET con extracto sonicado de conidias de *T. rubrum:* imágenes obtenidas por microscopía de fluorescencia (40X). (a). Neutrófilos sin estímulo (C-) . (b). Neutrófilos estimulados con PMA (C+). (c). Neutrófilos estimulados con conidias completas de *Trichophyton rubrum.* (d).Neutrófilos estimulados con extracto sonicado de conidias.

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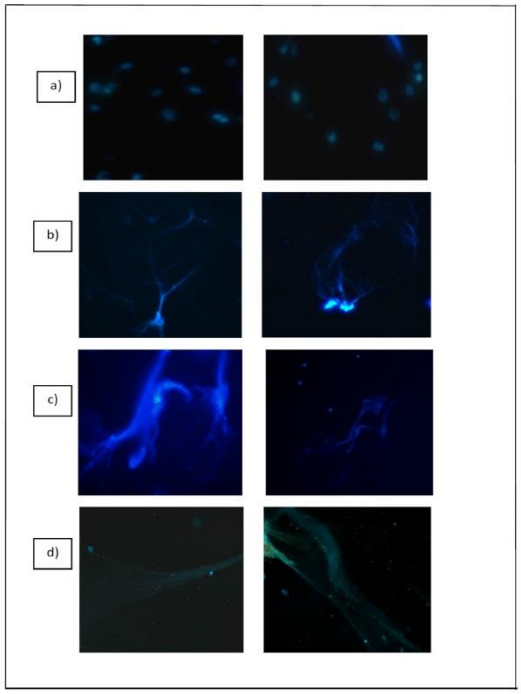

Figura 7 Inducción in vitro de NET con extracto sonicado de conidias de *T. rubrum:* imágenes obtenidas por microscopía de fluorescencia (40X). (a). Neutrófilos sin estímulo (C-) . (b). Neutrófilos estimulados con PMA (C+). (c). Neutrófilos estimulados con conidias completas de Trichophyton rubrum. (d).Neutrófilos estimulados con extracto sonicado de conidias.

Discussion

The nature of DNA as a polynucleotide that contains deoxyribose and complementary nitrogenous bases linked by phosphodiester bonds and hydrogen bonds within its structure, locate it as a hierarchical molecule, which contains the genetic information of all biological organisms. In this work, it was demonstrated that not only is it a molecule that stores and protects information, but it is also a utilitarian molecule since it participates in the protection of the organism against infectious agents, and can be released into the extracellular space of neutrophils and mast cells, in a of network in conjunction with antimicrobial accessory molecules [11, 50, 51].

Therefore the knowledge of this function, has demonstrated this novel role of DNA in granulocytes, and opens the possibility of new hypotheses for the realization of research on this mechanism different from apoptosis, called "netosis", developed evolutionarily as protection against infection, in different biological models of invertebrates and vertebrates as an adaptation mechanism.

Previous results of our work group revealed that human keratinocytes in vitro, respond to the antigenic stimulus of conidia and the homogenate of the fungus T. rubrum, proliferating and differentiating in response to this stimulus, also by means of TLR-2 receptors, TLR-4 and TLR-6 induce the production of antimicrobial peptides such as beta-defensin 2 and interleukins 1β and 8 [19]. So it shows local immunity.

Here it is shown that the neutrophils of human peripheral blood also respond to the stimulus of the complete conidia of T. rubrum, forming the DNA networks, but importantly, in this work it is demonstrated that the sonicate-homogenate of these conidia also induces the NET. Other works report that NET traps and eliminates bacteria. Using Staphylococcus aureus, it is reported that it reacts with neutrophils and these suffer netosis, subsequently releasing the NETs [17].

It has also been shown that DNA is the main component of the NET since in an experiment with Streptococcus pyogenes and DNAse treatments it is not possible to trap the bacteria, and therefore eliminate them, because the networks are disintegrated by the DNAse [12, 13]. The foregoing indicates the importance of this mechanism. Furthermore, interestingly, in other experiments with Staphylococcus aureus, the results showed that pathogens are trapped, but do not die by NETs, because they promote the premature death of the immune cell, as a mechanism of evasion [40].

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It is demonstrated that Streptococcus pneumoniae escapes NETs because they produce DNADs and allow the diffusion of pneumococci from the upper airways of the lungs and from the lungs into the bloodstream during pneumonia [6]. A similar study was conducted with Mycobacterium tuberculosis, and the result showed that mycobacteria stimulate the neutrophils that release the NETs but the networks fail to destroy the bacteria with their accessory molecules [36]. So this important mechanism can be evaded.

Several experiments have been carried out to directly or indirectly induce the NETs with different microorganisms, but it has never been done with the conidia or with any structure of T. rubrum, nor with the sonic extract of conidia, so this would be the main contribution of this work. It is likely that when the conidia are destroyed by sonication, they release the molecular patterns of the fungus, and in this way the ability of the fungus to be able to parasitize is lost.

That complete conidia is needed among other conditions in order to perpetuate its species. The above, together, allows us to hypothesize that the sonicate-homogenate of conidia can have an effect similar to a vaccine, since when homogenized by sonication, the conidia breaks and does not have the danger of parasitizing, but it does have the advantage of start an immune response, what which can be applied prophylactically in the case of the prevention of infections in the skin and other sites or of infectious diseases involving neutrophils.

5. Conclusions

The conidia and their sonicate-homogenate of the pathogenic microorganism Trichophyton rubrum were placed in contact with neutrophils isolated from human peripheral blood.

The above markedly induced in vitro the neutrophils of human peripheral blood to the production of NET. It is important to conclude that the homogenate of T rubrum conidia that does not cause infection could be an immunizing and therefore protective prophylactic agent in healthy subjects exposed to the causative agent of tinea Trichophyton rubrum.

References

[1].Aga, E., D.M. Katschinski, G. Van Zandbergen, H. Laufs, B. Hansen & K. Muller. 2002. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite Leishmania major. Journal Immunol 169: 898–905.

[2].Almeida S.R. 2008. Immunology of Dermatophytoses. Mycopathologia 166: 277-283.

[3].Appelberg R. 2006. Neutrophils and intracellular pathogens: beyond phagocytosis and killing. Trends Microbiology 15: 87–92.

[4].Arenas R. 2002. Dermatofitosis en México. Revista Iberoamericana de Micología 19: 63-67.

[5].Baker, VS, GE Imade, NB Molta, P Tawde, SD Pam, MO Obadofin. 2008. Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in Plasmodium falciparum infected children under six years of age. Malaria Journal 7: 41.

[6] Beiter, K. 2006. An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. Curr. Biology 16: 401–407.

[7].Bianchi, M, A Hakkim, V Brinkmann, U Siler, RA Seger, A Zychlinsky. 2009. Restoration of NETs formation by gene therapy in CGD controls aspergillosis. Blood 114: 2619–2622.

ISSN-On line: 2414-8849

[8].Binet F, S Chiasson, D Girard. 2009. Interaction between arsenic trioxide (ATO) and human neutrophils. Immunology Res 4: 25-61.

[9].Bonifaz A. Dermatofitosis. En: Méndez editores (Ed.). Micología Médica Básica. México, 2000: 35-95.

[10].Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science: 303. 2004; 1532–1535.

[11].Brinkmann V, Zychlinsky A. Beneficial suicide: Why neutrophils die to make NETs. Nat Rev Microbiol. 2007; 5: 577–582.

[12].Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, Kotb M. DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Curr Biol. 2006; 16: 396–4.

[13].Camicia G, De Larrañaga G. Trampas extracelulares de neutrófilos: un mecanismo de defensa con 2 caras. Med Clin (Barc). 2013; 140 (2): 70-75.

[14].Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med. 2007; 13: 463–469.

[15].Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. Blood. 2008; 112: 935–45.

[16].Desloges N, Schubert C, Wolff MH, Rahaus M. Varicella-zoster virus infection induces the secretion of interleukin-8. Med Microbiol Immunol. 2008; 197: 277–84.

[17].Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007; 176: 231–241.

[18] Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers JrDD. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci. 2010; 107: 15880–5.

[19] García-Madrid LA, Huizar-López MR, Flores-Romo L, Islas-Rodríguez AE. Trichophyton rubrum manipulates the Innate Immune functions of human keratinocytes. Cent Eur J Biol. 2011; 6, (6): 902-910.

[20].Guimaráes AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, Conceicao F. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci. 2009; 106: 6748–53.

[21] Hakkim A, Furnrohr BG, Amann K, Laube 1. B, Abed UA, Brinkmann V. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci. 2010; 107: 9813–8.

[22] Hawksworth D. The magnitude of fungal diversity: the1.5 million species estimate revisited. Mycol Res. 2001; 105: 1422–1432.

[23] Hoffbrand AV, Moss PAH, Pettit JE. 2006. Essential haematology (5th ed.). Malden, MA: Blackwell Publishing.

[24] Hohl TM, Rivera A, Pamer EG. Immunity to fungi. Curr Op Immunol. 2006; 18: 1-8.

[25] Islas-Rodríguez A, García-Madrid L. Keratinocytes from human skin respond as typical immune cells alter the stimulation with Trichophyton rubrum. Nat Prec. 2007; 930.1.

ISSN-On line: 2414-8849

[26] Kopp EB, Medzhitov R. The Toll-receptor family and control of innate immunity. Curr Opin Immunol. 1999; 11: 13-18.

[27] Linehan SA., Martinez L, Gordon S. Macrophage lectins in host defence. Microbes Infect. 2000; 2: 279-88.

[28] Manzenreiter R, Kienberger F, Marcos V, Schilcher K, Krautgartner WD, Obermayer A. Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. J Cyst Fibros. 2012; 11: 84–92.

[29] Mayorga J, Muñoz F, Barba J, Hurtado NA. Dermatofitosis: Estudio epidemiológico en el Instituto Dermatológico de Jalisco (1984-1992). Dermatología Rev Mex. 1995; 39: 18-21.

[30] Medzhitov R. Inflammation: new adventures of an old flame. Cell. 2010; 140: 771–776.

[31] Méndez LJ, López R, Hernández F. Actualidades en Micología Médica. Micosis superficiales: Dermatofitos. México, Facultad de Medicina UNAM, 2004: 109-142.

[32] Nathan C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 2006; 6: 173–182.

[33] Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. Immunol Rev. 2007; 219: 88–102.

[34] Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil enastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. Cell. 2010; 191:677–691.

[35] Papayannopoulos V, Zychlinsky A. NETs: a new strategy for using old weapons. Trends Immunol. 2009; 30: 513–21.

[36] Ramos V, Mondragón R, Mondragón M, González S, Muñiz S, Rojas O. Neutrophil extracellular traps are induced by *Mycobacterium tuberculosis.* Tuberculosis Edinb. 2009; 89: 29-37.

[37] Romani L. Immunity to fungal infections. Nat Rev Immunol. 2004; 43: 1-13.

[38] Segal A. How neutrophils kill microbes. Annu Rev.Immunol. 2005; 23: 197–223.

[39] Stein J, Sonoda KH, Faunce D, Zhang J. Regulation of adaptive immune responses by innate cells expressing NK markers and antigen transporting macrophages. J Leukoc Biol. 2000; 67: 488-94.

[40] Thammavongsa V, Missiakas VD, Schneewind O. Staphylococcus aureus Degrades Neutrophil Extracellular Traps to Promote Immune Cell Death. Science. 2013; 342: 863-866.

[41] Ulevitch RJ, Tobias PS. Receptor dependent mechanism of cell stimulation by bacterial endotoxin. Annu Rev Immunol. 1995; 13: 437-457.

[42] Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol. 2006; 8: 668–676.

[43] Von Kockritz M, Goldmann O, Thulin P, Heinemann K, Norrby A, Rohde M. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. Blood. 2008; 111: 3070–80.

[44] Wartha F, Beiter K, Albiger B, Fernebro J, Zychlinsky A, Normark S. Capsule and D-alanylatedlipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps. Cell Microbiol. 2007; 9: 1162–1171.

ISSN-On line: 2414-8849

[45] Woolhouse ME, Haydon T, Antia R. Emerging pathogens: the epidemiology and evolution of species jumps. Trends in Ecology and evolution. 2005; 20: 5: 238-244.

[46] Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F. Extracellular histones are major mediators of death in sepsis. Nat Med. 2009; 15: 1318–21.

[47] Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nat Med. 2008; 14: 949–953.

[48] Zhang Q, Itagaki K, Hauser CJ. Mitochondrial DNA is released by shock and activates neutrophils via p38 map kinase. Shock. 2010; 34: 55–59.

