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As a first chapter we present, *Process of technology inclusion in a forest SME*, by QUINTANAR OLGUIN, Juan, with adscription in INIFAP - CIRGO. CE San Martinito, as a second article we present, *Biochemical study of intracellular proteases from the phytopathogenic fungus Sporisorium reilianum*, by PÉREZ-RODRÍGUEZ, Joany, MENDOZA-MELGOZA, Cristina Guadalupe, HERNÁNDEZ-MONZALVO, Maricela, ANDUCHO-REYES, Miguel Angel and MERCADO-FLORES, Yuridia, with affiliation in the Universidad Politécnica de Pachuca, Universidad Autónoma del Estado de Hidalgo and the Instituto Politécnico Nacional, as the third chapter we present, *Telemetric system implementation for monitoring physical parameters within an entomological greenhouse*, by TOBÓN-GONZÁLEZ, Fernando, CUATEPOTZO-JIMÉNEZ, Vanessa, PÉREZ-JARILLO, Elsi, CERVANTES-CAMACHO, Ilse and ROBLES-CAMARILLO, Daniel, with affiliation at the Universidad Politécnica de Pachuca, as fourth article we present, *Electrical and Mechanical Analysis of a Natural Biopolymer*, by QUIROZ-VELAZQUEZ, Víctor Eduardo, PEREZ-RUIZ, Víctor Hugo and DIAZ-GUTIERREZ, Carlos Eduard or, with ascription in the Universidad Autónoma Metropolitana, as fifth article we present, *Effect of the fractions of beeswax hydrolysates as carbon source in the synthesis of short and medium chain polyhydroxyalkanoates (scl-mcl-PHA)*, by QUINTANAR-GÓMEZ, Samuel, TÉLLEZ -JURADO, Alejandro and VARGAS-HERNÁNDEZ, Genaro, with adscription at the Universidad Politécnica de Pachuca.

Content

Article **ECORFAN Journal-Ecuador**
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Process of technology inclusion in a forest SME

Proceso de inclusión de tecnología en una PyME forestal

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Abstract

Given the current need to have technology as a basis for economic development, acquiring technology appropriate to the needs of a microenterprise is a complex process that requires clear definitions, to ensure that both the bidder and the recipient of the technology clearly understand its implications. Traditionally, acquiring technology has been understood as the purchase of instruments, machinery or equipment, ignoring the acquisition of inherent knowledge, which is conceptualized as technology transfer, which is specifically what companies require to improve their production and competitiveness. In this context, it is urgent to change the paradigm of the process of acquiring equipment through the transfer of technology in its broader conceptualization. In the present work, a forestry SME acquires a technology applying a methodology based on the theory of open innovation (response to an identified need) and applying the concepts of continuous improvement, so that the technology to be acquired responds to its need, under the conditioning of "learning by doing", this so that users see "live" the benefits of the technology acquired, by creating new skills and new habits to consolidate changes based on the creation of new cultural values.

Process Technology Transfer, Continuous Improvement, Adoption Level

Resumen

Ante la necesidad actual de contar con tecnología como base del desarrollo económico, adquirir una tecnología apropiada a las necesidades de una microempresa es un proceso complejo que requiere definiciones claras, para asegurarse que tanto el ofertante como quien recibe la tecnología entienden claramente sus implicaciones. Tradicionalmente, adquirir tecnología se ha entendido como la compra de instrumentos, maquinaria o equipos, ignorando la adquisición del conocimiento inherente, que se conceptualiza como transferencia de tecnología, que es concretamente lo que requieren las empresas para mejorar su producción y competitividad. En este contexto, es urgente cambiar el paradigma del proceso de adquirir equipo por el de transferencia de tecnología en su conceptualización más amplia. En el presente trabajo, una Pyme forestal adquiere una tecnología aplicando una metodología basada en la teoría de la innovación abierta (respuesta a una necesidad identificada) y aplicando los conceptos de mejora continua, para que la tecnología a adquirir responda a su necesidad, bajo la condicionante de “aprender haciendo”, esto para que los usuarios vean “en vivo” las bondades de la tecnología adquirida, mediante la creación de nuevas competencias y nuevos hábitos para consolidar los cambios sobre la base de la creación de nuevos valores culturales.

Proceso Transferencia Tecnología, Mejora Continua, Nivel Adopción

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Introduction

In the current globalization, the notion of competitive advantage based on access to resources at lower costs, has become obsolete. Nowadays, the most competitive companies are not those with access to lower cost resources, but those that use the most advanced technologies and methods when using their resources. The term technology is a word composed of Greek origin formed by the words tekne ("art, technique or trade") and logos ("set of knowledge").

There is a number, definitions of this. Herrera (2006) defines technology as the set of specific knowledge and processes to transform reality and solve a problem. For Córdoba (2015), technology refers to the set of theories and techniques that allow the practical use of scientific knowledge, and the set of industrial instruments and procedures of a specific sector or product. Martinez (2017) defines it as the set of knowledge that makes possible the creation of products and the design of the processes that make this possible. This definition implies the need for man to have the knowledge and skills to know-how and know-how to use technology.

Traditionally in the agricultural and forestry sector of the country, it has been understood to acquire technology such as the purchase of instruments, machinery or equipment and the transfer of its technology, such as dissemination events, generation of publications or training and technical assistance. in which the acquirer of the technology, is a passive receiver who must apply the "new" technology as it is offered.

However, technology transfer is a concept that acquires ever greater relevance, with more reason in the framework of a globalized society and should be understood as the process that allows the transfer of technical, economic and commercial knowledge, between one organization and another, as well as the learning that stimulates the capitalization of the generated knowledge, that is, the applicability of it to configure it in economic benefits (De Ossa *et al*, 2018).

Therefore, when talking about technology transfer we refer to all those processes necessary for the productive sector to access and take advantage of existing technology. It should be understood that the acquisition and transfer of technology does not always have to come from an external entity, it can occur within the receiving economic actor from a group that improves a part of the process and extends it towards the whole production.

Therefore, the introduction of a technology to the company not only refers to its purchase and installation, but also includes the identification of the technological needs of the future users, as well as the learning and assimilation activities related to the acquired technologies (Velásquez y Medellín; 2005).

In academic terms, the concept of technology transfer is defined as the process of knowledge transfer necessary for the manufacture of a product, the management of a process or the provision of a service (González and Fernández, 2008, De Ossa et al, 2018). And it must be understood as an interactive sequence of activities that lead to the adoption of a new technique or knowledge and that involves dissemination, demonstration, training and other activities that result in the reduction of the uncertainty of innovation (Batista, 2009; Manjarres *et al*, 2013).

In addition, the definition of technology transfer differs substantially from one discipline to another. Economists tend to define technology based on generic knowledge, focusing on variables related to design and production. On the other hand, sociologists tend to unite technology transfer to innovation, including social technology. Anthropologists tend to consider the transfer of technology within a context of cultural change and the way in which technology affects this change.

It is also essential to distinguish between technology transfer and technology diffusion. The transfer is inherent in the existence of an agreement and an economic transaction, which is not included in the dissemination process, which consists of the disclosure of potentially innovative technological knowledge (Guede, 2011).

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According to the above, and identifying that the Mexican forestry sector is of a traditional type, characterized by the low proportions of use of advanced knowledge in production and that seeks to use those technologies that are in circulation even when they are not appropriate to the technological needs of their own development, rather they are technological packages of generic type and largely incorporated in equipment and / or productive processes (Quintanar, 2012). The objective of this study is to describe the process used by a forestry SME to acquire and include a new technology in its production process, from the moment the technological need was determined to its adoption.

Technology inclusion model

The model that was applied to acquire and include a process technology in a small wood furniture manufacturing company, located in Manzanillo, Colima, combines a series of proposals made by different authors (Halty and Martínez, 1973; Velásquez and Medellín, 2005). González, 2009, Suárez et al, 2012, Tejera, 2013, Rincóny Peláez, 2013, Sarmiento et al, 2018) and consists of six basic steps according to the sequence presented in Figure 1 and described below:

Step 1. Evaluation of the need to acquire a new technology. The process of acquisition and transfer of technology began with the determination and characterization of the current technology in use and evaluating the need to replace it with a new technology, foreseeing the costs of the replacement process. Also, the possibility of improving current technology was valued, with this the risk, scope and type of technology required was dimensioned.

Step 2. Detection of technological offer. As traditionally available technology is acquired, either by its proximity or by the novelty it represents, without an exhaustive search of options or offer of available technologies to cover or solve the detected need. In this step, the characteristics and costs of the possible technologies to be acquired should be clearly analyzed, in order to decide on the most appropriate, based on the principle that it should be better than the technology in current use.

There must also be clarity that these technologies must be "finished" technologies, *that is, that they are not a simple idea to be developed or tested.*

Step 3. Evaluation and selection of an appropriate technological alternative. For the selection of the technological alternative the following fundamental criteria must be taken into account:

- The technology selected as an alternative to be transferred must be a "finished" technology.
- The new technology must surpass the technology that is currently being used or that dominates the acquirer.
- Take into account the costs of the existing technological options.
- Have a budgetary provision for the application of the new technology.
- Take into account the new technological alternatives that are perceived in the short term.
- Find out what technologies are being developed or acquired by competitors.

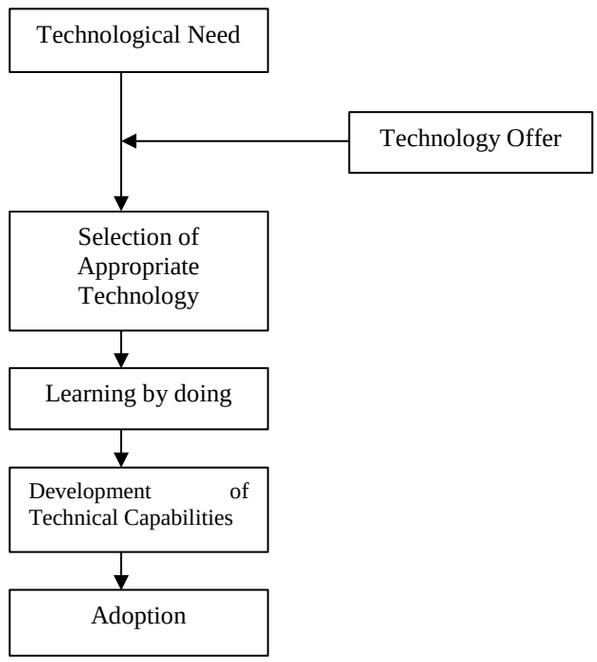

Figure 1 Model for acquisition and transfer of technology in the forestry industry

Step 4. Adaptation and implementation of the technological alternative. Once the appropriate technological alternative to current needs has been selected, for its implementation it is recommended that participants who receive the new technology analyze and determine the training needs, basically the critical variables of the new practice and that require immediate attention, generating a commitment to start improving those variables. Which, later will be evaluated by themselves, under the principle that small changes generate incremental achievements in traditional practices, optimizing the process over time.

Step 5. Development of new technical capabilities. As a fundamental part for the implementation of the new technology that is acquired, they must carry out training courses related to the knowledge and practice related to the technology acquired, in order to develop and strengthen the new technical capabilities required to operate it.

Step 6. Adoption of technology. Finally, to show the degree of mastery of the technology acquired, the participants demonstrate their new skills and competencies in situ through a public demonstration. Thus, the acquisition process and its transfer of technology ends at the moment that the recipients of the same, "apply" it on a routine basis. All under the premise, that the technology that is acquired or transferred must surpass the technology currently used by the acquiring company (Quintanar, 2015). In addition, it has passed the test of economic practice under the criteria of efficiency and profitability (Mendoza, 2010).

Results of the model application

The process of acquisition and inclusion of technology in a forestry microenterprise faces a series of problems or barriers. It should be considered that the introduction of a technology in a microenterprise not only refers to its purchase and installation, but also includes the identification of its technological need and the possible solutions of suppliers, as well as learning and assimilation activities for the use of the acquired technologies (Kearns et al, 2005).

The micro manufacturer of furniture with wood located in Manzanillo, Colima, for years has had a problem in the finished pieces, either furniture or white works (Table 1). Therefore, their need is the acquisition of a technology that allows them to reach and manage a moisture content in their raw material, appropriate to the final use of it (step 1).

When searching for specialized information on technological options for the wood drying process (step 2), the results show several options for managing wood moisture, from sophisticated vacuum technologies, conventional drying or solar drying technologies, between them there is a very high differential in the cost of infrastructure and its operation.