[49] Zhou J, Yang XQ, Fu Z, Zhao XD, Jiang LP, Wang LJ. Increased patogénesis and inflammation of airways from respiratory syncytial virus infection in T cell deficient nude mice. Med Microbiol Immunol. 2008; 197: 345–51.

[50] Yu Y, Kwon K, Tsitrin T, Bekele S, Sikorski P, Nelson KE, et al. Characterization of Early-Phase Neutrophil Extracellular Traps in Urinary Tract Infections. PLoS Pathog. 2017;13(1):e1006151.

[51] Campillo-Navarro M, Leyva-Paredes K, Donis-Maturano L, González-Jiménez M, Paredes-Vivas Y, Cerbulo-Vázquez A, et al. *Listeria monocytogenes* induces Mast Cell Extracellular Traps. Immunobiology. 2016.

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Article **ECORFAN Journal**
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A Case of Inactivated Commercial Vaccine Functionality against Infectious Coryza Serotypes A, B and C *(Avibacterium Paragallinarum)* in Birds of Combat

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Abstract

Infectious coryza remains a serious problem for the poultry industry in many countries, despite the widespread use of commercial vaccines. In order to reduce costs, caused by this disease, it was applied the vaccine against infectious coryza of serotypes A, B and C to 200 birds of combat. The lines studied were Leiper Hatch, Blueface, Yellow leg, Radio, Money and crosses of these in mixed flock. The vaccine was applied at three weeks of age with a booster application two weeks (5 weeks old). The route of administration was intramuscular in the breast, in doses of 0.5 ml/animal. The vaccine was stored at a temperature between 4 and 8 °C, avoiding exposure to sunlight. Before applying the vaccine was stirred until blended and applied to a temperature of 20 °C. Of the 200 animals that were administered the vaccine, we obtained a 69% mortality attributable to infectious coryza, but 5% of mortality from other causes. The survival rate was found 28.5% (27 females and 30 males), of which 5 females and 20 males were one-eyed.

Infectious Coryza, Vaccine, Combat Birds

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1. Introduction

Biosecurity is the set of management practices designed to prevent the entry and transmission of pathogens that may affect health on farms. It is a fundamental part of any poultry business, as it provides an increase in the productivity of the flock and an increase in economic performance (Ricaurte, 2006). Infectious coryza is an infectious-contagious respiratory disease of several species of birds, whose responsible etiological agent is the bacterium Haemophilus paragallinarum (Rajurkar et al., 2009).

The disease is characterized by producing conjunctivitis, sinusitis, periorbital inflammation and nasal discharge, being common the complications with independent gram-negative microorganisms, which facilitate the magnitude of the injuries to the whole respiratory system and other internal organs, in this situation a more severe disease, called complicated coryza, which can cause high mortality (García, 2000).

The economic impact of the infection by this bacterium lies in the losses that it causes to poultry, due to growth retardation, weight loss, increase in the number of birds eliminated and predisposition to complicated chronic respiratory disease (Vargas and Terzolo, 2004). This disease is a frequent clinical problem, especially in chickens that are intended for posture, while in classic, uncomplicated coryza, lesions are generally confined to the upper respiratory tract. Sinusitis may be associated with inflammation of chins, conjunctivitis or keratitis (Colas et al., 2011).

It seems inappropriate to pay attention to the old, tired and repeated problem of infectious coryza. However, the fact remains that it remains a serious problem for the poultry industries in many countries, despite the widespread use of commercial vaccines (Bragg, 2003).

Since the bacteria that cause this old disease continue to appear and worrying, although much more is known about it than when it was first described in 1931. Its weakness and extreme adaptation to the chicken, as well as its inability to survive outside the host, indicated a real way to control it (Bickford, 1979).

The protection and control of infectious coryza, in chickens mainly, is through the use of vaccines containing inactivated bacteria of the disease (Dávila, 2010). The vaccines that the different laboratories produce are subject to strict quality controls and have made an effort to offer the resource required by the different epidemiological conditions, whether they are alive or dead, alone or combined, of different degrees of invasion, with variants or simple, of standardized or autochthonous strains, cloned and without cloning; lyophilized, frozen, etc. This great variety makes it easier to choose the most appropriate one (Flor, 2007).

2. Medical history

The present work was carried out in the farm "Caminera" in Tepic Nayarit. The reason is that year after year the problem of infectious coryza has been presented, and although medicines such as tylosin, tiamulin and sulfas have been used among others, this causes excessive expenditure, so, to reduce economic losses, the The cost of the vaccine was the best option and it was expected that this would protect the flock, therefore it was decided to apply a commercial vaccine against infectious coryza. At the time of application no bird showed signs of the disease.

The macroscopic findings were tearing, inflamed eye orbits, loss of one eye being blind or blind, see Figure 1. On the other hand, nasal discharge, sneezing and facial inflammation were observed, as well as growth retardation, weight loss and predisposition to Complicated chronic respiratory disease (Welchman et al., 2010).

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The findings at necropsy were similar to those described by Welchman et al, (2010), such as mucus in the trachea, swelling of the infraorbital sinuses accompanied by congestion of the mucous membranes of the nasal cavity and paranasal sinuses and the formation of an excess of mucus.

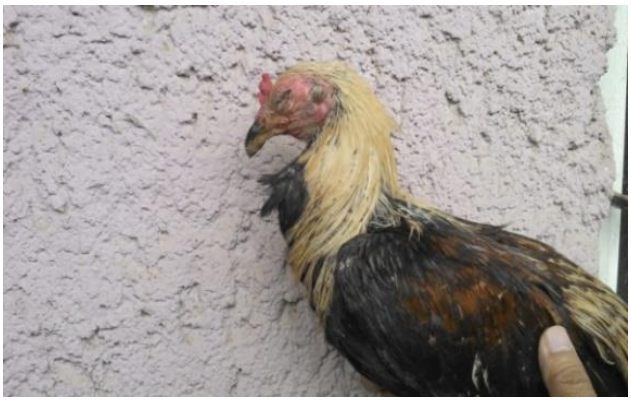

Figure 1 Fighting chicken with signs of infectious coryza

For the diagnosis, clinical signs and necropsy findings presented by the birds were taken into account together with the clinical history of the disease, which quickly spread in the geographical area where the infectious coryza is the main manifestation. In addition, microbial samples (exudates) were taken from the nasal sinuses of sick birds, using cotton swabs and subsequently inoculated, through nasal route, to 5 healthy birds (Calnek, 2000). The five birds were kept under observation and isolated from the rest of the group, and the coryza was produced 48 hours after inoculation.

As treatment, an avian bacterin in aqueous suspension was used, which contains cultures in artificial medium of avibacterium paragallinarum serotypes A, B and C, inactivated. This bacterin is used as an aid in the prevention of infectious coryza of commercial poultry. The route of administration was by intramuscular injection in the breast, in birds older than 3 weeks of age with doses of 0.5 ml per animal. The vaccine was stored at a temperature between 4 and 8 ° C.

It was applied at a temperature of 20 ° C, avoiding exposure to sunlight at all times. It was stirred well before being used until a homogeneous mixture was obtained. Two weeks after the first application, the reinforcement was administered. The vaccine was applied to 200 fighter birds of the lines Leiper-hatch, Blueface, Yellow leg, Radio, Gyros and crosses of these in mixed flock.

3. Discussion and Comments

Of the 200 animals that received the vaccine, a mortality of 69% attributable to the infectious coryza was obtained, plus a 5% mortality from other causes. The survival obtained was 28.5% (27 females and 30 males), of which 5 females and 20 males were one-eyed.

Rajukar et al (2009) mention that the complications of infectious coryza can be determined by stress factors. The natural temperament of fighter birds leads to a state of significant stress, which could determine the lack of effectiveness of the vaccine.

A possible complication of the infectious coryza with other bacterial or viral infections should also be taken (Welchaman et al., 2010), as in the complication with Mycoplasma Gallisepticum (Abd El-Ghany, 2011), which may have not allowed the effect favorable vaccine. On the other hand it can be thought that the presentation of the infectious coryza was caused by a local strain, against which the vaccine used had no effect. Blackall (1999) comments that commercial vaccines are produced in an international standard against inactivated strains of Avibacterium paragallinarum, but vaccines do not protect against local variants of Avibacterium paragallinarum, implying the need to produce vaccines specific to the region (-Ghany, 2011).

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4. Conclusions and recommendations

The vaccine did not meet the expectations expected in the protection against avian infectious coryza disease in fighting birds. However, it is important to check the cold chain to make sure that the vaccine management is adequate. It is recommended to apply the vaccine, experimentally, in a sample that is representative of the total number of birds and wait up to a week, and depending on the result, make the application to the entire flock, in order to reduce costs.

The birds of battle if they lose an eye are waste animals for which the economic losses in this operation were large.

5. References

ABD EL-GHANY, W.A. (2011). Evaluation of autogenous Avibacterium paragallinarum bacterins in Chickens. International Journal of Paultry Science. 10 (1): 56-61.

BRAGG, R.R. (2004). Limitation of the spread and impact of infectious coryza through the use of a continuous disinfection programe. Onderstepoort Journal of Veterinary Research. 71: 1-8.

BICKFORD A.A. (1979). Control del coriza en dos zonas afectadas. California Poultry Letter. 7: 1-3.

CALNEK B.W. (2000). Enfermedades de las aves. Editorial Manual Moderno. Segunda edición, México.

COLAS C.M; Lamazares M.C; Pérez G.L; Sosa T.I.M; Abeledo M.A; Merino L.A; Fuente D; Gómez Á.E. (2011). Evaluación epidemiológica de procesos respiratorios bacterianos en reemplazos de ponedoras. Rev. Salud Anim. 33 (3): 178-183.

DÁVILA G.E. (2010). Vacunas e inmunidad contra coriza infecciosa. Sistema de Revisiones en Investigación Veterinaria de San Marcos (SIRIVS). Universidad Mayor de San Marcos. http://veterinaria.unmsm.edu.pe/files/Articulo_davila_Final.pdf.

FLOR A.G. (2007). Vacunas y técnicas de vacunación. VII Congreso Nacional de Avicultura. http://www.amevea-ecuador.org/datos/GALO%20FLOR.PDF.

GARCÍA F.J.A. (2000). Estudio microbiológico de coriza infecciosa aviar en aves de postura comercial provenientes de la región de los altos de Jalisco, durante el periodo 1998-2001. Tesis de Maestría. Universidad de Colima. Tecomán, Colima.

RICAURTE G.S.L. (2006). Bioseguridad en granjas avícolas. http://www.engormix.com/MA-avicultura/sanidad/articulos/bioseguridad-granjas-avicolas-t868/165-p0.htm.

RAJURKAR G; Roy A; Mohan M.Y. (2009). An overview on epidemiologic investigations of Infectious coryza. Veterinary World. 2(10): 401-403.

ROCA C E. (1996). La patología aviar en el ultimo siglo. Universidad Autónoma de Barcelona. http://ddd.uab.cat/pub/selavi/selavi_a1996m5v38n5/selavi_a1996m5v38n5p29.pdf.

VARGAS E.S; Terzolo H.R. (2004). Haemophilus paragallinarum: etiología de la coriza infecciosa. Veterinaria México. 35 (3): 245-259.

WELCHMAN D.B; King S.A; Wragg P; *et al*. (2010). Infectius coryza in chickens in Great Britain. Veterinary Record. 912-913.

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Article **ECORFAN Journal**
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NBelyax nanoparticle test as a disinfectant agent, applied in the hands of nursing staff. Hospital de la Beneficencia Española and Hospital Nicolás San Juan, as case studies

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Abstract

This study identified the presence of pathogenic microorganisms on inert surfaces of different areas intended for direct medical care in two hospitals of the national public sector located in different places of Mexico. In these hospitals the effectiveness of the nanoparticle NBelyax as a soap and hand soft cream broad spectrum disinfectant agent was determined using the microbial process through the evaluation of the percentage reduction of Colony Forming Units (UFC). Obtaining results of 100% effectiveness for pathogenic microorganisms.

Nanoparticle, Nbelyax, Bacterial Challenge, Hospital, Hand, Handwashing, Nurses

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1. Introduction

Since the mid-nineteenth century, chemical products applied to the skin have been used in order to prevent infections. Semmelweis (1847) introduces the practice of washing hands with chlorinated compounds. Lister, years later; expanded the use of phenolic solutions, both in the hands and skin of patients and in the instruments used. (1-6)

At present, even when the use of antibiotics in clinical practice is very broad, the use of antiseptics has not been eliminated as a measure to prevent infections; On the contrary, the formulations of old substances such as Iodine have been perfected and other more elaborate formulations have appeared. (7-9). However last year, the Food and Drug Administration (FDA) of the United States, released a statement on a new regulation of the use of antiseptics. The rule, published in the Federal Register of the United States, "Safety and Effectiviness of Consumer Antiseptics; Topical Antimicrobial Drug Products for Over the Counter Human Use "(https://www.gpo.gov/fdsys/pkg/FR 2016-09-06 / pdf / 2016-21337.pdf) (10) establishes the prohibition of the use of antiseptic products, based on the recommendations of the Supervisory Committee of Drugs Without Prescription (NDAC, by its acronym in English).

The previous provision is based on the results obtained when evaluating the effectiveness of antibacterial products for washing, compared with the effectiveness of non-antibacterial products; the results showed that there are no significant differences in the effectiveness of both. However, when the effects of the ingredients of these products were evaluated it was found that they had a high risk of producing reproductive alterations and cancer. In addition to causing bacterial resistance.

The studies carried out were in vitro to evaluate the bacterial resistance and in vivo to evaluate the toxicity as endocrine and carcinogenic disruptors. The in vivo studies were of the topical or dermal type and the pharmacokinetics, dermal absorption, dermal carcinogenesis, reproductive and developmental toxicity, as well as the hormonal effects were evaluated.

In total there were 19 ingredients that presented these effects. The foregoing demonstrates the need for the use of new technologies to cover this field that remains deserted, NBelyax is a good alternative since it does not need the prohibited ingredients to be effective against pathogens. As always, NBelyax is at the forefront since it has been tested in dermal toxicity studies and does not present alterations, as well as its main asset in studies of chronic and subchronic toxicity and it does not present adverse effects when administered. At the moment more sophisticated studies are being developed to evaluate the reproductive and carcinogenic effects of our product.

1.1 Justification

There is documentary evidence of the importance of guaranteeing an inhospitable environment free of germs, pathogens or with maximum permissible levels (NMP) according to current regulations, so it is currently recommended to conduct environmental sampling to identify the existence of pathogenic microorganisms, before out-of-hospital outbreaks or in case of trying to incorporate new techniques or innovative products developed with Nanotechnology.

The case before us is to determine the antimicrobial effectiveness of the products: soap and surgical antiseptic cream for its possible incorporation in the process of asepsis, sanitization and disinfection of the hands of the nursing staff of both hospitals.

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LEON-GUTIERREZ, Gabriela, ALBARRAN, León, LEON-GUTIERREZ, Sergio and ARTEAGA-LOPEZ, Paola. NBelyax nanoparticle test as a disinfectant agent, applied in the hands of nursing staff. Hospital de la Beneficencia Española and Hospital Nicolás San Juan, as case studies. ECORFAN Journal-Republic of Guatemala. 2017.

1.2 Problem

Maintain safety and hygiene within the framework of the effectiveness of proper handwashing processes using nanotechnological innovation products that reduce the risk of intrahospital infections caused by the contact of infected material and the hands of nurses.

1.3 Objectives

1.4.1 General objectives

Identify the presence and control of pathogenic microorganisms in the hands of nurses at random.

1.4.2 Specific objectives

- Training and supervision of the proper hand washing process.
- Determine the quantitative value of germs before and after the application of surgical soap and antiseptic cream with the NBelyax nanoparticle.
- Determine the antimicrobial activity of the products with the NBelyax nanoparticle in hands, by applying the microbiology method.
- To determine the correct performance of the safety and hygiene processes as well as the effectiveness of products with the NBelyax nanoparticle.

2. Materials and methods

The process of applying soap and cream to the nursing staff was as follows:

Soap with NBelyax nanoparticle

1. Prior taking of the sample with isopos in the hands of the nursing staff.
2. Hand washing with the soap, which contains the NBelyax nanoparticle, with a duration of approximately 20 seconds.
3. Rinsing process and taking the second sample after washing.

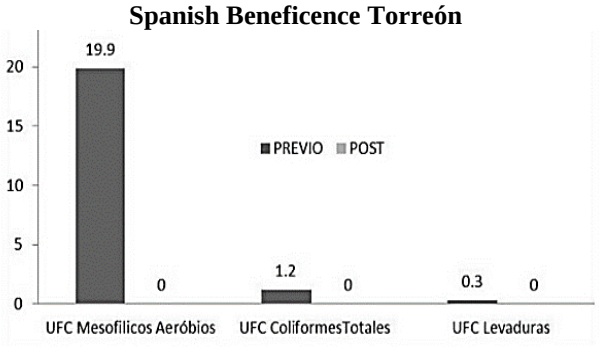
Cream with NBelyax nanoparticle

1. Prior taking of the sample with isopos in the hands of the nursing staff.
2. Application of the cream in hands containing the NBelyax nanoparticle, allowing it to be absorbed into the skin for approximately 20 to 40 seconds.
3. Process of taking the second sample after washing.

Evaluation by means of microbiological techniques and obtaining results.

3. Results

Hospital of the Spanish Beneficence of Torreón



Graph 1

Graph 1 shows the results of the effect of the use of surgical grade soap and antiseptic cream in hands. The results show the differences between the before and after use of both products on the final UFC count of mesophilic organisms, coliforms and yeasts present in the hands of the nursing staff of the Hospital de la Beneficencia Española in Torreón. It was observed that a decrease to zero of CFU is obtained after the application of both products.

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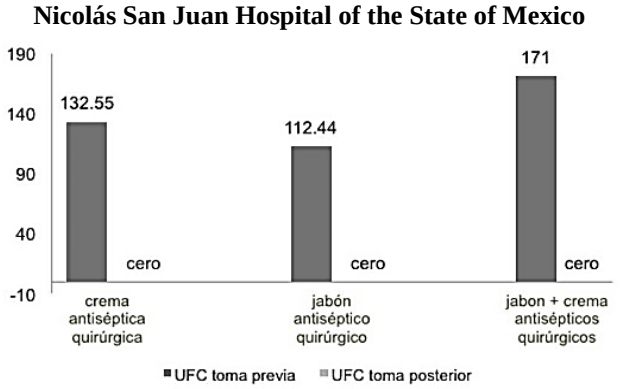

Graph 2

Graph 2 shows the results of the effect of the soap and surgical grade antiseptic cream in hands on mesophilic organisms. The results show the differences between the before and after use of both products on the final UFC count of mesophilic organisms, coliforms and yeasts present in the hands of the nursing staff of the Nicolás San Juan Hospital in the State of Mexico. It was observed that a decrease to zero of CFU is obtained after the application of both products.

4. Discussion and Conclusions

The results obtained in this study, give evidence of the bactericidal effect of the NBelyax nanoparticle in its modality as soap and cream development. The results of the sampling in two hospitals, evidence the reduction of CFU of mesophilic bacteria, coliforms and yeast present in the hands of the nursing staff of both hospitals. The results support the bactericidal effectiveness of the NBelyax nanoparticle.

It is concluded that the study objectives were fulfilled and the bactericidal effect of the NBelyax nanoparticle is based on the results of the microbiological study carried out. The study invites to develop later ones to broaden the microbiological spectrum of action.

New techno-scientific evidence was obtained as novel disinfection tools that can replace those products banned by the FDA. It is important to highlight the usefulness of reducing the presence of pathogenic organisms during the hand-washing process of medical personnel in general, particularly in hospital units that assist in procedures aimed at the prevention, control and epidemiological surveillance of Nosocomial infections.

5. References

[1] Nosocomial Infection Surveillance. 1984. MMWR CDC Surveill Summ 35 (No.1ss): 17ss, 1986. 20 -21.

[2] Wenzel RP, ed. Prevention and Control of Nosocomial Infections. 2nd ed. Baltimore: William & Wiekins; 1993. 15 -17.

[3] Edmond MB, Wenzel RP: Infection Control, Mandells Infections Diseases, Principles and Practice of Infection Diseases. 4th Edition. Churchill Livignstone Inc. 1995, New York. 102.

[4]Malagón-Londoño/Hernández-Esquivel. Infecciones Hospitalarias. Editorial Médica Panamericana. 2ª Edición. Bogotá.1999: pg 78.

[5] International Society for Infectious Diseases, Guía para el control de infecciones en el hospital, Boston MA. USA, 2000.pg 45.

[6] Haley RW, Shaberg DR et al. Estimating the extra charges and prolongation of hospitalization due to intrahospitalization infection: a comparison of methods. J. Infect. Dis. 1980;141-248

[7] Crede W, Hierholzer WJ Jr. Linking hospital epidemiology and quality assurance: seasoned concepts in a new role. Infect Control 1988;9:42.

ISSN-On line: 2414-8849

[8] Gaynes RP, & Horan TC. Surveillance of Intrahospitalaria Infections. In Mayhall CG. Hospital Epidemiology and Infection Control. Williams & Wilkins, Baltimore. 1996. Pp1017-1031.

[9] Edmond MB & Wenzel RP. Infection Control. In Mandell G, Bennett J, Dolin R. Principles and Practice of Infectious Diseases. Churchill Livingstone New York. 1995. pp2572-2575.

[10] Safety and Effectiviness of Consumer Antiseptics; Topical Antimicrobial Drug Products for Over the Counter Human Use (https://www.gpo.gov/fdsys/pkg/FR 2016-09-06/pdf/2016-21337.pdf)

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Article **ECORFAN Journal**
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Response of stomatal conductance and xylem sap abscisic acid concentration of *Sorghum bicolor* (L.) Moench cv. Tegemeo under re-watered and drought-stressed conditions

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Abstract

The sorghum plant grows in a wide range of soils and environments, and its agronomic and economic importance worldwide is incrementing yearly. This crop has several traits that make it a model for research for the study of C4 species and stress tolerance. In this research, three methods for creating a controlled environment to grow the sorghum plant using diverse substrates and nutrient solution combinations were tested. After trials, the method that was composed of sand and nutrient solution by infertile sandy soils was effective and sorghum plants were significantly bigger in height, and heavier in fresh and dry weight compared to those that were cultivated by other two methodologies. Using this artificial susbtrate, sorghum plants were grown-up and then were subjected to a drought conditions. Following the drought-stressed period, the stomatal conductance was significantly reduced and xylem sap abscisic acid concentration significantly increased by about 64% in plants developed in both substrates. This experimental system can be used for future research contributing to sorghum study of mechanisms in overcoming abiotic constrains such as drought and water relations and possible adaptation to tropical and subtropical climates.

Sorghum, Drought, ABA Xylem Sap, Stomatal Conductance

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1. Introduction

Sorghum is the fifth most important cereal in the world, and its relevance is increasing because of its low demand for water, coupled with its potential adaptation to global climate change (Tsuboi, 2017). Furthermore, sorghum is categorized as a competitive coarse grain in the world markets and as an important food supply to overcome the accelerated expansion of human population (Thomason *et al*., 2017). In the same line, sorghum planted area worldwide has been increased by 66% over the past 50 years, and the yield has improved by 2.4 fold (Qi *et al*., 2016).

This cereal is cultivated in at least 73 countries throughout Africa, America and Asia in tropical and subtropical regions (Upadhyaya *et al*., 2016). In the semiarid tropics of undeveloped countries with low income human populations, sorghum remains the primary source for human nutrition.

Countries such as the United States, Argentina, and Australia have been the major sorghum producers for primarily commercial use in livestock feed, bioenergy, and other non-food and industrial uses (Qi, 2016, O'Brien, 2016). Recently, sorghum has been useful for phytoremediation in poluted lands, whereby soil contaminants (usually heavy metals) are taken up by the plant and removed from the environment (Tsoboi, 2017).

Sorghum plants possess inherent abilities to survive and exceed under extreme temperatures and drought conditions, and it has also gained interest because contains most of the traits in a model plant species such as large embryos that are easy to rescue, plenty of seeds production, moderate genome size and available whole-genome sequences (Paterson *et al*., 2009; Calviño and Messing, 2012; Rizal *et al*., 2014).

Sorghum also has a high photosynthetic efficiency and use of water due to the effective C4 carboxylation pathway (Nielsen and Vigil, 2017) and harbors genes for higher biomass and other yield-related traits.

In spite of being one of the major crops in the world, the use of sorghum in scientific research lags far behind other cereals (Calviño and Messing, 2012). Moreover, the outcomes of sorghum research have not been used widely in crop improvement compared with other cereals like maize and rice (Izawa and Shimamoto, 1996; Rensink and Buell, 2004).

As a consequene, in order to promote sorghum as a model species it is necessary to get a better knowledge about sorghum responsivness to water deficit and drought starting with simple experimental model designs, and then scaling up to bigger trials to understand its adaptation under severe environmental conditions (Calviño and Messing, 2012; Neri-Luna *et al*., 2016; Chen *et al*., 2017).