Step	Description	Assessment of activities
1	Needs assessment	Need to achieve appropriate moisture contents in raw material
2	Detection of technological offer	Feasible drying methods: - Conventional - Solar - Fresh air
3	Evaluation and selection	Appropriate drying method for low volumes and low cost
4	Adaptation and implementation	Construction of solar dryer and testing with the type of raw material of the acquirers.
5	Development of capabilities	Training related to the handling of the dryer and the humidity in the wood.
6	Adoption	Demonstration and routine use of technology

Table 1 Process of inclusion of solar drying technology

Thus, once assessed their need, and given the low volumes of consumption of dry wood that normally have and not having financial resources for the acquisition of a conventional dryer, the proposal of appropriate solution to their current needs, was the acquisition of solar wood drying technology, through the installation of a solar dryer and its respective advice for its implementation (step 3).

Once the technological option to be acquired was defined, a greenhouse-type solar dryer was built and installed. A course-workshop was also held on basic concepts and practical issues of wood drying (step 4).

This was done to provide the necessary knowledge to the producers about the drying of the wood and that the participants have the same level of information about it and not to raise expectations higher than those inherent to the process itself.

Once built and installed, we proceeded to test it with wood that users provide. In addition, a new training was carried out for the user personnel, on the principles of solar drying, in addition, practices were carried out on stacking the wood, taking and recording the temperature and relative humidity data. Basic data to later propose drying routines with which the user can manage the dryer more efficiently (Step 5).

With the solar dryer in operation, a demonstrative event of the solar drying process was carried out, with an invitation to the general public (step 6).

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According to the sequential application of this methodology, the results are consistent with the analysis of the implementation of technology as a functional strategy to strengthen productive and competitive quality in SMEs (Córdoba, 2015). However, before starting the process of evaluating the need for technology in the microenterprise, the need to implement technology in entrepreneurs to improve profitability, reducing costs and increasing their productivity must be aroused (Zamora, 2013).

On the other hand, when the methodology is applied correctly and the technological need is dimensioned in a concrete way, this facilitates the selection of that technology that covers practically in its entirety the need of the acquirer of this technology (Figure 2). In addition, its use is immediate because it is a technology tailored to its requirements, therefore, the result of learning and adopting such technology is high and in a short period of time, it is used routinely in the productive process of the acquirer.

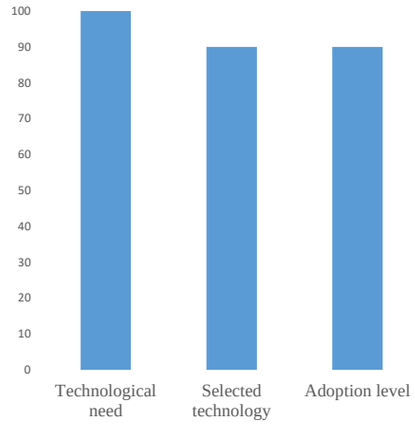

Figure 2 Expectation level of adoption of a selected technology using this methodology in relation to a technological need

Conclusions

- The transfer of technology applied under a methodical process, generates improvements in the productive processes of those who receive the transfer of a new technology.
- The appropriation of a technology requires the participation of the personnel that will use it, so that when selecting it, it will satisfy their technological needs.
- The process of technology transfer ends until the receiver of the same, "applies" it routinely or adopts it.

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Article **ECORFAN Journal-Ecuador**
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Biochemical study of intracellular proteases from the phytopathogenic fungus *Sporisorium reilianum*

Estudio bioquímico de proteasas intracelulares del hongo fitopatógeno *Sporisorium reilianum*

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Abstract

The intracellular proteolytic system of the phytopathogen *Sporisorium reilianum* is not well-understood. This work reports the presence of at least three intracellular proteases. The activity of dipeptidyl aminopeptidase (psrDAPi), carboxypeptidase (psrCPi) and proteinase A (psrPAi) were measured under several nutritional conditions. All enzymes were detected in the soluble and membrane fractions, and in membrane fractions. Zymographic analysis demonstrated that this fungus contains at least one soluble psrDAPi and two membranal isoforms. psrCPi was found to be regulated by an endogenous inhibitor. The effect of different proteases inhibitors was evaluated against the intracellular enzymes in study.

Head smut, Intracellular proteases, *Sporisorium Reilianum*

Resumen

El sistema proteolítico del hongo fitopatógeno *Sporisorium reilianum* no había sido estudiado. En este trabajo se reporta la presencia de al menos tres proteasas intracelulares. Las actividades de, dipeptidil aminopeptidasa (psrDAPi), carboxipeptidasa (psrCPi) and proteinasa A (psrPAi) fueron medidas bajo diferentes condiciones nutricionales. Se encontró que todas las enzimas fueron detectadas en la fracción soluble y asociadas a la membrana. El análisis zimográfico demostró que este hongo contiene al menos una isoforma soluble y dos en membranales. psrCPi se encuentra regulada por la presencia de un inhibidor endógeno. Se determinó el efecto de diferentes inhibidores de proteasas sobre las enzimas encontradas en este estudio.

Carbón de la espiga, Proteasas intracelulares, *Sporisorium Reilianum*

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Introduction

S. reilianum is the causal agent of head smut, a disease that affects corn worldwide. This basidiomycete infects corn plants during seed germination, but symptoms do not become visible until flowering time, with the presence of phyllody and carbonaceous masses of teliospores that invade the male inflorescences and cobs. These fungal structures are later released and can fall or be disseminated by wind or rain. Under optimal conditions, the teliospores geminate in the soil where they produce a basidium with four haploid basidiospores that bud in the same way as a yeast. In young plant tissues, two sexually-compatible basidiospores fuse to form an infective dikaryotic hypha that penetrates the plant's epidermis through an appressorium. Colonization is progressive and the mycelium can be observed in all plant tissues, though sporulation only occurs in the tassels and ears [1, 2, 3, 4].

Proteolysis is a vital process for cells due to the important functions that it performs, such as utilizing exogenous proteins as nutrients, eliminating non-functional proteins, maintaining amino acid pools, and post-translational control [5]. The survival of cells in their natural environment depends on their ability to adapt to frequent changes. Proteolysis plays an important role in responding to stress caused by nutrient starvation, variations in pH, temperature and UV radiation, and the presence of heavy metal ions or toxins. One example of proteolysis is spore formation in *Saccharomyces cerevisiae* when the carbon source available is poor. This process manifests high proteolytic activity that provides amino acids to the cells for new protein synthesis and energy generation [6].

S. cerevisiae has been employed as a study model to elucidate intracellular protease functions in eukaryotic cells, leading to the biochemical and genetic characterization of several of their proteolytic enzymes [5, 6, 7]. Intracellular proteolytic enzymes have also been identified in other yeasts of biotechnological interest, such as *Schizosaccharomyces pombe* and *Yarrowia lipolytica* [8, 9].

The intracellular proteolytic system of phytopathogenic fungi has only been reported for the basidiomycete *Ustilago maydis*, which produced the intracellular proteinases pumA and pumB, as well as aminopeptidase pumAPE and dipeptidyl aminopeptidase pumDAP [10]. In this fungus, the *pep4* gene that encodes a vacuolar proteinase, A, is involved in dimorphism and pathogenesis [11]. Also, the X-prolyl-dipeptidyl aminopeptidase encoded by *dapUm* gene has been cloned and transformed in *Pichia pastoris* for pharmaceutical purposes [12]. However, the intracellular proteolytic system of *S. reilianum* remains largely unknown. The study of its proteases localized in the interior of the cell will contribute to increasing our knowledge of this phytopathogenic fungus.

Materials and Methods

Throughout this study, the intracellular proteases from *S. reilianum* are identified by the prefix "psr" (for protease *S. reilianum*) while "i" indicates intracellular activity.

Microorganism and culture conditions

The *S. reilianum* diploid strain was provided by Dr. Santos Gerardo Leyva Myr of the Universidad Autónoma Chapingo, Mexico. It was isolated from corn crops in the state of Hidalgo in central Mexico. *Saccharomyces cerevisiae* DBY was used as the reference strain in this study. It was kindly provided by Dr. María Paz Suárez Rendueles of the Universidad de Oviedo, Spain. The strains were routinely maintained on YEPD plates (0.1% yeast extract, 2% peptone, 2% glucose, 2% agar) at 28°C. *S. reilianum* was conserved in inclined tubes with the same medium and mineral oil at room temperature, while *S. cerevisiae* stored in glycerol at 25% at -70°C.

S. reilianum was grown in liquid cultures in YEPD with minimal medium (0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate and 2% Glucose) [8] and different sources of nitrogen: 1% peptone, 0.5% proline, 0.5% ammonium sulfate or corn infusion (4 mg of protein/mL of medium). The corn infusion was obtained as described Mercado-Flores et al. [10].

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Production of intracellular proteases

The production of intracellular proteases was carried out in 250-mL Erlenmeyer flasks containing 25 mL of either of either media just described. A pre-inoculum of *S. reilianum* was prepared in each medium and incubated for 24 h at 28°C at 150 rpm, and then used to inoculate each flask until all were adjusted to a final absorbance of 0.2 at 600 nm. All cultures were incubated at 28°C at 150 rpm. Three flasks were taken as samples at 0, 6, 12, 24, 48, 72, 96 and 120 h.

The Enzymatic Crude Extract (ECE) was obtained as follows: the culture from each flask was centrifuged at 5,000 g at 4°C for 10 min. The biomass was collected, washed twice with distilled water and then transferred to a rupture mechanical in Vortex. The mixture contained 7.5g of glass beads (0.5 mm in diameter), 12.5 mL of 0.1 M Tris-HCl pH 7.5, and 5 g of biomass. Total cellular disintegration time was 20 min, vortexing lasted 1 min, and the mixture was then placed in ice for 1 min. The lysate was removed from the glass beads and centrifuged at 10,000 g at 4°C for 10 min. The supernatant (ECE) was collected and used to determine proteolytic activity. Culture growth was followed by absorbance at 600 nm.

Enzymatic assays and protein determination

In-plate testing to determine the intracellular presence dipeptidyl aminopeptidase (psrDAPi) was performed in four plates with YEPD, where *S. reilianum* and *S. cerevisiae* DBY were inoculated by closed stripe each one in the middle of the plate and incubated at 28°C for 48 h. The cultures in two plates were permeabilized with chloroform. The plates were used for each enzyme (two with permeabilized cellules, two with non-permeabilized cellules). The substrate lysyl-prolyl-ß-naphthylamide was used to determine psrDAPi. Their activity was revealed as described by Hirsh et al. [13].

The enzymatic activity of psrDAPi, carboxypeptidase (psrCPi) and proteinase A (psrPAi) were determined as described by Hirsh et al. [13]. The following substrates were used: L-alanyl-prolyl-4-nitroanilide for psrDAPi, N-benzoyl-tyr-4-nitroanilide for psrCPi, and acid-denatured hemoglobin for psrPAi.

To identify the presence of the endogenous inhibitor of psrCPi activity, the ECE was incubated with 50 µL of sodium deoxycholate (5%) at 37°C for 5 min, followed by the standard enzymatic assay [13]. Protein determinations were performed following Bradford [14] using bovine serum albumin as the standard.

Differential centrifugation, zymogram analysis and proteolytic inhibitor effect

To determine whether the proteolytic enzymes were soluble or associated with the membrane, the ECE obtained from the different culture media described above in which enzyme activity was highest were centrifuged at 100,000 g at 4°C for 1.5 h using a Beckman ultracentrifuge. The supernatant (soluble fraction) and precipitate (membrane fraction) were used as Enzymatic Extract to determine the different proteases. The membrane fraction was resuspended in 1 mL of Tris-HCl at 0.1M, pH 7.5. The enolase activity was used as intracellular marker [10]. psrDAPi activity was detected in situ using zymograms, as described by Suárez and Wolf [15]. The substrate lysyl-prolyl-ß-naphthylamide was used. The zymograms were incubated at 37°C until red bands appeared to indicate enzymatic activity.

The protease inhibitors Na_2EDTA, E64, 1-10 phenanthroline, bestatin, PMSF and pepstatin A were evaluated to ascertain their effect against the proteases studied. In this procedure, the ECE were pre-incubated with the respective inhibitor for 30 min at 37°C before conducting the standard enzymatic assay.

Results

In-plate testing of psrDAPi activity

The intracellular activity of psrDAPi was determined in plate. The enzyme was detected in permeabilized cellules, and also found in non-permeabilized cellules (Fig. 1).