Despite the known resilience of sorghum to drought stress, little is known about the responses to water deficit and its drought tolerance, specifically, the role of non-hydraulic root-to-shoot signalling (*i.e*. changes in concentration and flux rate of abscisic acid, ABA) considered significant in regulating shoot growth and water use when soil is drying, without any demonstrable change in shoot water or nutrient status (Hansen and Dörffling, 2003).

The xylem sap ABA concentration has a distinctive role in stomatal regulation (which is the main mechanism used by plants to control gas exchange and transpiration) of hydric status in water stress conditions (Dodd, 2003; Pospíšilová, 2003).

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NERI-LUNA, Cecilia, VILLARREAL-RUIZ, Luis, HUERTA-MARTÍNEZ, Francisco Martín and ROBLES-MURGUÍA, Celia. Response of stomatal conductance and xylem sap abscisic acid concentration of *Sorghum bicolor* (L.) Moench cv. Tegemeo under re-watered and drought-stressed conditions. ECORFAN Journal-Republic of Guatemala 2017.

The aims of this research are: (i) to compare three different methods for the establishment and growth of sorghum plants under controlled conditions in order to select the most suitable; (ii) to provide the optimum approach in order to evaluate the stomata responses to changes in the concentration of ABA in the xylem sap under a drought-stressed and re-watered period.

2. Materials and Methods

2.1. Experimental set up

In this research three different artificial shorgum culture methods suggested by Brundrett *et al*. (1996), INVAM (1997), YMRG (1996) were tested. Plastic pots (2 L, 15cm x 15cm x 12cm) were thoroughly washed with water, and disinfected with 1% (w/v) Virkon solution (Antec International Limited) and rinsed with destillated water before use (Vimard *et al*., 1999).

For each method, the pots (n= 7) were filled with the adequate substrate and the containers were watered to field capacity according to the appropriate nutrient solution (Table 1).

Sorghum bicolor (L.) Moench cv. Tegemeo seeds were washed with running tap water, and surface sterilized with 10% NaOCl for 20 min (Jarstfer and Sylvia, 1993) followed by a final rinse with running distilled water. The seeds were placed in a baker with autoclaved distilled water for 16 h in darkness to imbibe (Maiti, 1996).

Method	Substrate	[1]Particle size /disinfection	Nutrient Solution
(a) Brundrett *et al*. (1996)	Silica sand	(0.50-0.78 mm) autoclaved twice for 1 h at 121°C.	**Snowball and Robson (g/L):** (26) KH_2PO_4, (43) K_2SO_4, (21) NH_4NO_3/2 weeks, (43) $CaCl_2 2H_2O$, (6.4) $MgSO_4 7H_2O$, (4.3) $MnSO_4 H_2O$, (3) $ZnSO_4$ $7H_2O$, (1.3) $CuSO_4 5H_2O$, (0.2) H_3BO_3, (0.1) $CoSO_4 7H_2O$, (0.05) $Na_2MoO_4 2H_2O$. Plants watered twice a week with nutrient solution
(b) INVAM (1997)	Silica sand: loamy soil (2:1 v/v).	Soil (1-0.5 mm) steamed twice for 1 h at 85°C. Sand (0.50-0.78 mm) autoclaved twice for 1 h at 121°C.	**Long Ashton (g/L):** (50.6) KNO_3, (80.25) $Ca(NO_3)_2 4H_2O$, (52) $NaH_2PO_4 2H_2O$, (46) $MgSO_4 7H_2O$, (6.7) FeNaEDTA Plants watered twice a week with 50% full strength of nutrient solution
(c) YMRG (2001)	Silica sand: calcined attapulgite clay soil conditioner (50:50) + bone meal	Sand:calcined attapulgite clay soil conditioner (0.50-1.00 mm) autoclaved twice for 1 h at 121°C.	**Rorison (g/500 mL):** (62.01) $MgSO_4 7H_2O$, (119.02) $Ca(NO_3)_2 4H_2O$, (57.69) $K_2HPO_4 3H_2O$, Trace elements: (6.250) FeEDTA, (0.560) $MnSO_4 H_2O$, (0.716) H_3BO_3, (0.046) $(NH_4)_6Mo_{24} 4H_2O$, (0.110) $ZnSO_4$ $7H_2O$, (0.099) $CuSO_4 5H_2O$ Plants watered twice a week with half to one fifth strencgth of nutrient solution

[1]Gaur and Adholeya (2000).

Table 1 The three tested methods for shorgum growth under greenhouse conditions controlled conditions

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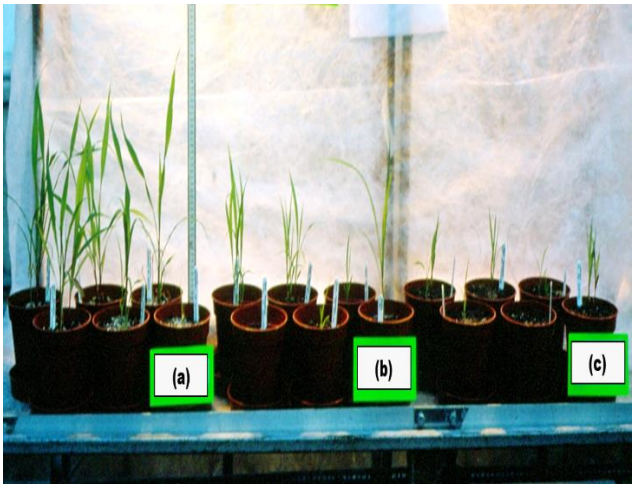

Figure 1 Experimental set up with sorghum seedlings growing with three differents methods under greenhouse controlled conditions. (a) Brundrett et al. (1996). (b) INVAM (1997). (c) YMRG (2001).

After imbibition, the sorghum seeds were distributed on a damp layer of tissue paper and placed in an envelope of aluminium foil and stored in a dark cupboard for 48 h to allow germination (Mace, 1999). After germination 5 sorghum seeds with a uniform radicle were planted in each container and covered with the substrate.

Finally, the planted pots were placed on a greenhouse bench and randomly arranged every week (Figure 1). After 2 weeks, the sorghum seedlings were thinned to 1 plant per container and received the nutrient solution and water appropriate to the corresponding treatment. Sorghum plants were cultivated in the greenhouse under controlled conditions with a photoperiod of 16h light/8h dark and a Photosynthetic Active Radiation (PAR) of 300-800 μmol m^{-2} s^{-1}. The temperature ranged from 16°C to 26°C with a relative humidity (rh) between 45-70%.

A destructive harvest was performed at 9 weeks after planting. First, the height of each plant was recorded, and the shoot components (leaves, stem, and ligules) were separated and the fresh weight (FW) was recorded.

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The plant samples were placed in paper bags, and dried at 60°C for 48 h and allowed to cool in a perspex single door desiccator chamber with silica gel and subsequently the dry weight (DW) was recorded using a Mettler AJ100 analytical balance. Statistical analyses were carried out using SPSS® v24 package.

All data was verified for normality using a Kolmogorov-Smirnov Test and for homogeneity of variances using the Levene Test. Plant parameters at harvest were compared by One-way ANOVA (Dytham, 2011). Based on these results, the following experiment was designed in order to test the sorghum response to stomatal conductance and variations in xylem sap abscisic acid concentration ([ABA_{xyl}]).

2. Stomatal conductance measurements and xylem sap collection

PVC pipe cylinders (10.5 cm diameter X 18cm height) designed to fit inside a pressure chamber were disinfected as mentioned before. Each cylinder was sealed at the base with three layers of plant fleece attached with waterproof tape. The cylinders (n=8) were packed with sand (S) substrate and watered with the nutrient solution proposed by Snowball and Robson (1984) and using the best sorghum growing system in previous experiment (Brundrett, 1996). In order to validate this artificial system, cylinders (n=8) were packed with natural field soil (NS) for comparative purposes. The natural soil is a sandy clay loam with a pH 5.9 in H_2O and 5.5 in $CaCl_2$, CEC 2.4 cmol kg^{-1}, base saturation 88.4, texture loamy sand (sand 73.9%, silt 20%, clay 6.1%), organic matter 6.4%, C_{org}3.5% and N_{org} 449.6 mgN/100g.

Sorghum seeds were washed, surface sterilized and germinated as explained before. Three germinated seeds were planted in the middle of each cylinder and the planted cylinders were placed on a greenhouse bench and randomly re-arranged every week.

After emergence, the seedlings were thinned to one per cylinder. Plants cultivated in S were watered twice per week with 120 ml of the nutrient solution (Snowball and Robson, 1984) and with deionised water at intervals (twice a week) of nutrient additions. The plants growing in NS were watered with tap water as required. They were kept in the greenhouse under the following environmental conditions: $T°C_{min}$ 23°C/$T°C_{max}$ 30°C, 16-48% rh and a mean PAR of 200 μmol m^{-2} s^{-1} at plant level for 14 h d^{-1}.

Drought period: Eight weeks after sowing, the water was withheld from half of the plants (n=4 in each substrate (S or NS). Firstly, the planted cylinders were watered to field capacity and allowed to drain overnight. In the morning, the sorghum plants were re-watered and allowed to drain until they stopped dripping, then the drought-stressed period was induced.

The initial weight of each cylinder was recorded using a digital balance (Ohaus, 5 kg capacity). This step was repeated every 4 h throughly, until the plants stopped losing weight, and at this point they were moved to the lab for xylem sap collection.

Throughout the drought-stressed period, the stomatal conductance (gs) was recorded several times using a Diffusion Porometer (AP4, ©Delta-T Devices, Cambridge, UK). Finally, the control plants (n=4) for each susbtrate, were watered as required.

Xylem sap was collected based on the method described by Seel and Jeschke (1999). The plants were sealed in the special lid of the pressure vessel (Figure 2) with the two-component silicon–based dental impression material Blend-a-gum (Coltène® PRESIDENT fast microsystem™ regular body).

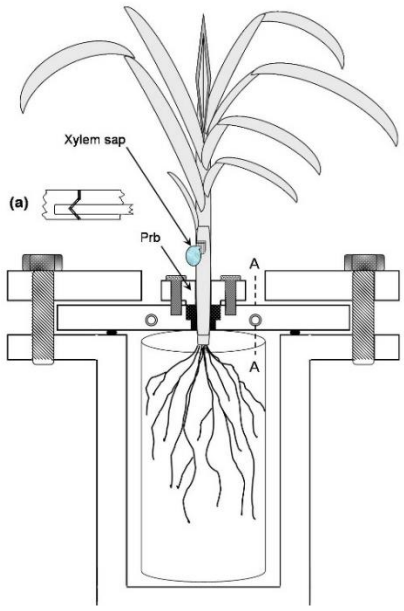

Figure 2 Schematic representation of the pressure chamber. Inset (a) shows a section through the lid along the line A-A with the V-shaped joint sealed with high vacuum grease. Sealing of the stems within the lid also is achieved by silicon-based dental impression material which is, after sealing, compressed by the pressurizing blocks (Prb)

A segment of 1 cm was cut out in the stem base with the lower cut sloping downwards at 60° to the centre to retain exuding sap.

After sealing the plants into the pressure vessel, the pneumatic pressure was raised gradually until the first drops of xylem liquid appeared; this balancing pressure *sensu* Passioura and Munns (1984) was recorded. Subsecuently, the xylem sap flow was induced by increasing the pressure to 100 kPa. Xylem sap samples (*c*. 500 μL) were collected and stored in the dark at -80°C for further analysis.

The ABA concentration was analysed in crude samples of xylem sap by the radioimmunoassay technique described by Quarrie *et al*. (1988).

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After thawing, 200 μL 50% PBS were put into 2 mL plastic centrifuge tubes. Subsequently 50 μL of ABA standard or xylem sap sample was added carefully to each tube. This solution was mixed with 100 μL of ^{3}H-ABA in γ-globulin solution (ca 9,000-10,000 cpm per vial) and 100 μL MAC252 antibody solution. The solution was mixed briefly by inverting tubes and then spun down in a microcentrifuge (Centurion 8080, Norlab) for 30s.

The assay mixture was incubated at 4°C in darkness for 45 min to allow binding to take place. Subsecuently, a saturated solution (500 μL) of $(NH_4)_2SO_4$ was added to each tube in order to precipitate the ABA-antibody complex and, after a brief shaking, the mixture was left in the dark at room temperature for 30 min. Free antibodies and those that bound to ABA in the reaction mixture were precipitated and pelleted by centrifugation for 4 min at 1400g, then the supernatant was discarded.