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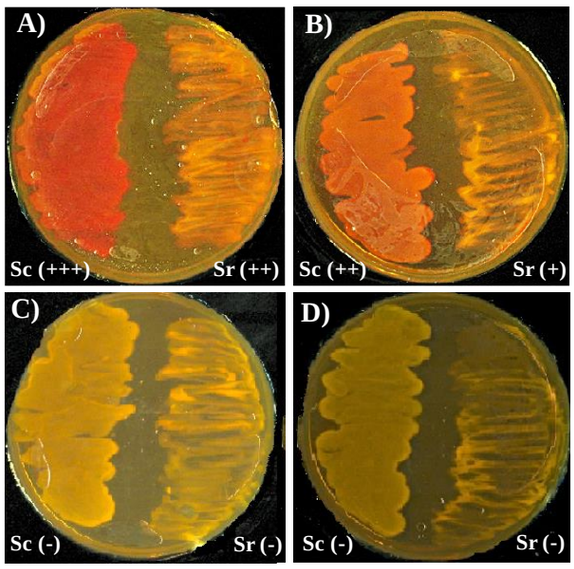
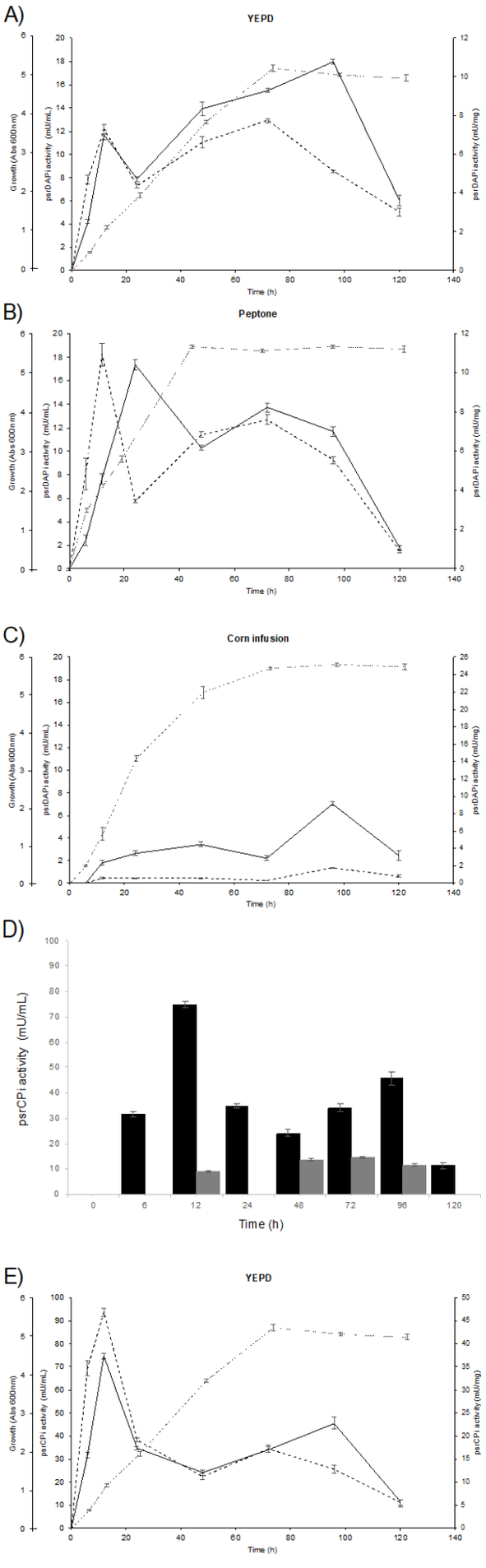


Figure 1 Plate test of psrDAPi activity. A) Activity in permeabilized cellules. B) Activity in non-permeabilized cellules. C) Control without substrate in permeabilized cellules. D) Control without substrate in non-permeabilized cellules. Sc= S. cerevisiae, Sr= S. reilianum. The symbols + and – indicate positive and negative tests, respectively. The cellules were permeabilized with chloroform as described in the Materials and Methods section.

Production of intracellular proteases of S. reilianum in different nitrogen sources

psrDAPi activity achieved its greatest activity in the YEPD medium and its minimal level with peptone, but was also detected in the minimal medium with the corn infusion (Fig. 3A-C). However, it was not found in the media that contained proline and ammonium sulfate.

In order to reveal psrCPi activity, it was necessary to add sodium deoxycholate, as shown in Figure 3D. This made it possible to determine its production in the YEPD and minimal peptone media, the corn infusion, and with ammonium sulfate. The highest activity levels were observed at 12h in YEPD and in the medium with ammonium sulfate (Fig. 3E-G). Meanwhile, psrPAi was found in all the culture media, reaching its highest activity levels in the YEPD medium at 48 h (Fig. 3H-L).



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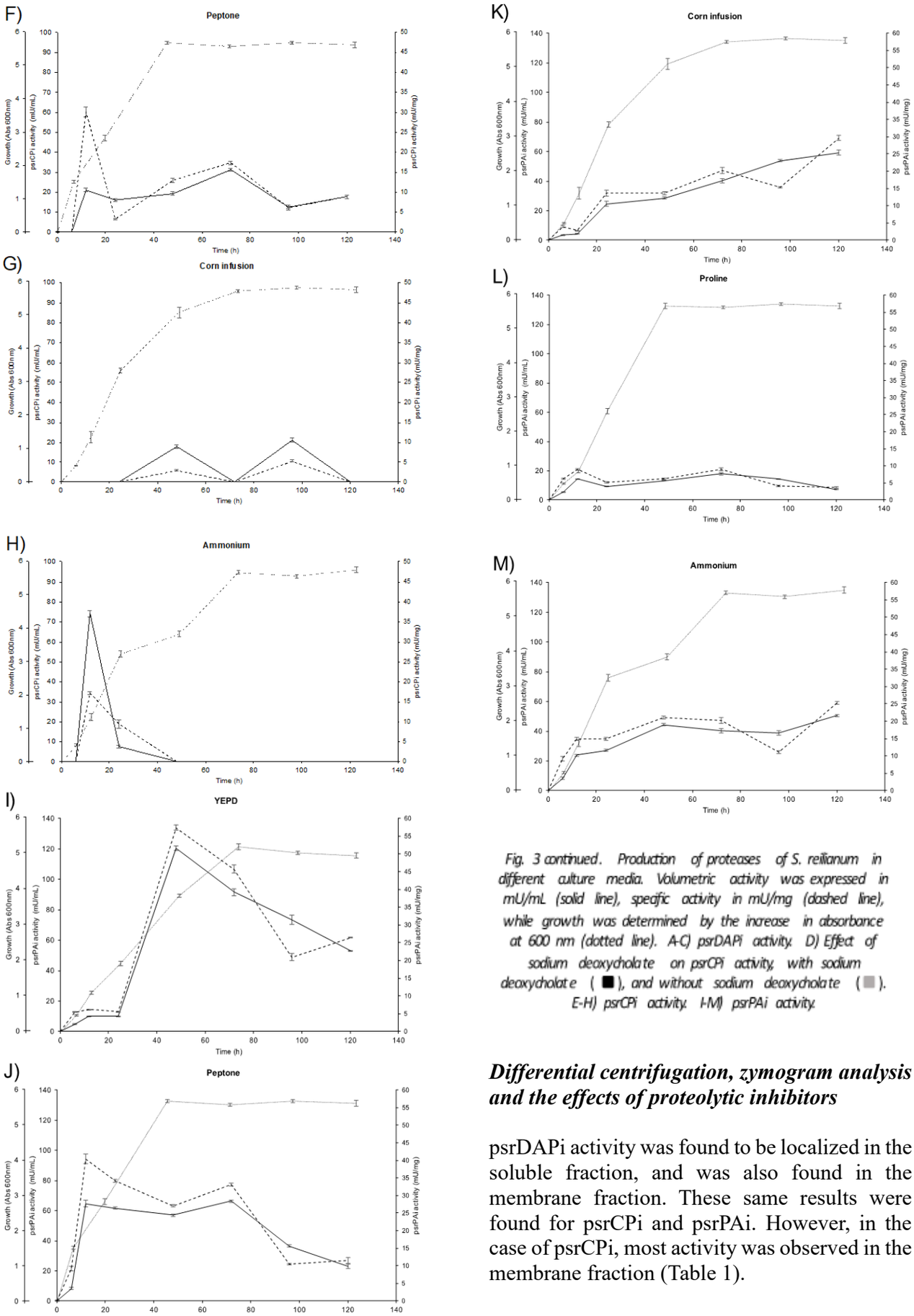

Fig. 3 continued. Production of proteases of S. reilianum in different culture media. Volumetric activity was expressed in mU/mL (solid line), specific activity in mU/mg (dashed line), while growth was determined by the increase in absorbance at 600 nm (dotted line). A-C) psrDAPi activity. D) Effect of sodium deoxycholate on psrCPi activity, with sodium deoxycholate (■), and without sodium deoxycholate (■). E-H) psrCPi activity. I-M) psrPAi activity.

Differential centrifugation, zymogram analysis and the effects of proteolytic inhibitors

psrDAPi activity was found to be localized in the soluble fraction, and was also found in the membrane fraction. These same results were found for psrCPi and psrPAi. However, in the case of psrCPi, most activity was observed in the membrane fraction (Table 1).

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Cellular fraction	Activity (%) psrDAPi	psrCPi	psrPAi	Enolase
ECE	100 ± 0.8	100 ± 0.5	100 ± 0.5	100 ± 2.4
Soluble fraction	63.7 ± 3.2	29.1 ± 0.4	73.0 ± 1.7	92.3 ± 4.4
Membrane fraction	35.6 ± 0.7	65.6 ± 0.7	24.6 ± 0.9	0.0

The enzymatic extracts were obtained from a culture of 96 h in YEPD

Table 1 Distribution of enzyme activities in cellular fractions of S. reilianum

Upon elaborating zymograms to reveal the isoenzyme profiles of the protease psrDAPi, the study found only one band of activity in the soluble fraction, but two bands of in the membrane fraction (Fig. 4).

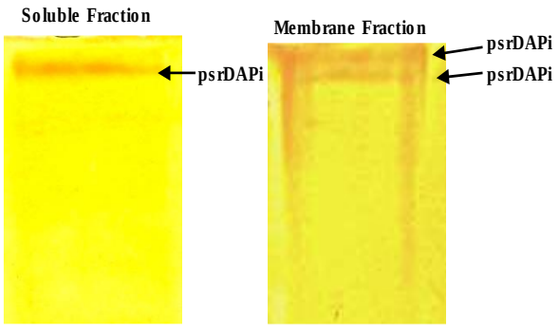

Figure 4 psrDAPi zymograms. The red bands indicate proteolytic activity

The evaluation of the effect of different protease inhibitors on the enzymes studied showed that the psrDAPi from the soluble fraction was only affected by E-64, in contrast to its activity in the membrane fraction, which was inhibited by PMSF. In the case of psrCPi activity in the soluble and membrane fractions, it decreased considerably upon adding PMSF, whereas the psrPAi from the same fractions was highly-affected by pepstatin A. Finally, the acid protease of the membrane fraction was also inhibited by PMSF, EDTA and E-64 (Table 2).

Discussion

The study of the biology of *S. reilianum* has been limited to determining its life cycle, mechanisms of pathogenicity, control, and some extracellular enzymes [2-4, 16, 17]. However, the intracellular proteolytic activity of this fungus had not been studied previously.

The present study found that *S. reilianum* produces psrDAPi. Similar activity has been reported for *U maydis* involving an enzyme called pumDAP, though there are observable differences in its production, since pumDAP is produced only in a minimal medium with proline and a corn infusion as the source of nitrogen [10], while psrDAPi is produced in YPD medium and a minimal medium with peptone, with low levels found in a medium with a corn infusion.

The best-characterized dipeptidyl aminopeptidases of fungal origin come from *S. cereviseae* and are called yscDAP A and yscDAP B. The first is located in the membrane of the Golgi apparatus and participates in processing the precursor of sexual factor α [18, 19]. yscDAP B, meanwhile, is found bonded to the vacuolar membrane.

Over-expression of yscDAP B in mutants that lack yscDAP A results in the maturation of sexual factor α [20]. For *S. reilianum*, studies have described that the sexual complementation of this fungus requires the production of pheromones [21]. In this case, psrDAPi is found in association with the soluble (psrDAPs) and membrane (psrDAPm) fractions. It is likely that the latter is an activity with functions similar to those of the membrane dipeptidyl aminopeptidases of *S. cereviseae*. This basidiomycete's genome has been shown to contain 5 genes that codify for possible dipeptidyl aminopeptidases; one of which is located in the membrane [16].

S. reilianum also produces of psrCPi activity, which is located in both the soluble and membrane fractions. The first is considerably inhibited by PMSF (serine protease inhibitor) and by metalloprotease inhibitors, which may indicate a mixture of enzymes that share the same catalytic activity. For the yeast *S. cerevisiae*, studies have described two carboxypeptidases of vacuolar location, denominated yscY and yscS. The first is a serine protease, the second, a zinc-dependent metalloprotease [5]. It has been shown that this yeast produces a polypeptide of cytoplasmic location called Ic, which is a specific inhibitor of the carboxypeptidase yscY. Determining its activity in cellular lysates requires adding sodium deoxycholate or previous incubation at acid pH to effectuate the dissociation of the endogenous inhibitor and the protease [22].

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This is also true for *S. reilianum*, because detecting psrCPi activity required adding sodium deoxycholate to the reaction mixture. This suggests the existence of an endogenous inhibitor that regulates this enzyme's activity.
The genome of this fungus contains 10 genes that codify for possible carboxypeptidases, six of thempossibly with vacuolar location [16].

Our study also found an additional activity of membrane-located psrCPi in *S. reilianum* that, in contrast to soluble psrCP, saw its activity reduced by PMSF and metalloprotease inhibitors, as well as by E-64 (cysteine protease inhibitor).

Meanwhile, descriptions of *S. cereviseae* mention a carboxypeptidase located in the membrane, denominated yscα, which requires both a serine and a cysteine group at the active site in order to perform catalysis [5]. In contrast to *S. reilianum*, carboxypeptidase activity at the biochemical level has not been found in *U. maydis* [10].

The present study found that *S. reilianum* has intracellular acid protease activity (psrPAi) that is likely similar to that reported for *U. maydis* (pumAi) [10]. Like psrPAi, production of the intracellular acid protease pumAi occurred in all culture media evaluated, and with every nitrogen source (peptone, ammonium, corn infusion, proline); however, in the case of proline it was associated with a logarithmic phase of growth [10].

Also, while in *U. maydis* the greatest specific activity of pumAi was obtained in a medium with proline [10], in that same medium, psrAi presented its lowest levels of activity. aspartyl protease, since it was inhibited by pepstatin A. The same results were found for pumAi from *U. maydis* [10]. Soluble intracellular aspartyl proteases have also been reported in such fungi as *S. cerevisiae*, *Aspergillus niger*, *Neurospora crassa*, *Coccidiodes immitis* and *Candida albicans* [23-27].

Inhibitor	Concen_ tration	Percent activity (%) psrDAPi Soluble fraction	Membrane fraction	psrCPi Soluble fraction	Membrane fraction	psrAi Soluble fraction	Membrane fraction
	Without inhibitor	100 ± 3.2	100 ± 0.7	100 ± 0.4	100 ± 0.7	100 ± 1.7	100 ± 0.9
Bestatin	100 mM	100 ± 0.4	100 ± 0.6	100 ± 3.5	100 ± 1.6	100 ± 0.9	100 ± 0.1
	200 mM	100 ± 0.7	100 ± 0.1	100 ± 7.1	100 ± 3.1	100 ± 1.8	100 ± 0.3
Pepstatin A	5 µM	100 ± 0.1	100 ± 0.8	72 ± 0.5	83 ± .3	0 ± 0	0 ± 0
	25 µM	100 ± 0.5	100 ± 0.4	65 ± 2.9	46 ± 0.1	0 ± 0	0 ± 0
PMSF	1 mM	100 ± 0.1	100 ± 0.6	28 ± 3.2	87 ± 0.3	56 ± 4.9	0 ± 0
	5 mM	100 ± 0.1	76 ± 3.1	14 ± 1.1	4 ± 0.4	6 ± 0.3	0 ± 0
EDTA	1 mM	100 ± 0.1	100 ± 0.3	100 ± 0.1	81 ± 0.3	85 ± 0.4	21 ± 0.7
	10 mM	91 ± 0.1	100 ± 3.2	100 ± 0.5	19 ± 1.1	79 ± 2.4	20 ± 4.7
1-10-phenantroline	1 mM	100 ± 0.1	100 ± 0.1	100 ± 0.9	90 ± 1.4	ND	ND
	10 mM	75 ± 1.4	100 ± 7.3	100 ± 2.4	83 ± 0.55	ND	ND
E-64	10 µM	100 ± 0.9	100 ± 0.9	100 ± 0.1	72 ± 0.4	100 ± 0.9	28 ± 1.6
	50 µM	8 ± 3.2	100 ± 3.2	100 ± 3.5	58 ± 0.1	100 ± 0.9	27 ± 0.8

Table 2 Effect of proteolytic inhibitors on *S. reilianum* intracellular proteases

The activity of psrPAi turned out to be a soluble In the case of *S. cerevisiae*, it knows that the soluble aspartyl protease yscA is found in association with the vacuole [5], an organelle that presents acid pH values and is the site of important cellular functions, including autophagia [28, 29]. The fact that psrPAi is active at acid pH values strongly suggests that it is of vacuolar location. In *U. maydis*, the gene pep4 codifies for the vacuolar acid proteinase PrA, which plays an important role in that fungus' morphogenesis and virulence [11]. It is probable that psrPAi performs the same function in S. *reilianum.*

Upon performing cellular fractioning, our study found acid protease activity in the membrane and in the soluble fraction. Both were inhibited by pepstatin A, however, there is a difference in sensitivity to other inhibitors, since psrPAi of membrane is totally inhibited by E-64 and strongly inhibited by PMSF and EDTA, while soluble psrPAi is not inhibited by E-64 and only weakly inhibited by PMSF and EDTA. These findings could indicate that they are distinct enzymes that share the same catalytic activity, but are located in different sites in the cell. No pumAi activity has been reported in the membrane fraction of *U. maydis* [10]; an important difference with respect to *S. reilianum*.

The database for the genome of *S. reilianum* reports 8 genes that codify for possible aspartyl proteases [16]. One of these has already been associated with the aspartyl protease Eap1, codified by the gene with access key sr11394 [30], but the location of the others remains unknown. During the present study, we found that the activity of psrCP showed a significant decrease upon adding pepstatin A (an aspartyl protease inhibitor).

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Studies of the yeast *S. cerevisea* have reported that the vacuolar proteinase yscA activates other vacuolar proteases [5], so it may be that adding pepstatin A to the reaction mixture inhibits psrPAi affecting the activity of the protease psrCPi.

Conclusion

The results presented constitute the first report on the intracellular proteases psrDAPi, psrCPi and psrPAi of *S. reilianum*. It is probable that these enzymes have important functions during the life cycle and/or pathogenesis of this basidiomycete.

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Telemetric system implementation for monitoring physical parameters within an entomological greenhouse

Implementación de un sistema telemétrico para el monitoreo de parámetros físicos dentro de un invernadero entomológico

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Abstract

The controlled breeding of *Leptophobia aripa* has become in a sustainable activity in Latin America countries where this species is endemic. Producers interest is establishing predictivity criteria related with the organism and installed infrastructure, in order to cuantify presicely the availability of its host plant and insects for subsequent sale. In this paper is described the implementation of an embedded electronic system designed to read and log the micro-weather parameters inside the enthomological greenhouse and generated models to explain the cicle of life for *Leptophobia aripa*, growing in this environment. The calculated model characterizes the growing rate of *Leptophobia aripa* across its life stages.

Leptophobia Aripa, **Embedded System, Telemtric System**

Resumen

La cría controlada de *Leptophobia aripa* se ha convertido en una actividad sustentable en países de América Latina donde la especie es endémica. Un interés de los productores es establecer criterios de predictividad asociados con el organismo y la infraestructura instalada, con la finalidad de cuantifiacar con precisión la sensible disponibilidad de la planta hospedera y los organismos para su venta posterior. En el presente trabajo se describe la implementación de un sistema electrónico embebido diseñado para realizar lecturas y registros de los factores microclimáticos dentro del invernador entomológico y los modelos generados para el ciclo de vida de *Leptophobia aripa* cuando se cría en dicho ambiente. El modelo caracteriza la tasa de crecimiento de la *Leptophobia aripa*, en todos sus estadios de vida.

Leptophobia Aripa, **Sistema Embebido, Sistema Telemétrico**

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1. Introduction

In recent years, new solutions have been proposed for the sustainable exploitation of non-timber biological resources in different countries of Latin America (Peña 2015). The telemonitoring and analysis of physical parameters such as temperature, relative humidity, insolation, fluid level, etc., applicable to industrial, biotechnological, agricultural, and other processes (Montesinos-Patiño 2002); It has been resolved through electronic devices and expert systems that centralize information by computer means, allowing to analyze the relationship between physical parameters with the development and production of *Leptophobia aripa* and Lepidum savitium (Bustillo 1975), which has facilitated the analysis of data to establish a growth model of the *Leptophobia aripa*, presented in this work.

2. Development

The proposed telemetric system design (Solarte 2011) and its subsequent implementation is shown as a scheme in Figure1, and was developed in four stages:

- Stage 1. Technological development.
- Stage 2. Acquisition of data.
- Stage 3. Data transmission.
- Stage 4. Monitoring and visualization of data.

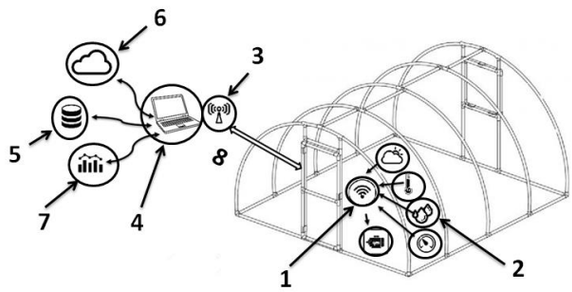

Figure 1 Diagram of the designed telemetric system

Components	
1	Electronic telemetry prototype
2	Physical parameters inside the greenhouse
3	Communication module
4	Embedded system
5	Database
6	Access from the internet
7	Web service
8	Wireless data transmission

Table 1 Components of the telemetric system designed

2.1 Technological development

For the development of the prototype the evolutionary process methodology was used (Sommerville 2005) which consists of 5 steps for the development of the prototype which will allow the acquisition of data.

Step 1. Communication

The objective of what the developed prototype has to do is defined, which consists of "Developing a telemetric system that allows compiling the physical parameters of the entomological / agricultural greenhouse, which allows consultation through a web service in real time"

Step 2. Quick plan

The needs and variables to be measured are identified, such as insolation, temperature and relative humidity, as well as implementing the appropriate wireless communication protocol for the transmission of information.

Step 3. Modeling and rapid design

The design of the prototype / hardware is quickly planned, the components are identified for the manipulation, communication and collection of physical parameters of the systems.

Step 4. Construction of the prototype

The prototype is developed which allows to take readings of the physical parameters already mentioned, by means of the different sensors, depending on the request that the user requires, this is done from an embedded system, which chooses the transceiver that will respond to reading requests enabling wireless communication implemented with modules under protocol 802.15.4.

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Figure 2 Design and prototype assembly

Step 5 Deployment delivery and feedback

The prototype was implemented in an agricultural / entomological production greenhouse of 40 m3, where communication tests were carried out and analog reading sensors were calibrated.

2.2 Data acquisition

For the acquisition of the corresponding data (temperature, relative humidity, insolation), inside the entomological greenhouse was used the DHT22 sensor for temperature and relative humidity (Figure 3) and a solar cell to monitor the insolation (Figure 4). As an embedded system, a Raspbery Pi 3B was used (Figure 5), in addition a software routine was developed which allows making the connection between the telemetric prototype and the system, which makes requests via wireless to obtain the parameters; which are stored in a database, which in turn makes a mirror copy to a web server through the SSH (Secure Shell) protocol, data storage tests were performed on the embedded system and on the server managed through a web page to display neatly the collected data.

Figure 3 DHT22 sensor

Figure 4 Solar cell

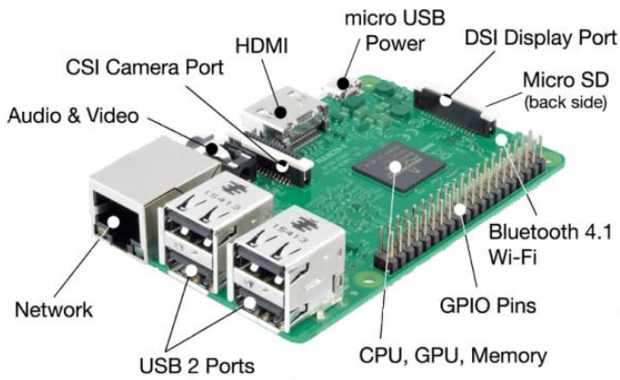

Figure 5 Raspberry PI 3B

2.3 Data transmission

The data transmission system consists of two Xbee S2 modules (Figure 6) that work under the 802.4.15 protocol in the free 2.4 GHz band.