The pellet was washed by suspending in 1 mL of 50% saturated $(NH_4)_2SO_4$ solution in order to remove the excess unbound radioactivity (the total pellet was re-suspended by shaking the rack of vials). The tubes were centrifuged again for 4 min at 1400g and the supernatant was discarded carefully and the pellet resuspended in 100 μL of deionized water.

The tubes were again spun for 30 s and 1.25 mL of scintillation cocktail (Ecoscint-H) was added to all tubes and the content was mixed thoroughly. Finally, the tubes were put inside the glass scintillation vials and the radioactivity was quantified in a liquid-scintillation counter (Packard 4430, Berlshire, England), the tubes where count up (on protocol 8), three times for 5 min on ^{3}H setting.

Concentrations of ABA were calculated from the radioactivity (cmp) present in the pellets. A series of ABA standards was included in each batch of assays in order to construct a calibration curve. This was usually linearised by subtraction of B_{min} and plotting log-transformed counts against the natural logarithm (ln) of the unlabelled ABA present per vial, where:

$$\text{Log}\,(B/B_{max}) = \ln [B/B_{max} / 1-(B/B_{max})] \quad (1)$$

B= Corrected cpm bound in the presence of an ABA standard = (cpm-B_{min})
B_{max}= The maximal bound radioactivity when only ^{3}H-ABA reacted with MAC252 (H_2O standards).
B_{min}= The minimal bound radioactivity when a large excess of non-labelled ABA was added to the vial.

Sample ABA concentrations were calculated from this formula and the final result was expressed in pmol per mL. Statistical analyses were carried out using SPSS® v24 package. All data were verified for normality using a Kolmogorov-Smirnov Test and for homogeneity of variances using the Levene Test. An independent sample t-Test was performed in order to compare means between watered *vs.* droghted plants treatments (Dytham, 2011).

3. Results

3.1. A comparison of methods using artificial substrates

The effect of different non-natural substrates and diverse nutrient solutions on sorghum´s development was variable. For instance, sorghum plants grown using Brundrett (1996) method, were significantly bigger in height, and heavier in fresh and the dry weight compared to those that were cultivated by other methods (Table 2). It is important to pointed out that the sorghum germination process was not affected in any of the three methods used (P=0.319).

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3.2. Response of stomatal conductance and changes of [ABA_{xyl}]

The main reduction in stomatal conductance (gs) of sorghum leaves was at 53 h and 99 h after the water was withheld for plants cultivated in S and NS substrates respectively (Figure 3a). It appears that when stomatal closure (within the first 100 h after the water was suspended) the weight loss from containers stopped (Figure 3b).

The combination of stomatal conductance measurements and weight loss data clearly indicated that plants were suffering water stress. Daily weather conditions in the greenhouse during the drought-stressed period were: mean PAR of 152±8 μmol m^{-2} s^{-1} at plant height, $T°C_{min}$ 23.5°C/ $T°C_{max}$ 31°C, 16-39% rh and vapor pressure deficit between 1.7-3.4 kPa.

The mean balancing pressure (ρ_o) needed to obtain xylem sap from the stems of sorghum plants under re-watered conditions was ~150±0 kPa in both S and NS substrates. The sorghum plants subjected to a drought-stressed period needed higher balancing pressure were before xylem fluid appeared. For instance, ~525±43 kPa in those plants grown-up in sand and ~300±0 kPa in plants cultivated in NS were needed.

Following the drought-stressed period, the gs was significantly (P=0.006) reduced and [ABA_{xyl}] significantly increased (P=0.003) by about 64% in plants developed in both substrates (Figure 3c-d). During the first 8 h after withholding water, the sand substrate allowed more water loss (P<0.05) than the NS substrate, and were not observed significant differences until 76 h, when the plants developed in NS substrate lost more water.

By the end of drought-stressed period, no significant differences were found (P>0.05). It appears that sand substrate permitted more rapid gs reduction than NS, but the differences were not significant (P>0.05).

4. Discussion

Sorghum can be used as a model system for research because of its: (1) agronomic importance, (a) C4 photosynthesis, (b) low input levels of nutrients, (c) adaptation to stresses due to its reservoir of genes for tolerance to high salt, pH, drought and heat; (2) increase of its economic significance in areas as a valuable source of food, biofuel and phytoremediation (Tsuboi *et al.*, 2017); (3) representative of large genomes like sugarcane and switchgrass (Paterson *et al*., 2009); (4) ease to produce different types of breeding materials (Upadhyaya *et al*., 2016); (5) the big plant size

Plant parameters	Brundrett *et al.*	INVAM	UYRG	P
Germination (%)	77.7 ±7.02 a	49.9±16.6 a	55.5±14.0 a	NS
Height (cm^{-1})	32.19 ±3.52 b	30.2±3.55 b	18.57±2.13 a	**
Leaves				
FW (g)	1.55±0.41 b	0.50±0.08 a	0.31±0.10 a	**
DW (g)	0.44±0.09 b	0.14±0.02 a	0.07±0.01 a	**
Stem				
FW (g)	0.85±0.25 b	0.23±0.04 a	0.14±0.06 a	*
DW (g)	0.19±0.04 b	0.05±0.009 a	0.02±0.007 a	***
Total				
FW	2.41±0.67 b	0.74±0.12 a	0.46±0.16 a	**
DW	0.63±0.13 b	0.19±0.02 a	0.10±0.02 a	***

(§) Plants were harvested 9 weeks after plantation. Means ±1SE (n=7). Significance values were calculated using One-Way ANOVA at 95% CI and Student-Newman-Keuls Test. *P≤0.05; **P≤0.01; ***P≤0.001, NS= Not significant. **FW**= Fresh Weight; **DW**= Dry Weight.

Table 2 Parameters of sorghum plants (§) using three different techniques

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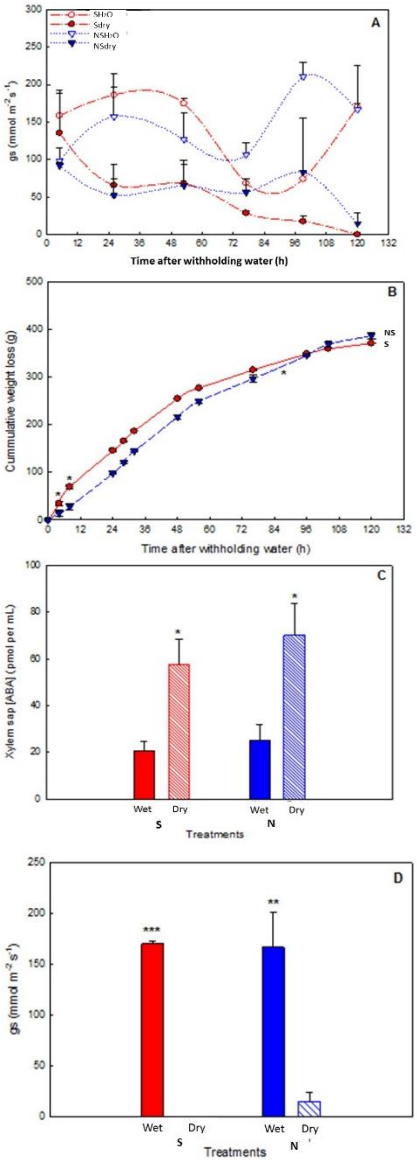

Figure 3 Response of sorghum plants under re-watered and drought-stressed conditions grown in S and NS substrates. (A) gs in leaves. (B) Cumulative weight loss. (C) Xylem sap ABA concentration. (D) gs in leaves before harvest (gs in drought-stressed plants cultivated in sand was zero). Data represent ±1SE of the mean (n=4). Significance values were calculated using a t-Test. *P≤0.05; **P≤0.01; ***P≤0.001.

With wide leaves, make it suitable for physiological measurements, leaf anatomical analysis, growth and development studies (Roozeboom and Prasad, 2016).

However, despite all this, other crop plants like maize and rice have still remained considered the primarly models for the study of cereals and C4 species (Izawa and Shimamoto, 1996; Strable and Scalon, 2009). Surprisingly, the outcomes of *Arabidopsis* research have been used widely in crop improvement (Rensink and Buell, 2004).

Therefore, it is important to consider sorghum, for biological research on stress tolerance from harsh environmental conditions due to its valuable agronomic traits (Rizal *et al*., 2014). Given the multifunctionality responses by plants to environmental factors, some researchers have pointed out the need of studies under controlled conditions in order to evaluate the results more carefully (van der Heijden and Kuyper, 2001; Lendzemo *et al*., 2005).

Therefore, in this research to get a better knowledge about sorghum responsiveness to water deficitency and drought tolerance, diverse artificial procedures (instead of the complicate use of natural soil) were tested. Our results indicate that the substrate and nutrient solution suggested by Brundrett *et al.* (1996) was effective and allowed sorghum to grow well under the greenhouse controlled environmental conditions.

Furthermore, in order to validate this experimental system, the sensitive parameters such as stomatal conductance and [ABA_{xyl}]) suggested in other drought experiments (Dodd, 2003; Hansen and Dörffling, 2003; Pospíšilová, 2003) were chosen to detect sorghum plants response when subjected to a drought-stressed period.

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The results shown that stomatal response and changes in xylem sap concentration were detectable and there were not different from those obtained using field natural soil, thus, confirming that using sand as substrate with the proper nutrient solution is suitable for further research.

Because, the germplasm of sorghum is extraordinarily diverse (Upadhyaya *et al*., 2016), could be a key species for the expanding knowledge about the functions of beneficial crops in agriculture, especially in hot and dry regions of the world.

For instance, the method used for growing sorghum under controlled conditions could be useful for trials involving sorghum germoplasm collections in order to identify new sources of variations for stress resistance, phenology, seed yield and quality, and for bioenergy uses (Upadhyaya *et al*., 2016). In addition, this system can be useful to know: (1) how sorghum proceeds from one stage to another, (2) how it accumulates dry matter along the way, and (3) how it partitions the dry matter to tissue and grain, essential for understanding how a sorghum plant is likely to respond to environmental factors (Roozeboom and Prasad, 2016).

On the other hand, a common problem in trying to scale up results from greenhouse to natural environment is the uncertainty about whether the effects that take place in these unrealistic conditions occur in field conditions (Rillig, 2004). Furthermore, in nature, plant biodiversity, fitness and adaptation rely on the development of roots in combination with soil microorganisms with complementary functions in order to promote plant growth and health. Therefore, it could be important to generate proposals involving soil microorganism that may influence water relations or lessen drought straining in sorghum plants.

5. Conclusion and future research

The method proposed by Brundrett (1996) was proven as a good alternative for sorghum development under greenhouse controlled conditions.

Data from sorghum plants grown-up in this system related to stomatal conductance and xylem sap ABA concentration subjected to a drought-stressed period were successfully measurable and were not different from those obtained using field natural soil.

Therefore, this experimental system seems to be reliable for future research in supporting the idea of sorghum as a model cereal crop. Most importantly, sorghum can be study in order to understand the plant advanced mechanisms in overcoming abiotic constrains such as water relations and drought in its development of strategies for crop stress tolerance and adaptation to harsh environmental conditions around the world.

6. References

Brundrett M, Bougher N, Dell B, Grove T and Malajczuck N. 1996. Working with mycorrhizas in forestry and agriculture. ACIAR Monograph 32: 374 p.

Calviño M and Messing J. 2012. Sweet sorghum as a model system for bioenergy crops. Current Opinion in Biotechnology 23: 323–329.

Chen J, Chopra R, Hayes C, Morris G, Marla S, Burke J, Xin Z, and Burow G. 2017. Genome-Wide Association Study of Developing Leaves' Heat Tolerance during Vegetative Growth Stages in a Sorghum Association Panel. Plant Genome 10 (2): 1-15.

Dytham C. 2011. Choosing and using statistics: a biologist's guide. 3rd Edition. Blackwell Publishing. United Kingdom. 292 p.

ISSN-On line: 2414-8849

Dodd IC. 2003. Hormonal interactions and stomatal responses. Journal of Plant Growth Regulation 22: 32-46.

Gaur A and Adholeya A. 2000. Effects of the particle size of soil-less substrates upon AM fungus inoculum production. Mycorrhiza 10: 43-48.

Hansen H and Dörffling K. 2003. Root-derived trans-zeatin riboside and abscisic acid in drought-stressed and rewatered sunflower plants: interaction in the control of leaf diffusive resistance. Functional Plant Biology 30: 365-375.

INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi at West Virginia University, USA). http://invam.caf.wvu.edu/.