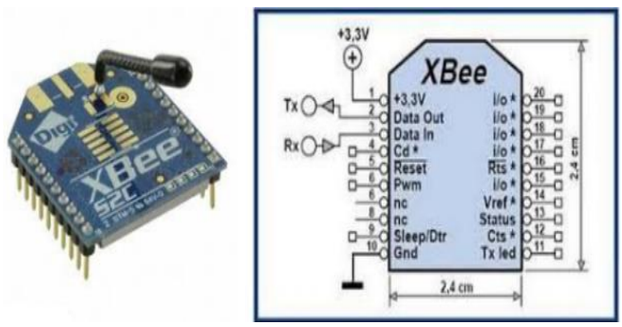

Figure 6 Xbee S2

For the selection of the equipment, the low cost of the equipment was considered and its wide use as a solution to establish point-to-point, point-to-point wireless links that allow a full-duplex communication where a module is configured as a *coordinator*; is in the embedded system and allows requests to each telemetry equipment *end device* of the network, they respond to the request by sending the requested parameters.

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The technical characteristics of the Xbee module are shown in Table 2. The communication tests were performed to verify the efficiency of response between the embedded system and the telemetric prototype, which allows requests to be made every 5 seconds with the Xbee S2 modules.

Xbee modules		
Features	**Xbee S2**	**Xbee Pro**
Power	3.3V	3.3V
Speed	250Kpbs 1mW	250Kpbs 60mW
Power	100m. Local o inalámbrica	1000-1500m Local o inalámbrica

Table 2 Characteristics of Xbee modules
(www.digi.com)

2.4 Monitoring and visualization of data

The information that is acquired through the telemetric system is stored in a database created in MySQL within the embedded system and, as already mentioned, makes a mirror copy using the SSH protocol to a web server, which allows visualizing the information as it is shown in Figure 7, where the temperature, relative humidity and insolation record is shown in real time, recording the moment of reading, these parameters will allow to analyze ideal conditions for its development, growth and sustainability of the breeding process.

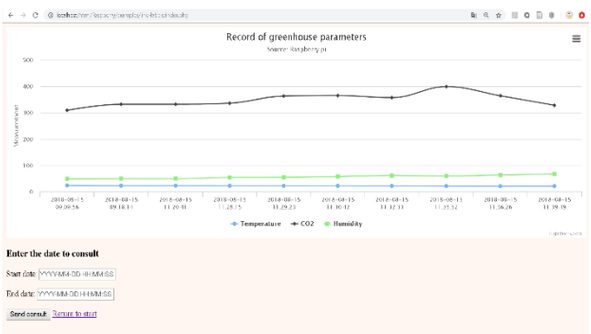

Figure 7 Graphic of the registered physical parameters, consulted from the web service

3. Developed Methodology

The generated model consisted of nine laying sites (nests) of *Leptophobia aripa* within the instrumented greenhouse, monitored at the time of greatest presence of the species in the months of April and May, registering the presence of 338 eggs, which were followed stage by stage until his development as an adult. The data obtained were recorded as shown in Table 3.

With the data obtained, the time for the average development cycle of the *Leptophobia aripa*, which is 24.75 days, was calculated for the present experiment. The modeling was carried out applying the linear least squares regression method, to calculate the growth curve of the *Leptophobia aripa* in the referred conditions.

4. Results

The average length of each nest was recorded per larval stage (Table 4). Sanchez indicates that the average cycle is 27.2 days under controlled conditions in Mesa Cundinamarca in Colombia, this work was done from five nests with a temperature that fluctuates between 16 and 23 ° C, having a percentage of 80% of butterflies successful adults (Sánchez 2004).

While the study of Bustillo and Bertha report that the average development cycle is 21.5 days. In controlled conditions in Colombia with a temperature that goes from 16 and 30 ° C (Bustillo 1975).

With the monitoring of physical parameters within the greenhouse and analyzing the data in Table 3, it was compared with previous studies on the life cycle of the *Leptophobia aripa*, where the temperature does influence its life cycle, since when analyzing the development behavior shown in Table 3 and the percentage of organisms that reach the adult stage, it is concluded that when the temperature is higher the development cycle is shorter otherwise when the temperature is higher The development cycle is shorter (Campos - Nava 1992), this can be known and compared with the studies carried out by Sánchez.

His study indicates that the average temperature of his experiment is 19.5 ° C (Sánchez 2004), this temperature is the lowest of the 3 studies carried out, so the average life cycle is 27.2 days, while in the study de Bustillo records an average temperature of 23 ° C and records that the life cycles are 21.5 days (Bustillo 1975). The average temperature recorded in the present study was 21.28 ° C for the development cycles, taking an average of 24.75 days.

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Nests									
Period	Nest1	Nest2	Nest3	Nest4	Nest5	Nest6	Nest7	Nest8	Nest9
Eggs	42	32	38	41	37	45	39	41	29
Larva	39	30	36	38	36	43	36	38	27
Pupa	32	24	27	33	31	36	26	33	20
Adults	32	24	27	33	31	36	26	33	20
Duration of the cycle (days)	24.65	24.5	24.88	24.5	24.96	24.52	24.76	24.95	25.05
% individuals	76.19%	75 %	71.05 %	80.48 %	83.78 %	80 %	66.66 %	80.48 %	68.96 %
Average temperature during the cycle	20.5°C	19.5°C	18.5°C	24.1°C	25°C	23°C	18°C	24°C	19°C

Table 3 Number of individuals obtained from the nests of "Leptophobia aripa" for the present experiment, by larval stage, duration of the stage and physical parameters recorded

			Nidos				
Nests	Egg	Hatching	Status1	Status 2	Status 3	Prepupa	Pupa
Nest 1	1.1	2.0	5.0	11.1	19.0	19.7	19.0
Nest 2	1.3	2.0	5.0	10.9	18.6	19.7	18.4
Nest 3	1.4	1.9	4.9	11.1	19.0	20.1	18.5
Nest 4	1.6	1.9	5.0	11.1	19.1	19.6	19.3
Nest 5	1.4	2.2	4.8	10.9	19.0	19.7	19.4
Nest 6	1.3	2.3	4.7	10.9	19.0	19.2	19.6
Nido7	1.3	2.0	4.9	11.1	18.7	19.3	19.4
Nest 8	1.2	2.0	4.8	11.2	19.3	20.1	19.3
Nest 9	1.8	2.2	5.0	11.0	19.0	20.0	19.3

Table 4 Average length in millimeters per larval stage

	Coefficients of the linear model							Y(X)=A0X0+A1X1+A2X2+…+AnXn		
Grado del polinomio	A0	A1	A2	A3	A4	A5	A6	Error estándar	Coeficiente de correlación	Coeficiente de determinación
Primero	1.1286	3.1821						3.89944	0.888	0.78855
Segundo	-8.6571	9.706	-0.8155					2.24545	0.97155	0.94391
Tercero	0.2429	-0.4302	2.1512	-0.2472				1.52386	0.99027	0.98062
Cuarto	13.2286	-21.0237	12.0708	-2.0836	0.1148			0.7088	0.9986	0.99721
Quinto	1.7571	1.6353	-3.1375	2.4282	-0.4936	0.0304		0.60861	0.99948	0.99897
Sexto	-32.6	80.905	-70.3028	29.8979	-6.3215	0.6471	-0.0257	Error	1	1

Table 5 Coefficients obtained by the linear least squares regression method, for the growth model of "*Leptophobia aripa"*

When analyzing the data of table 5, it can be observed that the polynomial degree of the fifth degree has a percentage of data correlation of almost 100% and has the minimum standard error among the other polynomials, establishing the behavior curve for the growth of the *Leptophobia aripa* as shown in figure 8.

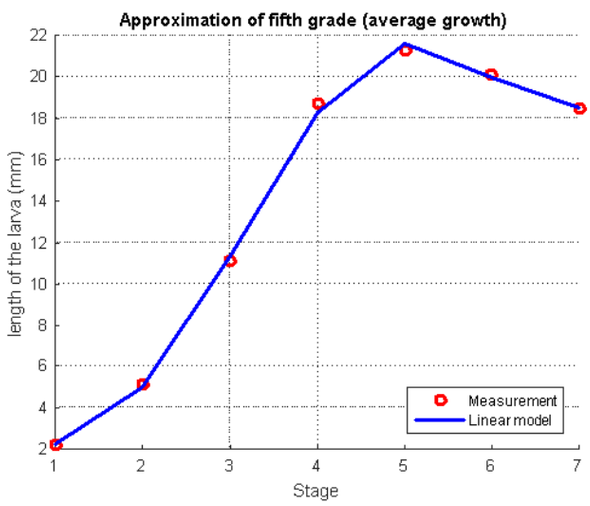

Figure 8 Model that represents the growth behavior of the *Leptophobia aripa*

5. Conclusions

The comparison between the previously published results (Sáncez 2004, Bustillo 1975) and those obtained in this work, it is possible to affirm that the life cycle of Leptophobia aripa has a duration proportional to the temperature of its breeding environment.

The present investigation in comparison to that of Sánchez (Sánchez 2004) and Bustillo and Bertha (Bustillo 1975), allows defining a growth model in this case of the Leptophobia aripa, in addition, it allows real-time monitoring of parameters physical and control of these to have optimal conditions within the greenhouse.

By means of the developed telemetric system, correlation models can be defined between the host plant and the insect to ensure a continuous and sustainable production of both species. It is also possible to propose control models to optimize the production with precision agriculture techniques.

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Electrical and Mechanical Analysis of a Natural Biopolymer

Análisis Eléctrico y Mecánico de un Biopolímero Natural

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Abstract

This work presents the formulation of a polymer based on biodegradable materials. The polymer formulation consists on the selection of appropriate percentages of the combination of starches of natural origin, plasticizers and waste of fruits, such as pineapple, orange, chilacayote, guava, lemon, mango, apple, pineapple, watermelon and banana. The resulting polymer characteristics are studied in mechanical and electrical analysis. The test probes of the polymer are films, molds and test wires. The conducted experimentation includes the doping and electropolymerizing processes with different compounds and different electrical parameters respectively. The changes in the two processes aim to modify the physical and electrical intrinsic properties of the polymer. The test molds are used accordingly to the ASTM standard that provides the guidelines for mechanical tests. The resistance measurements are performed on the polymer wire to determine its resistivity. The electrical and electronic instrumentation is developed to perform an electropolymerization process by a pulsed power supply that provides variable electrical parameters at the output, namely voltage (1-24V), current (1-3A), frequency (10-1000Hz) and pulse widths (10-100 μs).

Biopolymers, Doped, Characterization

Resumen

En este trabajo se presenta la formulación, análisis mecánico y eléctrico de películas, probetas y alambres de un polímero hecho a base de material biodegradable. La formulación consiste en la selección del porcentaje más adecuado de la combinación de almidones de origen natural, plastificantes y desechos de frutas tales como la piña, naranja, chilacayote, guayaba, limón, mamey, mango, manzana, sandia y el plátano. La experimentación realizada es sometida a un proceso de dopado y electropolimerizado con diferentes compuestos y con diferentes parámetros electricos respectivamente, acciones realizadas para modificar las propiedades intrínsecas (físicas y eléctricas) del polímero. Se utilizan moldes de prueba de acuerdo a la norma ASTM para realizar ensayos mecánicos y para determinar la resistividad se realizan mediciones de resistencia en el alambre del polímero obtenido. Se desarrolla la instrumentación eléctrica y electrónica para realizar el proceso de electropolimerización mediante una fuente pulsada que proporciona a la salida parámetros electricos variables, tales como voltaje (1-24V), corriente (1-3A), frecuencia (10-1000Hz) y ancho de pulso (10-100μs).

Biopolimero, Dopado, Caracterizacion

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Introduction

Before the plastic age, mankind used the directly accesible earth resources as the only source to get tools (Garcia, S., 2009), instruments and everyday use devices (Rodríguez, A. 6, 2008). Recently, the consumption needs of products pushed the industry to create cheaper materials with physical properties like those available in nature (Hottle, T., 2013). Plastic is a petrochemical derivate developed for mass usage. Today plastic has innumerable applications. Relevant plastic applications are emerging for biomedical purposes (Bret, D., 2011). The spread of plastic all over the world is mainly due to its low cost. Nevertheless, thousands of tons of plastic are discarded daily. The huge amount of waste is a problem since plastic is not biodegradable.