Izawa T and Shimamoto K. 1996. Becoming a model plant: the importance of rice to plant science. Trends in Plant Science 1(3): 95-99.

Jarstfer AG and Sylvia DM. 1993. Inoculum production and inoculation strategies for vesicular-arbuscular mycorrhizal fungi. *In*: Metting FB Jr (Ed.). Soil microbial ecology: Applications in agricultural and environmental management. Marcel Dekker, New York. pp. 349-377.

Jarstfer AG and Sylvia DM. 1993. Inoculum production and inoculation strategies for vesicular-arbuscular mycorrhizal fungi. *In*: Metting FB Jr (Ed). Soil microbial ecology: Applications in agricultural and environmental management. Marcel Dekker, New York. pp. 349-377.

Lendzemo VW, Kuyper TW, Kropff MJ and van Ast A. 2005. Field inoculation with arbuscular mycorrhizal fungi reduces *Striga hermonthica* performance on cereal crops and has the potential to contribute to integrated *Striga* management. Field Crops Research 91: 51-61.

Mace YJ. 1999. When the going gets tough: the responses of *Sorghum bicolor* L. cv. Tegemeo seedlings to soil penetration resistance. BSc. Thesis. Department of Plant and Soil Science. University of Aberdeen, UK. pp. 28-31.

Maiti R. 1996. Sorghum Science. Science Publishers, Inc. USA. 352 p

Neri-Luna C, Villarreal-Ruiz L, Huerta-Martínez FM and Robles-Murguía C. 2016. Response to drought of sorghum (*Sorghum bicolor* (L.) Moench cv. Tegemeo) grown with a split-root system: a climate change perspective. UTSOE-Journal of Multidisciplinary Science 3 (6): 54-72.

Nielsen DC and Vigil MF. 2017. Defining a Dryland Grain Sorghum Production Function for the Central Great Plains. Agronomy Journal 109: 1582–1590.

O'Brien D. 2016. Domestic and International Sorghum Marketing. *In*: Ciampitti I. and Prasad V. (Editors). Sorghum: State of the Art and Future Perspectives. Agronomy Monograph 58. doi:10.2134/agronmonogr58.2014.0079.

Passioura JB and Munns R. 1984. Hydraulic resistance of plants. II. Effects of rooting medium, and time of day, in barley and lupin. Australian Journal of Plant Physiology 11: 341-350.

Paterson AH, Bowers JE, Bruggmann R, Dubchak I, Grimwood J, Gundlach H, Haberer G, Hellsten U, Mitros T, Poliakov A. 2009. The *Sorghum bicolor* genome and the diversification of grasses. Nature, 457: 551-556.

ISSN-On line: 2414-8849

Pospíšilová J. 2003. Participation of phytohormones in the stomatal regulation of gas exchange during water stress. Biologia Plantarum 46(4): 491-506.

Qi G, Li N, Sun XS and Wang D. 2016. Overview of Sorghum Industrial Utilization. *In*: Ciampitti I. and Prasad V. (Editors). Sorghum: State of the Art and Future Perspectives. Agronomy Monograph 58. doi:10.2134/agronmonogr58.2014.0070.

Quarrie SA, Whitford PN, Appleford NEJ, Wang TL, Cook SK, Henson IE and Loveys BR. 1988. A monoclonal antibody to (S)-abscisic acid: its characterization and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. Planta 173: 330-339.

Rensink WA and Buell CR. 2004. Arabidopsis to Rice. Applying Knowledge from a Weed to Enhance Our Understanding of a Crop Species. Plant Physiology 135(2): 622-629.

Rillig MC. 2004. Arbuscular mycorrhizae and terrestrial ecosystem processes. Ecology Letters 7: 740-754.

Rizal G, Karki S, Alcasid M, Montecillo F, Acebron K, Larazo N, Garcia R, Slamet-Loedin IH, and Quick WP. 2014. Shortening the Breeding Cycle of Sorghum, a Model Crop for Research. Crop Science 54: 520–529.

Roozeboom KL and Prasad P.V.V. 2016. Sorghum Growth and Development. *In*: Ciampitti I. and Prasad V. (Editors). Sorghum: State of the Art and Future Perspectives. Agronomy Monograph 58. doi:10.2134/agronmonogr58.2014.0062.

Seel WE and Jeschke WD. 1999. Simultaneous collection of xylem sap from *Rhinanthus minor* and the hosts *Hordeum* and *Trifolium*: hydraulic properties, xylem sap composition and effects of attachment. New Phytologist 143: 281-298.

Snowball K and Robson AD. 1984. Comparison of the internal and external requirements of wheat, oats and barley for copper. Australian Journal of Agricultural Research 35: 359-365.

Strable, J and Scalon MJ. 2009. Maize (*Zea mays*): a model organism for basic and applied research in plant biology. Cold Spring Harb Protoc. doi:10.1101/pdb.emo132.

Thomason W, Chim BK, Holshouser D, Behl H, Balota M, Xia K, Frame W and Black T. 2017. Comparison of Full-Season and Double-Crop Soybean and Grain Sorghum Systems in Central and Southeastern Virginia. Agronomy Journal 109: 1532–1539.

Tsuboi K, Shehzad T, Yoneda J, Uraguchi S, Ito Y, Shinsei L, Morita S, Rai H, Nagasawa N, Asari K, Suzuki H, Itoh R, Saito T, Suzuki K, Takano I, Takahashi H, Sakurai K, Watanabe A, Akagi H, Tokunaga T, Itoh M, Hattori H, Fujiwara T, Okuno K, Tsutsumi N, and Satoh-Nagasawa N. 2017. Genetic Analysis of Cadmium Accumulation in Shoots of Sorghum Landraces. Crop Science 57: 22–31.

Upadhyaya HD, Dwivedi SL, Wang Y-H and Vetriventhan M. 2016. Sorghum Genetic Resources. *In*: Ciampitti I. and Prasad V. (Editors). Sorghum: State of the Art and Future Perspectives. Agronomy Monograph 58. doi:10.2134/agronmonogr58.2014.0056.5.

Van der Heijden EW and Kuyper TW. 2001. Does origin of mycorrhizal fungus of mycorrizal plant influence effectiveness of the mycorrhizal symbiosis? Plant and Soil 230(2): 161-174.

ISSN-On line: 2414-8849

Vimard B, St-Arnaud M, Furlan V and Fortin JA. 1999. Colonization potential of *in vitro*-produced arbuscular mycorrhizal fungus spores compared with a root-segment inoculum from open pot culture. Mycorrhiza 8: 335-338.

YMRG (York Mycorrhiza Research Group). 2001. Techniques in arbuscular mycorrhiza research. Laboratory Manual. Department of Biology. The University of York. UK. 51 p.

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Optimization and characterization of the immobilization process of trypsin in calcium alginate beads

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Abstract

The aim of this study was to optimize a procedure for the immobilization of trypsin on calcium alginate (Ca-alginate) and to compare the structure of the Ca-alginate beads based on their stability, porosity and enzymatic activity using bovine serum albumin (BSA) as a source of hydrolyzable protein. The optimal reagent concentrations used in the immobilization process were determined using a central composite routable design. The sodium alginate ($C_6H_7O_6Na$) and calcium chloride ($CaCl_2$) concentrations that yielded the highest enzymatic activity and immobilization rate were 2.92% (w/v) and 1.28 M, respectively. The beads generated under these conditions had a pore size of 10 μm. The stability of immobilized trypsin under these optimized conditions was evaluated using residual enzyme activity assays. There was a 30% decrease in enzymatic activity after 8 rounds of reuse, indicating that this immobilization process could produce a stable biocatalyst. The optimum pH and temperature profile were at pH 8.5 and 50 ºC, in addition, the thermal and storage stability of the trypsin was increased upon immobilization. The kinetic parameters K_m and V_{max} were 5.46 mM/min and 6100 μmol/mL/min, respectively.

Trypsin, Immobilization, Repeated Batch, Biocatalyst, Kinetic Parameters

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Introduction

Alginate is a naturally occurring structural component of marine brown algae and the capsular polysaccharide of some soil bacteria. It is a linear binary copolymer consisting of β-(1,4)-linked D-mannuronic acid (M) and α-L-guluronic acid (G) residues. The physical properties of alginate depend on the sequence of M and G residues as well as the average molecular weight and molecule-weight distribution of the polymer.

Alginate has some unique properties with respect to non-toxicity, biocompatibility, biodegradability, chelating ability, and hydrophilicity and is currently being used as an important biopolymer in the production of microcapsules designed for drug delivery, tissue engineering (Chico, Camacho, Pérez, Longo, Sanromán, Pingarrón & Villalonga, 2009; Sun, Wang, Zhong & Jiao, 2008; Wang, Wenk, Hu, Castro, Meinel, Wang, Li, Merkle & Kaplan, 2007) and the immobilization of enzymes, such as β-galactosidase, *Saccharomyces cerevisiae* invertase or fungal phenol oxidase (Quiroga, Llanes, Ochoa & Barberis, 2011; Won, Kim, Kim, Park & Moon, 2005; Taqieddin & Amiji, 2004).

Ca-alginate beads are one of the most widely used carriers for enzyme immobilization because they offer several advantages over enzymes in free solution including facilitation of the recovery and reuse of enzymes. Enzyme immobilization can be an alternative for the use of enzymes in food applications, including the use of trypsin, which can hydrolyse proteins into bioactive peptides (Muro-Urista, Álvarez-Fernández, Riera-Rodriguez, Arana-Cuenca, Téllez-Jurado, 2011), or in analytical applications, including biosensors, which are an essential quality control method used in medicine and food production.

In general, the trypsin immobilized can be consumed directly, improving blood circulation thereby increasing the supply of immune substances in the affected tissue (Srivastava & Singh, 2013).

Trypsin is the most widely used enzyme in protein fragmentation and selectively cleaves at lysine and arginine residues, typically resulting in peptides that have a mass range suitable for high-resolution/high-sensitivity mass mapping using mass spectrometry. The concept of using immobilized enzymes in protein analysis has received much attention in recent years because it offers several advantages over previous protocols (Kakuta, Takashima, Nakahata, Otsubo, Yamaguchi & Harada 2013; Ma, Zhang, Liang, Zhang & Zhang, 2007) including larger enzyme to substrate ratios, higher digestion efficiencies, and the possibility of repeated use.

Moreover, the rate of enzyme denaturation or inactivation is lower with immobilized enzymes than with free enzymes, which frequently lose their catalytic activity fairly quickly, leading to a lack of reproducibility. Immobilized trypsin can be used in batch-wise experiments or packed into columns and used in flow systems, such as immobilized-enzyme reactors (IMERs) (Ma *et al.* 2007).

Currently, immobilized proteins/enzymes are used routinely in the medical field to diagnose and treat various diseases. For example, immobilized antibodies, receptors, or enzymes are used in biosensors and ELISAs to detect various bioactive substances diagnostic of different disease states. Encapsulated enzymes are also used in bioreactors to remove waste metabolites and to correct inborn metabolic deficiencies (DiCosimo, McAuliffe, Poulose & Bohlmann, 2013).

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Although several supports have been tested for immobilizing this enzyme including polyelectrolyte microcapsules (PEMC), silica gel-supported macroporous chitosan beads, magnetic glycidyl methacrylate-based nanoparticles and ether sulfone and vinylidene fluoride membranes (Shen, Guo, Qi, Qiao, Wang & Mao, 2013; Starke, Went, Prager & Schulze, 2013; Garbers, Mitlöhner, Georgieva & Bäumler, 2007; Xi, Wu, Jia & Lin, 2005), Ca-alginate beads are advantageous because they increase enzyme stability compared to other supports (Quiroga *et al.* 2011) and have not been studied previously.

The concentrations of $CaCl_2$ and $C_6H_7O_6Na$ should be taken into account to avoid the diffusion of nutrients and product through the porous matrix. Ca-alginate beads have a wide pore-size distribution due to the open lattice structure of the matrix, which results in the release of biomacromolecules from these matrixes during storage. Alginate beads are also used to control enzyme diffusion into the surrounding solution (Ding, Shan, Xue, Zhu & Cosnier, 2009).

The aim of this present study was to optimize the concentrations of $CaCl_2$ and $C_6H_7O_6Na$ for trypsin immobilization and to compare the structure of the Ca-alginate beads based on their stability, porosity and enzymatic activity using bovine serum albumin (BSA).

Methodology

Materials

Sodium alginate ($C_6H_7O_6Na$), trypsin from bovine pancreas, bovine serum albumin (BSA) and $CaCl_2$ were obtained from Sigma-Aldrich. The viscosity of the 1 % (w/w) aqueous $C_6H_7O_6Na$ solution at 20 ºC was measured with a Rapid Visco-Analyzer (RVA, 3C, Newport Scientific PTY Ltd., Sydney, Australia) following the instruction manual, as 720 cps.

Experimental Design

A response surface method central composite routable experimental design was applied to determine the relationship between the independent variables ($C_6H_7O_6Na$ and $CaCl_2$ concentrations) and their responses as well as their interactions in an effective model. According to the model, it contains four full factorial design points, four axial points, and five central points. Two variables and two responses were involved in the experimental design.

The dependent variables measured were enzymatic activity and immobilization rate. The independent variables are the sodium alginate concentration and the $CaCl_2$ concentration. Preliminary trials were conducted before applying the design to determine the conditions under which the process yielded beads. The process variables and their respective levels are listed in Table 1. The data were analysed using the program Statistic 7.0 for Windows.

Preparation of Beads

The immobilization procedure was performed using the drip method reported by Ding *et al.* (2009). Different concentrations (Table 1) of $C_6H_7O_6Na$ and $CaCl_2$ were evaluated. The beads were prepared by dripping $C_6H_7O_6Na$ into a solution (10 mL) containing trypsin (1.37 U/mg protein). The resulting beads were stored in a $CaCl_2$ solution at 4 °C for 2 h to complete gel formation. The solution was then decanted, and the beads were collected and washed with deionized water for subsequent use.

Determination of enzyme activity

The enzyme activity was determined in triplicate using the method described by Kunitz (1965) modificated, as substrate was used as a BSA.

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The reaction mixture was made up of 100 μL of the free enzyme or two beads in a solution of 100 μL Tris-HCl buffer and 200 μL of the BSA dissolved in Tris-HCl 0.05 M, pH 8. These were incubated at 37 ºC for 1 h, and then the reaction was stopped by adding 500 μL of 10% trichloroacetic acid (TCA) (J. T. Baker). This was centrifuged at 13000 rpm for 5 min.

One unit of activity was defined as the amount of enzyme required to release 1 μmol of tyrosine per minute, under the assay conditions described.

Determining the immobilization rate

The enzyme:substrate complexes immobilized were filtered using quantitative cellulose filter paper (Whatman) under a vacuum, and washed three times with 50 mL of distilled water. The filtrates and flow-through were collected to determine the unabsorbed enzyme content using the Lowry method as modified by Peterson (1977) with BSA as a standard. The same method was used to quantify the protein in the enzymatic solution. The quantity of immobilized enzyme was calculated based on the difference between the proteins content in the enzymatic solution and the content in both the filtrates and flow-through combined. The immobilization rate was calculated using equation (1):

$$Immobilized\ enzyme\ (\%) = \frac{\text{Amount of immobilized protein}}{\text{Amount of initial protein}} * 100 \quad (1)$$

Determination of the optimal pH and temperature for the immobilized enzyme

The optimal pH of free and immobilized trypsin was carried at pH range 4.0-9.0 at 37 ºC using the proteolytic activity assays according to the method of Kunitz (1965) modificated, as substrate was used as a BSA. The optimal temperatures of the free and immobilized enzyme were determinate in the range 20-60 ºC at their optimal pH value.

Stability studies

The thermal stability of free and immobilized trypsin was determined by measuring the residual enzymatic activity of two different temperatures (55 y 65 ºC) in phosphate buffer (0.1 M, pH 7.5) for 150 min. After every 30 min time interval, a sample was removed and assayed for enzymatic activity as describe Bayramoğlu, Yilmaz & Arica, (2004). The results were given as percentage activity. Activity of the free and immobilized trypsin was expressed as a percentage of this residual activity compared to that of the initial activity.

Repeated use studies

The reusability of trypsin immobilized was evaluated by measuring its enzymatic activity before and after up to 20 repeated uses using the Lowry method as modified by Peterson (1977).

The immobilized enzyme activity (U/mg protein) was defined as amount of immobilized enzyme required to catalyse the hydrolysis of 1 μmole of BSA per minute. The reusability study was performed in triplicate.

Determination of the kinetic parameters

The kinetic studies of the free and enzyme and immobilized enzyme were conducted by measuring the activity with BSA as the substrate in accordance with the activity assay described above; the substrate concentration varied from 1.25 to 2.50 mg/mL. The apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of immobilized trypsin, were calculated using the Lineweaver and Burk method and compared with the free enzyme.

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Scanning Electron Microscopy

The beads were mounted on brass stubs using double-sided adhesive tape, and Micrographs were taken with a scanning electron microscope (JSM- 6610LV, Jeol Ltd, Tokyo, Japan) at the required magnification (x1000) at room temperature. An acceleration voltage of 20 kV was used, and the image was detected with the secondary electron.

Results

Optimization

A central composite design was used to identify the optimal conditions for trypsin immobilization including the $C_6H_7O_6Na$ and $CaCl_2$ concentrations. After defining the experimental matrix, the experiments were conducted, and the initial activity (Y) of the immobilized enzyme was determined as shown in Table 2.

The variables that displayed a significance level lower than 0.05 (Prob>F) in these experiments were most relevant to the model. The results indicate that the $CaCl_2$ concentration (B), the square of the $C_6H_7O_6Na$ concentration (A^2), the $C_6H_7O_6Na$ concentration (A) and the square of the $CaCl_2$ concentration (B^2) were significant, as shown in equation (2):

$$EA = 39.33 + 3.94 * A + 63.73 * B + 69.25 * A^2 + 3.86 * B^2 \quad (2)$$

Where A and B are the coded values of the concentrations of $C_6H_7O_6Na$ and $CaCl_2$ concentrations, respectively, as shown in Table 2 and EA is enzyme activity.

The coefficient of determination (R^2) of the equation was 97 %, indicating that the experimental data were adequately adjusted and that these variables affect enzyme activity.

Therefore, 97 % of the variability in the experimental data could be explained by the empirical equation. Three additional experiments were performed under optimal conditions to validate the model, and the average enzymatic activity of these tests was 121.4 ± 0.42 U/mg protein. Based on the model [Equation (1)], the maximum enzyme activity was 128.9 ± 1.7 U/mg protein when 3 % $C_6H_7O_6Na$ and 1.36 M $CaCl_2$ were used (Treatment 4). To illustrate the effects of these variables on enzymatic activity, the response surface of these variables was constructed and is presented in Graphic 1.

The region of maximum enzymatic activity had higher alginate and $CaCl_2$ concentrations, therefore, the different concentrations of $C_6H_7O_6Na$ showed different levels of substrate diffusion into the beads. This is due to cross-linking, which is the main problem associated with the use of immobilized enzymes, especially when enzymes are acting on macromolecular substrates (Bencina, Bencina, Podgornik & Strancar, 2008). Because access of the substrate to the enzyme active site is limited, the accessible surface groups of the substrate also limit enzymatic activity.

This steric hindrance may consequently change the characteristic enzymatic cleavage patterns of the products derived from macromolecular substrates (Brena & Batista-Viera, 2006) and the immobilization rate was determined in order to examine these changes in our system. The results of the tests were compared with those reported by Ertan, Yagarb & Balkan (2006) who described that the optimum $C_6H_7O_6Na$ and $CaCl_2$ concentrations needed to achieve maximum enzymatic activity for *Aspergillus sclerotiorum* α-amylase immobilization were 3 % (w/v) y 0.3 M, respectively.

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Potumarthi, Subhakar, Pavani & Jetty (2008) described a statistically optimized calcium-alginate immobilization method for the production of *Bacillus licheniformis* NCIM-2042 alkaline protease in which they found that the optimum $C_6H_7O_6Na$ and $CaCl_2$ concentrations were 2.78 % (w/v) and 0.2 M, respectively. Similarly, a thermostable *Bacillus subtilis* α-amylase was immobilized by entrapment in Ca-alginate gel capsules and repeatedly used in batch processes of starch hydrolysis. The authors of this study found that the optimum $C_6H_7O_6Na$ and $CaCl_2$ concentrations for immobilization were 2 % (w/v) and 0.45 M, respectively (Konsoula & Liakopoulou-Kyriakides, 2006).

Thus, the maximum enzymatic activities for the different enzymes were achieved at $C_6H_7O_6Na$ concentrations of approximately 3 % (w/v). However, the $CaCl_2$ concentrations in the previous reports cited were lower (between 0.2 and 0.45 M) than those used in the present study (1.36 M), which may be due to the size of the trypsin substrate. Wang and Caruso (2005) reported that it is important to control the pore size of the beads and to treat the beads with an agent that encapsulates the enzyme (for example: polyvinyl diallylammonium, poly (allylamine hydrochloride) or $CaCl_2$ to form cross-links that prevent enzyme leakage. The inherent nature of the interactions in this method of entrapment provides high levels of activity and resistance to pH and temperature changes (Volodkin, Larianova & Sukhorukov, 2004).

Graphic 2 shows the influence of the $C_6H_7O_6Na$ and $CaCl_2$ concentrations on the immobilization rate (%) of trypsin, illustrating that at higher $C_6H_7O_6Na$ and $CaCl_2$ concentrations, the immobilization rate of trypsin increases.

These data are likely attributable to more complete gelation of the matrix due to the increased concentration of guluronic acid residues, which react with the calcium ions to form a matrix structure that is firmer. Sankalia, Mashru, Sankalia & Sutariya, (2006) reported that the degree of crosslinking is higher at higher $C_6H_7O_6Na$ concentrations given that increased contact time favours the efficiency of encapsulation. Likewise, when the $C_6H_7O_6Na$ concentration is low, the immobilization efficiency and substrate diffusion into the beads decreases. These data are confirmed by the steric restrictions mentioned above (Mahajan, Gupta & Sharma, 2010).

With the use of response surface methodology and overlapping surfaces, the optimal level of enzyme immobilization was calculated to find the optimal enzyme activity and immobilization rate values that would maximise enzymatic activity.

The conditions predicted by this model (Equation 2) for trypsin immobilization were then used to experimentally validate the model. We initially selected the central point of the optimal concentrations to ensure data reproducibility. The concentrations of the variables at the maximization point were: X_1= 2.92 % $C_6H_7O_6Na$ (w/v) and X_2= 1.28 M $CaCl_2$ (Graphic 3). Under these conditions, we achieved an enzymatic activity of 122 U/mg protein and an immobilization rate of 95 %, defined as the ratio of the enzymatic activity of the immobilized enzyme and the total activity of the soluble enzyme.

Effect of pH and temperature on activity

The effect of pH on the activity of free and immobilized trypsin was carried out in the pH range 4.0-9.0 and the results are presented in Graphic 4.

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Optimal conversion was obtained at pH 8 for the free enzyme, and optimum pH value for the immobilized enzyme was shifted to more alkaline region about pH 8.5. The immobilized trypsin has also a different pH activity profile and was broadened both in alkaline and acidic region. This displacement toward to more alkaline region could be possibly due to the secondary interaction between the enzyme and polymeric support (ionic interaction). Other researchers have reported similar observations upon immobilization of trypsin and other enzymes (Bayramoğlu, 2004).

The temperature dependence of the activities of the free and immobilized trypsin was studies in the temperature range 20-60 ºC (Graphic 5). The date revealed bell-shaped curves with optimum activity at 37 ºC for the free and at 50 ºC for the immobilized enzymes. The increase in optimum temperature was cause by the changing physical and chemical properties of the enzyme.

Thermal stability of free and immobilized trypsin

Thermal stability experiments were carried out with the free and the immobilized trypsin, which were incubated in the absence of substrate at two different temperatures. The Graphic 6 shows the heat inactivation curves at 55 and 65 ºC for the free and immobilized enzyme, respectively. The pattern of heat stability indicated that a smaller rate of thermal inactivation was observed for the immobilized enzyme.

At 55 ºC, the free enzyme retained 38% of its initial activity after 120 min of heat treatment, while the adsorbed enzyme showed significant resistance to thermal inactivation, retaining about 81 % of its initial activity after the same period.

At 65 ºC, the free enzyme retained 10 % of its initial activity after a 120 min of heat treatment, on the other hand, the immobilized trypsin was inactivated at much slower rate than the native form and the immobilized form showed significant resistance to thermal inactivation because retain about 63 % of its initial activity after the same time period.

These results suggest that the thermo stability of adsorbed trypsin becomes significantly at higher temperature. It should be noted that the higher stability of immobilized trypsin could partly be caused by the limitation of autolysis and partly by the protein conformational sytabilization. The extent of autolysis in the inactivation of trypsin is difficult to estimate in the native enzyme.