Plastic decomposition lasts hundreds of years causing considerable environmental pollution (Vroman, Isabelle, 2009). It is important to find alternatives that largely resolve the pollution derived from plastic waste (Kumar, A., 2011). Research and development of plastic allows the incorporation of biodegradable material reducing the disintegration time (Tanaka, M., 2015). However, it is a big challenge to generate biodegradable plastics with the same characteristics of common polymers, such as Polystyrene (PS), Polyurethane (PU), Polypropylene (PP) or polyvinyl chloride (PVC) among others (Mohanty, AK, 2000). The constraints are the physical, mechanical or electrical requirements of plastic products. It is important to study and develop polymers with a base different from petrochemical (Iwata, T., 2015). The following sections describe a novel polymer formulation with water base, starch and fruit peels. Mechanical and electrical tests are performed on the new material after doping and electropolymerizing processes.

Formulation

There are two main biodegradable plastic types described in the literature, the so-called Hydro-biodegradable (HBP) and Oxo-biodegradable (OBP). These biodegradable plastics are made of at least one of the following: starch, vegetable waste, aliphatic polyesters, orange husks, tree leaves or wool (Patel, Parth N, 2011).

Each of these ingredients gives the polymer a structure that characterizes its final physical form. In this work, the polymer formulation is studied considering the effect of the components in the the bioplastic, 1) the plasticizer: that destroys the secondary and tertiary structures of the macromolecules, 2) the gelatinizer: that causes the molecule crystallinity loss, 3) the additive: that provides mechanical and electrical properties to the final product and, 4) the temperature: that affects the polymer physical texture.

To determine the optimal percentages of the components, the process formulation is designed by trial and error looking for electrically and mechanically stable materials. Figure 1 shows the methodology with the best results in both the formulation and the experimental test phases. The methodology starts with the fruit shell waste selection (1), of which 25 g are used and weighed on a digital scale (2). The selected fruit waste is placed in a crusher (3) and mixed with 100 ml of water (4). The mix is filtered (5) to obtain 75 ml of a concentrate juice (6) to 0.25 g / ml, which has the plasticizer function. Then, 15 g of starch are added to the solution (7) as a polymeric base. The gelatinizer is 5 ml of glycerin (8). Finally, 10 ml of vinegar (9) is used to stabilize the solution pH.

Figure 1 Diagram of the Natural Biopolymer Formulation Methodology
Source: Own Elaboration

The final mixture is considered to be the basis of the formulation tests, and it is classified according to its treatment as natural BP (BPN), doped (BPD) or electropolymerized (BPE). In the BPN, the corn starch molecules, amylose or amylopectin, form the polymer main chain (Guzmán, A. and Gnutek, N, 2011). Figure 2 shows the structure of the BPN main chain.

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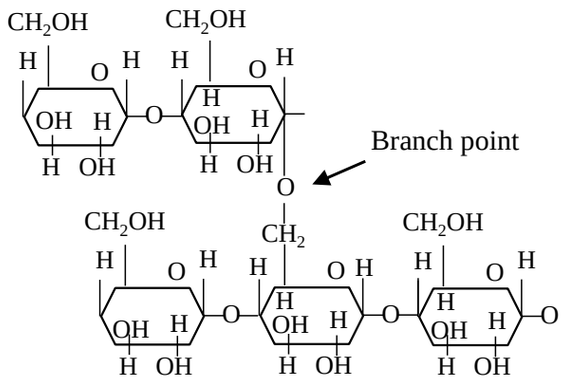

Figure 2 Chemical amylopectin structure.
Source: Own Elaboration

In the natural biopolymer there is no agent that modifies the physical or chemical properties of the bioplastic obtained. The doped biopolymers (BPD) are analog to electric semiconductors. The addition of external elements modifies their properties. Ascorbic acid ($C_6H_8O_6$) and zinc oxide (ZnO) are used in BPD to propitiate the generation of chemical agents in the reaction and therefore to cause the modifications in the BP characteristics (Tassew, A. 2014). Figure 3 shows the chemical structure of the dopants. The doping process is considered as a treatment applied to the final mixture and corresponds to the Point 11 shown in the methodology of Figure 1. Here, 1 g of $C_6H_8O_6$ and ZnO are added to the mixture.

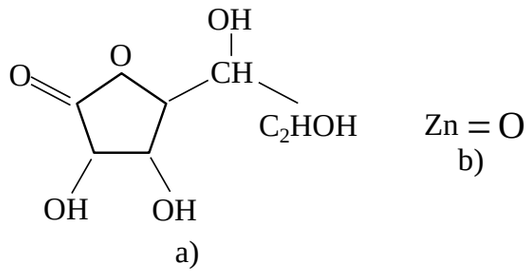

Figure 3 Chemical structure of the usual dopants for the BPD, a) ascorbic acid and b) Zinc oxide.
Source: Own Elaboration

Finally, the BPE undergoes an electrical treatment (Point 10 in Figure 1), in which a pulsed electrical current circulates through the polymer during formation. The method consists in introducing two electrodes (positive and reference ground) to the container with the mixture inside. The electrodes are connected to a pulsed power supply of potential 5 V, current 0.5 A, frequency 1000 Hz and pulse width 100 μs. Figure 4 shows the trigger circuit.

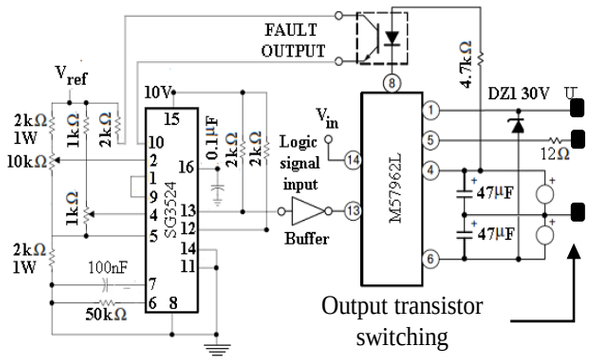

Figure 4 Electric diagram of the pulsed power supply trigger circuit
Source: Own Elaboration

Table 1 shows temperatures and the transition times during the reaction of the BP in the three forming processes, as well as for each of the fruit waste used in the formulation.

Process (N,D y E)	Forming temperature [ºC]			Forming time [min]		
Fruit	N	D	E	N	D	E
Chilacayote	70	71	71	5	5	4.5
Guava	72	71	72	4.3	4.2	3.8
Lemon	73	73	72	4.5	4.4	4
Mamey	72	72	71	5.1	5.1	4.5
Mango	72	73	72	4.9	4.8	4.1
Orange	70	72	70	5.5	5.5	4.9
Apple	72	73	72	5.1	5	4.4
Banana	72	73	71	5.4	5.4	5
Pineapple	72	72	70	5.1	5.2	4.6
Watermelon	71	70	70	5.5	5.3	4.7

Table 1 Typical temperatures and times of biopolymer formation under natural (N), doped (D) and electropolymerized (E) conditions
Source: Own Elaboration

The transition temperatures shown in Table 1 for the natural processes (N), doped (D), and electropolymerized (E), are between 70-73 ºC, however the reaction time for BPE it is a little lower, since electrical processes imply thermic addition to the forming temperature.

The physical forms of test tubes and films obtained during experimentation (see Figure 5) change their morphology according to the process. The BPN is more flexible and sensitive to touch. On the other hand, the doped material is more solid, more uniform and more elastic. BPD are less sensitive to ruptures and the density loss during the process is lower than the natural ones.

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BPE are apparently resistant to mechanical traction, some are elastic and others are fragile and rigid, but with smooth texture. Each one of the BP obtained in the experiments has different physical characteristics that depend on 1) the formulation, 2) the doping percentage and 3) the power pulsed supply electrical parameters. It was noted that the density loss (ρ) depends directly on the specimen thickness. For the test tubes the physical tests results were favorable, however after a few days their density decreased by 40%. This effect was less dramatic with film specimens, in which the density only decreased 10%.

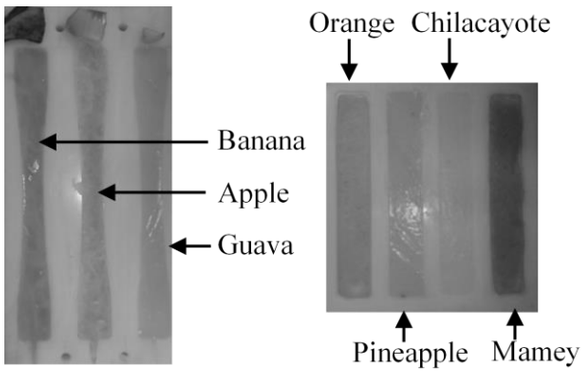


Figure 5 Films and test tubes under norm ASTM D882-12 and D638-02a respectively
Source: Own Elaboration

Electrical Testing

During the doping (D) and electropolymerized (E) processes, some bonds of the polymeric base of the starch break apart, generating free radicals (OH) and some free electrons (Fernández, T, 2003). The BP electrical resistivity changes depending on the amount of free charge carriers. To measure the resistivity we take electrical resistance measurements on the obtained material films with thicknesses less than 1 mm. The method used to determine the resistivity of the films is called four-point method or the Kelvin method (Severin, J., 1971). The measurement method is illustrated in Figure 6.

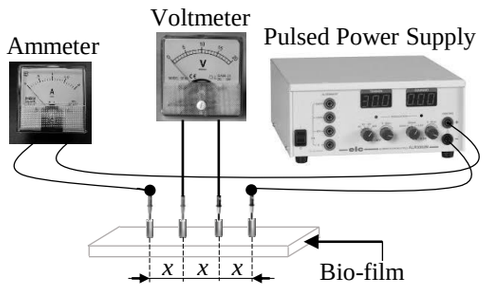
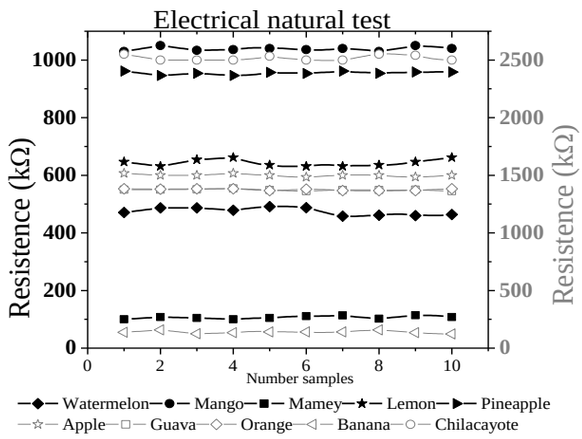
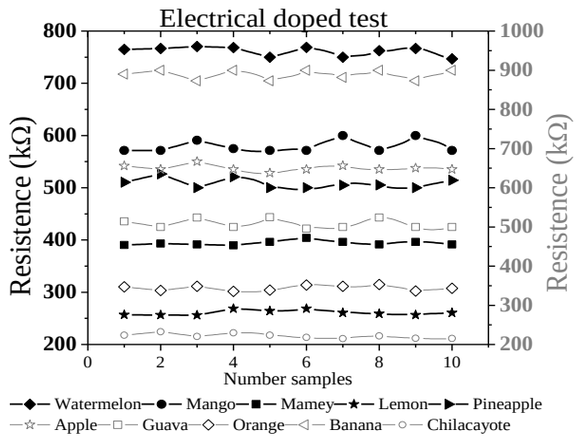


Figure 6 The four-point resistance measurement method
Source: Own Elaboration

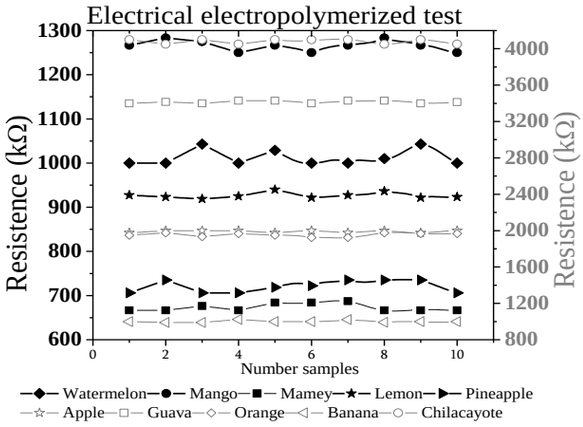
With this method, the characteristic electrical values of bio-films are obtained. The instrumentation consist of digital multimeter UNI-T UT139C, with micro measurements scales. The following graphs show the concentration of the average ohmic values for the natural, doped and electropolymerized process respectively.