Moreover, in these experiment calcium ions were added to buffer solutions to limit auto destruction of enzyme. If the heat stability of enzyme increased upon adsorption, the potential application of this enzyme would be extended. Increased thermal stability has been reported for a number of adsorbed enzymes, and the polymer network and multi-point attachment in ionic adsorption method are supposed the tertiary structure of trypsin (Bayramoğlu, 2004).

Repeated use studies

The reusability studies were performed using the optimal conditions for trypsin immobilization, and the results are shown in Graphic 7. The relative activity for the rounds of reuse was calculated relative to the first batch, which was designated to have 100 % relative activity. There were no significant differences in the enzymatic activity of immobilized trypsin after 4 rounds of reuse, and the activity decreased by 30 % after 8 rounds of reuse.

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These results can be explained by the mechanical damage caused by rupturing the beads and thus releasing the enzyme from the beads. Similarly, Won *et al.* (2005) reported a 72 % loss of activity after 3 rounds of reuse for a lipase immobilized in alginate beads. These data are also consistent with the reports by Konsoula & Liakopoulou-Kyriakides (2006), who found that α-amylase immobilized on Ca-alginate beads retained 90 % of its initial efficiency after 20 batches of starch hydrolysis when immobilized with 2 % (w/v) and 0.45 M of $C_6H_7O_6Na$ and $CaCl_2$, respectively.

The results in the present study indicate that $C_6H_7O_6Na$ is a better immobilization substrate for trypsin than silica gel-supported macroporous chitosan beads when only retains 70 % of its activity after 4 rounds of reuse (Xi *et al.* 2005), whereas it retained 70 % of its activity after 8 rounds of reuse in our study.

Free and immobilized enzyme kinetic parameters

Kinetic analyses of the free enzymes and immobilized enzyme were conducted at optimal pH (8.5) and temperature (50 ºC) using the proteolytic activity assay with BSA as substrate. The Michaelis-Menten constant was determined using double-reciprocal plots showing $1/V$ *vs* $1/[S]$.

The kinetic parameters (K_m y V_{max}) for the free enzyme the K_m value was found to be 12.75 mM/min, and the V_{max} was calculated to be 5621 μmol/mL/min. K_m value was found to be 5.46 mM/min for the immobilized enzyme and the V_{max} was calculated to be 6100 μmol/mL/min. The K_m for the immobilized trypsin was significantly greater compared with the free enzyme, which might be due to the diffusional resistance of the gel. Similar changes in the K_m were also reported for Wang, Chen, Wang & Xing (2014).

Scanning electron microscopy studies

The scanning electron microscopy study was performed to topographically characterize the internal structure of the Ca-alginate beads containing trypsin and evaluate the relationship between the bead structure and trypsin activity. As shown in Figure 1, the Ca-alginate beads have irregular forms while maintaining a constant lattice-form array on the surface of the beads. In addition, when the concentrations of $C_6H_7O_6Na$ and $CaCl_2$ are increased, the surface has fewer folds.

The reduction in the number of folds is likely due to an increase in the viscosity and a delay in the penetration of calcium into the beads, resulting in a decline of cross-liking, roughness and porosity on the surface of the beads (Sankalia *et al.* 2006). Kakuta *et al.* (2013) reported that the structure, porosity and Ca-alginate size of the beads are influenced by the pore size used to extrude the alginate solution and its viscosity. These characteristics are very important because the pore size of the beads should be suitable enough for the substrate to enter and hydrolysis to occur (Potumarthi *et al.* 2008; Wu, Wang, Li, Lin & Wei, 2010).

Wu *et al.* (2010) also reported that the structure, porosity and Ca-alginate bead size are influenced by the opening used to extrude the $C_6H_7O_6Na$ solution and its viscosity, and in a decrease in the enzymatic activity of immobilized enzyme relative to soluble enzyme. Figure 2 shows a photomicrograph of the cross-section of the Ca-alginate beads containing trypsin under optimum conditions (trypsin immobilized activity of 120.87 U/mg protein using $C_6H_7O_6Na$ and $CaCl_2$ concentrations of 2.92 % (w/v) and 1.28 M, respectively) an x1000 magnification.

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The beads had an irregular lattice structure with an approximate pore size of 10 μm, which would allow the substrate to enter the beads and hydrolysis to occur, which is shown by the fact that there was measurable enzymatic activity.

Conclusions

The results of this study indicate that the optimal conditions for trypsin immobilization were 2.92 % $C_6H_7O_6Na$ and 1.28 M $CaCl_2$. These conditions resulted in pore sizes of 10 μm, which provides an optimal immobilization rate and maximum enzymatic activity. The enzyme immobilized under these conditions retained 70 % of its initial activity after 8 rounds of reuse. The optimum pH and temperature profile were at pH 8.5 and 50 ºC, in addition, the thermal and storage stability of the trypsin were increased upon immobilization. The kinetic parameters K_m and V_{max} were 5.46 mM/min and 6100 μmol/mL/min, respectively.

References

Bayramoğlu, G. Yilmaz, M. Arica, M.Y. (2004). Immobilization of a thermostable a-amilase onto reactive membranes: Kinetics characterization and application to continuous starch hydrolysis, Food Chem, 84, 591-599.

Bencina, M., Bencina, K., Podgornik, A., Strancar, A. (2008). Monolithic bioreactors for macromolecules, Method Mol. Biol, 421, 257-274.

Brena, B.M., Batista-Viera, F. (2006). Immobilization of enzymes and cells. In: Methods in Biotechnology. Guisan JM (ed). 2nd Humana Press Inc., Totowa, NJ, pp. 15-30.

Chico, B., Camacho, C., Pérez, M., Longo, M., Sanromán, M.A., Pingarrón, J.M., Villalonga, R. (2009). Polyelectrostatic immobilization of gold nanoparticles-modified peroxidase on alginate-coated gold electrode for mediator less biosensor construction, J. Electroanal Chem, 629, 126-132.

DiCosimo, R., McAuliffe, J., Poulose, A.J., Bohlmann, G. (2013). Industrial use of immobilized enzymes, Chem. Soc. Rev, 42, 6437-6474.

Ding, S.N., Shan, D., Xue, H.G., Zhu, D.B., Cosnier, S. (2009). Glucose oxidase immobilized in alginate/layered double hydroxides hybrid membrane and its biosensing application, Anal. Sci, 25, 421-1425.

Ertan, F., Yagarb, H., Balkan, B. (2006). Optimization of α-amylase immobilization in calcium alginate beads, Prep. Biochem. Biotech, 37, 195-204.

Garbers, E., Mitlöhner, R., Georgieva, R., Bäumler, H. (2007). Activity of immobilized trypsin in the layer structure of polyelectrolyte microcapsules (PEMC), Macromol. Biosci, 7, 1243-1249.

Kakuta, T., Takashima, Y., Nakahata, M., Otsubo, M., Yamaguchi, H., Harada, A. (2013). Preorganized hydrogel: self-healing properties of supramolecular hydrogels formed by polymerization of host-guest-monomers that contain cyclodextrins and hydrophobic guest groups, Adv. Mater, 25, 2849-2853.

Konsoula, Z., Liakopoulou-Kyriakides, M. (2006). Starch hydrolysis by the action of an entrapped in alginate capsules α-amylase from *Bacillus subtilis*, Process Biochem, 41, 343–349.
Kunit, N. (1965). Methods of enzymatic analysis. 2nd ed. Velag, Chenie. Acad. Press N.Y. London, pp. 807- 814.

ISSN-On line: 2414-8849

Ma, J., Zhang, L., Liang, Z., Zhang, W., Zhang, Y. (2007). Monolith-based immobilized enzyme reactors: Recent developments and applications for proteome analysis, J. Sep. Sci, 30, 3050-3059.

Mahajan, R., Gupta, V.K., Sharma, J. (2010). Comparison and suitability of gel matrix for entrapping higher content of enzymes for commercial applications, Indian J. Pharm. Sci, 72, 223-228.

Muro-Urista, C.R. Álvarez-Fernández, F. Riera-Rodríguez, Arana-Cuenca, A. Téllez-Jurado, A. (2011). Review: Production and functionality of active peptides from milk, Food Sci. Technol. Int, 17, 293-317.

Peterson, G.L. (1977). A Simplification of the protein assay method of Lowry *et al*. which is more generally applicable, Anal. Biochem. 83, 346-356.

Potumarthi, R., Subhakar, C.H., Pavani, A., Jetty, A. (2008). Evaluation of various parameters of calcium-alginate immobilization method for enhanced alkaline protease production by *Bacillus licheniformis* NCIM-2042 using statistical methods, Bioresource Technol, 99, 1776–1786.

Quiroga, E., Llanes, C.O., Ochoa, N.A., Barberis, S. (2011). Performance improvement of araujiain, a cystein phytoprotease, by immobilization within calcium alginate beads, Process Biochem, 46, 1029-1034.

Sankalia, M.G., Mashru, R.C., Sankalia, J.M., Sutariya, V.B. (2006). Papain entrapment in alginate beads for stability improvement and site-specific delivery: Physicochemical characterization and factorial optimization using neural network modeling, *AAPS* Pharmscritech, 6, 209-222.

Shen, Y., Guo, W., Qi, L., Qiao, J., Wang, F., Mao, L. (2013). Immobilization of trypsin via reactive polymer grafting from magnetic nanoparticles for microwave-assisted digestion, J. Mater. Chem. *B*, 1, 2260-2267.

Srivastava, P.K., Singh, S.A. (2013). Review: Immobilization and applications of glucose-6-phosphate dehydrogenase, Prep Biochem Biotech, 43, 376-384.

Starke, S., Went, M., Prager, A., Schulze A. (2013). A novel electron beam-based method for the immobilization of trypsin on poly (ethersulfone) and poly (vinylidene fluoride) membranes, React. Funct. Polym, 73, 698-702.

Sun, W., Wang D.D., Zhong, J.H., Jiao, K.J. (2008). Electrocatalytic activity of hemoglobin in sodium alginate/SiO_2 nanoparticle/ionic liquid BMIMPF6 composite film, J. Solid State Electr, 12, 655-661.

Taqieddin, E., Amiji. M., (2004). Enzyme immobilization in novel alginate–chitosan core-shell microcapsules, Biomaterials, 25, 1937-1945.

Volodkin, D.V., Larianova, N.I., Sukhorukov, G.B. (2004). Protein encapsulation via porous $CaCO_3$ microparticles templating, Biomacromolecules, 5, 1962-1972.

Wang, W.Q., Wenk, E., Hu, X., Castro, G.R., Meinel, L., Wang, X., Li, C.M., Merkle, H., Kaplan, D.L. (2007). Silk coatings on PLGA and alginate microspheres for protein delivery, Biomaterials, 28, 4161-4169.

Wang, Y. Caruso, F. (2005). Mesoporous silica spheres as supports for an enzyme immobilization an encapsulation, Chem. Mater, 17, 953-961.

ISSN-On line: 2414-8849

Wang, Y., Chen, H., Wang, J., Xing, L. (2014). Preparation of active corn peptides from zein through double enzymes immobilized with calcium alginate–chitosan beads, Process Biochem, 49, 1682-1690.

Won, K., Kim, S., Kim, K.J., Park, H.W., Moon, S.J. (2005). Optimization of lipase entrapment in Ca-alginate gel beads, Process Biochem, 40, 2149-2154.

Wu, J., Wang, J.L., Li, M.H., Lin, J.P, Wei, D.Z. (2010). Optimization of immobilization for selective oxidation of benzyl alcohol by *Gluconobacter oxydans* using response surface methodology, Bioresource Technol, 101, 8936-8941.

Xi, F., Wu, J., Jia, Z., Lin, X. (2005). Preparation and characterization of trypsin immobilized on silica gel supported macroporous chitosan bead, Process Biochem, 40, 2833-2840.

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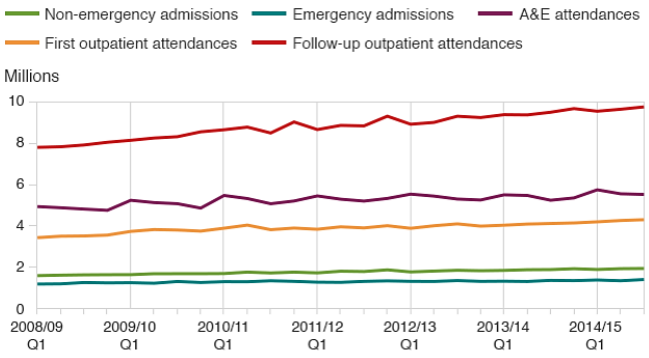

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