Graph 1 BPN Ohmic values
Source: Own Elaboration



Graph 2 BPD Ohmic values.
Source: Own Elaboration



Graph 3 BPE Ohmic values.
Source: Own Elaboration

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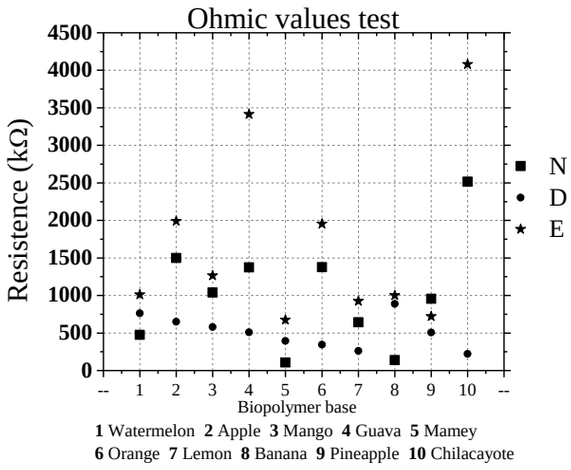
In Graph 1 it is observed that the lower ohmic values are characteristic for the mamey and the banana. The maximum resistive values are present in the chilacayote and the apple. On the contrary, in Graph 2 we can see that the banana and the chilacayote have reversed roles, now the BP made with chilacayote has the least resistance value and the banana shows one of the highest values. In these tests, an important aspect to comment is the resistance value range because is less to 1 MΩ. We can note that while some compositions increase their ohmic values, others decrease it until reaching the mentioned interval. The doping elements influence the BP conductivity, so now this depends largely on the percentage of zinc oxide and ascorbic acid added to the formulation and at a lower rate to the fruit waste intrinsic characteristics.

In Graph 3 we notice that the ohmic values have increased notoriously. Nevertheless, the mamey and the chilacayote maintain properties of low and high resistance in comparison with the others test within the same experiment, which indicates that probably the current action in the process directly magnifies the fruits conductive properties. The conductivity increment is observed because there is no dopant. Therefore, according to the electrical parameters of the pulse applied the ohmic values vary. Graph 4 shows a comparison of the resistive values measured in the BP subjected to the three different processes.



Graph 4 Comparison of ohmic values between the natural (N), doped (D) and electropolymerized (E) tests
Source: Own Elaboration

In Graph 4 it is observed that except for the watermelon, mamey and banana, a pattern is followed in which the resistance increases in the order of the test D, N and E. It is also observed in the test that the resistance value most significant change is present in the polymer with residues of chilacayote and guava. On the other hand, the less representative variations are found in those of watermelon, mamey and pineapple. We conclude that the type of fruit waste used in the formulation essentially provides the final electrical resistance of the bioplastic.

It is verified that the final conditions during doping depend directly and almost exclusively on the aggregate material percentages (the resistance decreasing in almost all cases). In the electropolymerized process, the pulsed electric current acting during the formation of the bioplastic strengthens the polymer bonds making it less conductive.

Mechanical Tests

Mechanical tensile tests are performed for the BPN, BPD and BPE films on the Universal Instron machine (see Figure 7) using the Bluehill software. The main objective is to characterize the BP according to the analysis of its stress-strain characteristic curve (Ward I., 2013). The results obtained define whether the material is fragile or not. In the same way, the interpretation of the graph results specifies if the BP is suitable for elastic or plastic applications and shows how deformed the material can be (Mendoza, R. 2011).

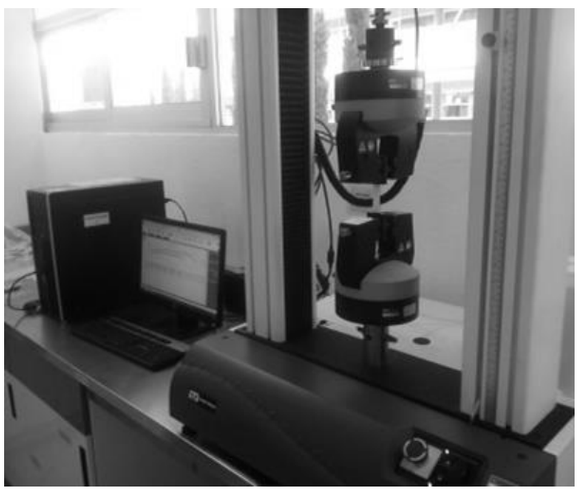


Figure 7 Tensile test in the biopolymers.
Source: Own Elaboration

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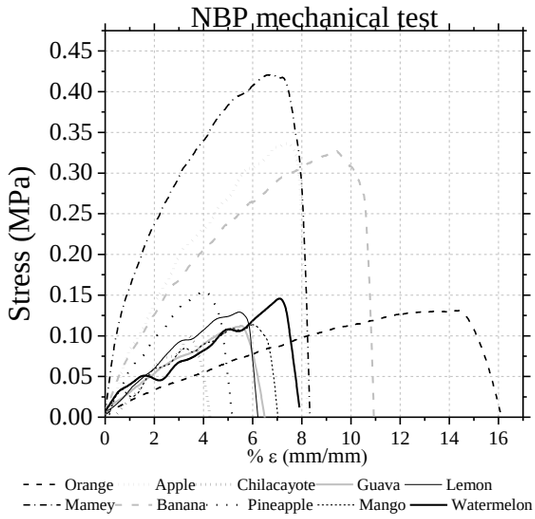
Tensile test	
Column 1	Young module
Column 2	Creep load
Column 3	Breaking load
Column 4	Traction stress
Sampling	100 ms
Width	17.2 mm
Lenght	91 mm
Thickness	0.56 mm
Geometry	Rectangular

Table 2 Tensile test characteristics
Source: Own Elaboration

The sample geometry and the most important parameters for the bio-polymer characterization considered in the stress test, are shown in Table 2 and taken from the ASTM D882 standard for thin films (tensile properties of thin plastic sheeting).

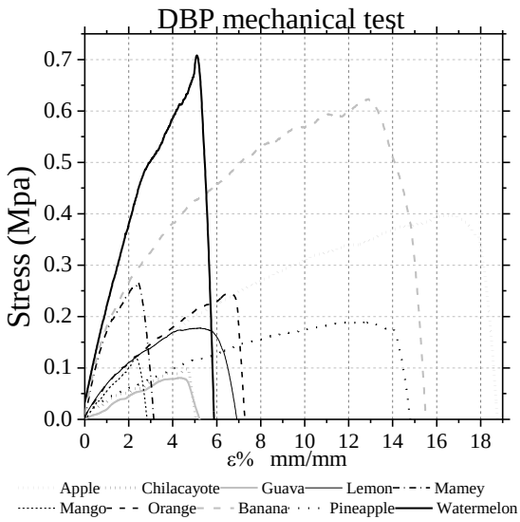


Graph 5 Natural bio-polymers stress test results
Source: Own Elaboration

Graph 5 shows the NBP formulation stress test results. The material that has a larger deformation (~ 16 mm/mm) is the formulation based on orange peel. Here the break is not abrupt, that means the material is not very fragile. The break occurs when the final elongation corresponds to ~ 8 mm, with a strain around ~ 130 kPa.

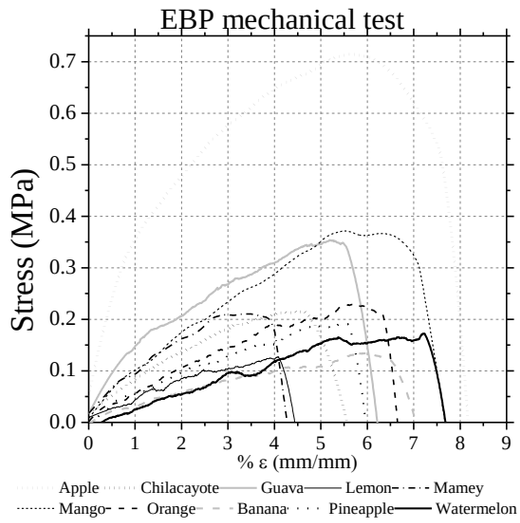
The material based on mamey presents the bigger value of the Young's modulus (inclination slope). This material is capable of supporting more effort (~ 420kPa). The elastic zone slope is almost directly proportional for values deformation less than 2mm, this mean that it supports large loads at the beginning of the traction compared to the others, however it is not very elastic.

In the test there are two other formulations with similar behaviors that are those of apple and banana base, noting that the latter has greater elongation capacity. For the other BPN, the behavior between them is very similar, due to the fact that they support loads of the order of 120kPa and deform ~ 3mm.



Graph 6 DBP tension tests
Source: Own Elaboration

The BPD traction test results are shown in Graph 6. The watermelon formulation is fragile when reaching a stress close to 700kPa. Note that the apple-based material is the most elastic with an elongation around ~ 9.5 mm when supporting a strain of ~ 400 kPa.



Graph 7 EBP tension tests
Source: Own Elaboration

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Graph 7 shows the mechanical tests results of the formulation subject to pulsed electrical discharges. Here the apple film has the highest deformation percentage which corresponds to approximately 4 mm, generated when reaching a maximum stress of ~ 700 kPa. There are two other similar behavior cases, that of guava and mango, in these the stress is near to 350 kPa with deformations of 3 and 4 mm respectively. For the rest of the tests with the other fruits not mentioned, the behavior is similar between them, a maximum stress is ~ 200 kPa with a deformation of between 2 and 4 mm.

Optical Microscopy

A simple analysis is carried out using an AxioCam ERc 5s digital optical microscope (Zeiss), shown in figure 8. 200x lenses and immersion oil are used to maintain the refractive index of the uniform light (which does not change angle), which generates more light reaching the object. A sample of natural, doped and electropolymerized orange film is analyzed as shown in the following figure.

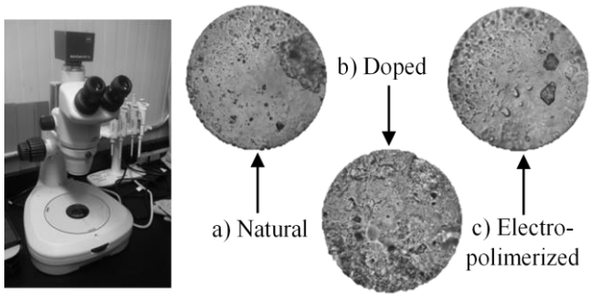

Figure 8 Optical microscopy test
Source: Own Elaboration

In figure 8 it is noted that the biopolymer surface structure changes form of a) natural formulation to b) doped, where it is notable that the structure is less uniform, this is due to the action of the oxides present in the process. On the other hand, for the electrically treated sample, we observed that there is not a very significant change with respect to the EBP.

Conclusions

It is important to consider new material options to replace the petroleum-based plastics. We introduced new formulations to make hydro-biodegradable films and tubes test.

In the new materials, the starch provides the polymer characteristics base and the residues of fruit peels add particular characteristics that are modified in posterior doping and electro polymerization processes. It was observed that the electrical characteristics of the doped biopolymers are apparently uniform, within a defined interval, but their mechanical characteristics are not uniform. The mechanical properties of electropolymerized biopolymers are more stable with respect to the two posterior processes, since the properties only change slightly irregardless of the type of fruit used.

We conclude that to obtain a more conductive BP, it is necessary to use the doped formulation, and for mechanically resistant materials, the electrical process is the option. A combination of doping with electrical treatment will most likely obtain a BP with suitable mechanical and electrical characteristics, as electrical conductor as specified according to the dopant and so mechanically resistant according to the pulsating source electrical parameters. A remarkable observation is that the EBP electrical resistance is higher than for a plastic straw, with a similar texture (significant example the banana EBP). Also, the DBP are more flexible and have a latex-like behavior that adheres to the container and takes its shape.

For future works, it is proposed to combine simultaneously the doping and electropolymerized processes. It will be necessary to conduct a study using an electron scanning microscope to get insight into the internal structure of the obtained BP. Another property to be measured is the flow index (MFI) with a plastometer, to determine if the BP is suitable for extrusion or for injection. Further experiments varying the electrical parameters such as voltage, current, frequency, and pulse width of the applied electrical signal, will fully characterize the electrical and mechanical effects in the BP. Further work is to find the most appropriate application options for each obtained material.

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Article **ECORFAN Journal-Ecuador**
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Effect of the fractions of beeswax hydrolysates as carbon source in the synthesis of short and medium chain *polyhydroxyalkanoates* (scl-mcl-PHA)

Efecto de las fracciones de hidrolizados de cera de abeja como fuente de carbono en la síntesis de *Polihidroxialcanoatos* de cadena corta y media (scl-mcl-PHA)

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Abstract

This study analyzed the synthesis of short and medium chain length polyhydroxyalkanoates (scl-mcl-PHA) from fractions of beeswax hydrolysates (Hw). The fermentations were carried out in batch-fed cultures of three stages with a strain of *Cupriavidus necator*. Glucose and ammonium sulfate were used as carbon and nitrogen sources in the first and second stage of culture. During the third stage (mcl-PHA production), fresh culture medium was fed with fractions of Hw (5 g L^{-1}) as a cosubstrate, in addition of Triton X-100 (3 CMC). The accumulation of scl-mcl-PHA from the fraction of waxy alcohols of Hw (OH Hw) was 20.85 g L^{-1} (90.19 % w/w) while from the fraction of fatty acids of Hw (AG Hw) was 5.57 g L^{-1} (67.28 % w/w). ANOVA of one factor was performed to quantitatively compare the intracellular amount of synthesized scl-mcl-PHA. Dunnett's multiple comparisons test established the time intervals, in which *C. necator* was able to produce a higher percentage of polymer. The presence of 3-hydroxybutyrate (3HB) and 3-hydroxydecanoate (3HD) in the scl-mcl-PHA synthesized was determined by GC.

Biopolymers, *Cupriavidus Necator*, Fed batch

Resumen

Este estudio analizó la síntesis de polihidroxialcanoatos de cadena corta y media (scl-mcl-PHA) a partir de fracciones de hidrolizados de cera de abejas (Hw). Las fermentaciones se llevaron a cabo en lotes alimentados de tres etapas con *Cupriavidus necator*, usando como fuentes de carbono y nitrógeno, glucosa y sulfato de amonio en la primera y segunda etapa de cultivo. Durante la tercera etapa (producción de mcl-PHA) se alimentó medio de cultivo con fracciones de Hw (5 g L^{-1}) como cosustrato, en adición de Tritón X-100 (3 CMC). La acumulación de scl-mcl-PHA a partir de la fracción de alcoholes céreos de Hw (OH Hw) fue de 20.85 g L^{-1} (90.19 % p/p) mientras que a partir de la fracción de ácidos grasos de Hw (AG Hw) fue de 5.57 g L^{-1} (67.28 % p/p). Se realizó un ANOVA de un factor para comparar cuantitativamente la cantidad de scl-mcl-PHA sintetizado. La prueba de Dunnett, estableció los intervalos de tiempo, en que *C. necator* produce mayor porcentaje de polímero. Se determinó por CG, la presencia de 3-hidroxibutirato (3HB) y 3-hidroxidecanoato (3HD) en los scl-mcl-PHA sintetizados.

Biopolímeros, *Cupriavidus Necator*, Lote alimentado

Citation: QUINTANAR-GÓMEZ, Samuel, TÉLLEZ-JURADO, Alejandro and VARGAS-HERNÁNDEZ, Genaro. Effect of the fractions of beeswax hydrolysates as carbon source in the synthesis of short and medium chain polyhydroxyalkanoates (scl-mcl-PHA). ECORFAN Journal-Ecuador. 2018, 5-9:30-36.

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† Researcher contributing as first author.

Introduction

Polyhydroxyalkanoates (PHA) are microbial polyesters produced as energy reserve materials when there is an imbalance of nutrients (nitrogen, phosphorus, magnesium, potassium, sulfur and oxygen) and an increase in the concentration of carbon in the culture medium (González-García et al. ., 2013). These materials are biodegradable and also have thermoplastic and elastomeric characteristics similar to petroleum-based plastics.

Structurally, they are classified based on the number of carbon atoms in the length of the aliphatic chain of their monomers, such as short chain PHA (scl-PHA) with 3-5 carbon and medium chain PHA (mcl-PHA) with 6 - 14 carbons. (González-García et al., 2013). The bacteria producing mcl-PHA can synthesize them from fatty acids or their salts (Impallomeni et al., 2011), aliphatic alkanes, alkenes (Kim et al., 1995) or agroindustrial residues (Lerch et al., 2011). *Cupriavidus necator (formerly Wautersia eutropha> Ralstonia eutropha> Alcaligenes eutrophus)* PHA form with yields of 80 - 90% of accumulated polymer in relation to the weight of its biomass in dry basis (Lerch et al., 2011).

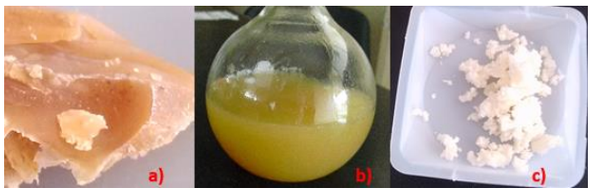
There are few reports that native C. necator (ATCC 17699) can synthesize mcl-PHA from fatty acids and derivatives, because their PHA synthase is strictly specific to obtain scl-PHA monomers (Slater et al., 1992). Verlinden et al., (2011), synthesized PHB from C. necator H16 (ATCC 17699) fed with residual frying oils, obtaining 1.2 g L-1 in 72 h of culture. Jain et al., (2013); confirmed that C. necator H16 can synthesize copolymers such as P (3HB-co-3HHx) from crude kernel palm oil (abundant in palmitic acid), as the sole carbon source in concentrations of 5 g L-1 under the limitation of nitrogen and without precursor compounds.

The polymer obtained represented 63% of the dry weight in the form of P (3HB-co-3HHx), with 4 mol% of 3HB. Regarding the carbon source, some substrates, such as beeswax, have not been reported for PHA synthesis. Beeswax is a renewable source used as an additive for ointments, creams and ointments. However, the residual waxes of unpurified bee are used for the molding of jewelery, the manufacture of adhesive tapes and insulators (León et al., 2014) that are generally discarded after their use.

Chemically, beeswax is composed of waxy esters that can be hydrolyzed with alkalis to obtain fatty acids (such as palmitate) (Buchwald et al., 2009) and long-chain monohydric alcohols (Maia and Nunes, 2013), which can be used as substrates for PHA synthesis. The objective of this work was to evaluate the yields of synthesis and incorporation of mcl-PHA monomers synthesized from separate fractions of bee wax hydrolysates (Hw), during the accumulation phase of a crop per batch fed, of three stages using C. necator.

Methodology

Microorganism and culture medium. C. necator ATCC 17699 was grown in Luria Bertani broth (LB) at 30 ° C and 150 rpm. This culture was used as a seed flask to inoculate 10% (v / v) of the reactor. The cultures were performed in triplicate in a 5 L fermentation unit (FA-5000, VICHI). The temperature was maintained at 30 ° C and the pH was controlled with 2 M NaOH and 0.47 M HCl..



Figureure 1 Fractions obtained from the alkaline hydrolysis of beeswax: a) beeswax b) fatty acids, c) waxy alcohols

Samples were taken every 3 h to quantify the concentrations of glucose, residual ammonium, biomass and PHA production. The fermentations were made from the modified method of López-Cuellar et al., (2011). To assist the bioavailability of the Hw fractions, 0.43 g L-1 of Triton X-100 was added to 3 CMC (Budde et al., 2011).

Alkaline hydrolysis of beeswax

The beeswax was previously treated to alkaline hydrolysis by the method described by Wás et al., (2014). Hydrolysis was carried out by placing 20 g of beeswax at reflux (24 h at 100 ° C), 10% w / v of sodium hydroxide (Bonaduce et al., 2004) and 300 mL of a tetrahydrofuran (THF) mixture.) - methanol 1: 1 as a solvent (Wang and Advincula, 2001). Once the fractions were obtained, they were separated by filtration while still hot.

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The solid corresponded to the fraction of long-chain alcohols of waxy esters (OH Hw) (Bonaduce et al., 2004). To the remaining NaOH solution (salts of fatty acids) was added HCl (ratio 2: 1) until obtaining a pH of 6.0 (Buchwald et al., 2009), forming a yellow product corresponding to the fraction of fatty acids of the esters waxy (AG Hw) (Figure. 1). Both fractions were dried for 24 h at 105 ° C.

Analytical procedures

The concentrations of cell mass (X) and residual biomass (rX) were determined by gravimetry (Budde et al., 2011). The glucose and ammonium measurements were made using the dinitrosalicylic acid (DNS) methods (Miller 1959) and Weatherburn (1967) respectively. The amount of Hw was determined according to the method described by Quintanar-Gómez et al., (2017).

Purification of scl-mcl-PHA

The scl-mcl-PHA were purified by extraction with chloroform at reflux for 10 minutes and precipitated with cold hexane after filtering the cellular debris (Rozsa et al., 2004). The procedure was repeated three times to avoid the presence of residual cellular and residual metabolites of Hw.

Chemical characterization of scl-mcl-PHA

The scl-mcl-PHA samples were subjected to prior methanolysis to obtain the fatty acid monomers (Impallomeni et al., 2013). A Thermo Scientific GC model Trace 1310 was used, equipped with a flame ionization detector and a Thermo scientific capillary column (30 m × 0.32 mm, a film thickness of 1.0 μm). The carrier gas was He (flow of 1 mL / min). The programmed temperature ramps were: initial temperature of 80 ° C for 4 min., Followed by an increase to 260 ° C with a ramp of 8 ° C / min, staying for 23 min. Volumes of 1 μL of the organic phase were injected (10: 1 division ratio) (Rathinasabapathy et al., 2013).

Statistic analysis

The obtained values were subjected to a statistical analysis of variance (ANOVA) of a factor to quantitatively compare the intracellular amount of scl-mcl-PHA between synthetic treatments from the Hw fractions.

A Dunnett's test was complemented to analyze the difference of inter and intra-treatments means and determine the stage of highest production of mcl-PHA. The statistical software (IBM SPSS Statistics version 21.0) was used, establishing a value of $\alpha = 0.05$.

Results and Discussion

Alkaline hydrolysis of beeswax

The yield obtained for the fraction soluble in organic solvent corresponded to 28.5% grams of dry matter (% gms) of saponifiable fatty acids, which were calculated from the weight difference between the original sample and the hydrolyzate obtained.

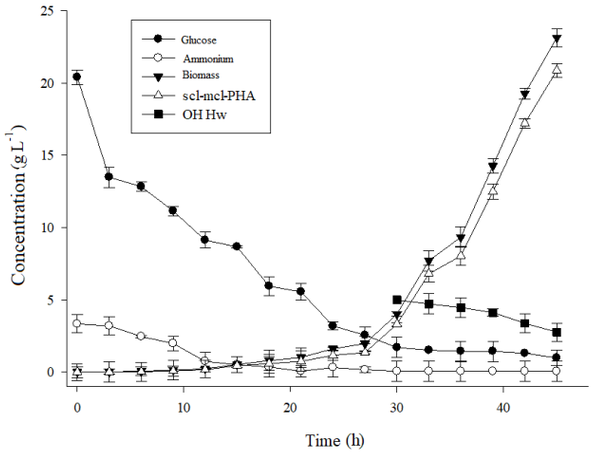

Figure 2 Consumption of glucose, ammonium, OH Hw, biomass production and scl-mcl-PHA with C. necator

The non-polar phase was separated by vacuum filtration, forming a white solid (Figure. 1), corresponding to the fraction of long-chain alcohols of beeswax esters (71.5% gms) (Bonaduce et al., 2004).

Synthesis of scl-mcl-PHA from fractions of OH Hw

Figureure 2 shows the kinetic profile of the fermentation with the fraction of OH Hw. C. necator reached a concentration of 1.02 g L^{-1}, a lag phase of 15 h and a maximum specific growth rate (μ) of 0.21 h^{-1}.

The initial ammonium concentration in the culture was 3.38 g L^{-1}, with a consumption rate of 0.52 g L^{-1} h^{-1} (21 hours) (Khanna and Srivastava, 2007). Once the ammonium was exhausted, the first batch was fed (second stage of cultivation) with glucose and ammonium as sources of carbon and nitrogen (ratio C / N = 12).

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The feeding rate was maintained at 1.46 g h-1, increasing the cell density from 1.02 to 4.02 g L^{-1} (21 to 30 h of culture respectively). The glucose and ammonium consumption rates were 0.21 and 0.13 g L^{-1} h^{-1}. After 30 hours of culture, the residual glucose and ammonium concentrations decreased to 1.68 g L^{-1} and 0.69 g L^{-1} respectively (Figure. 2).

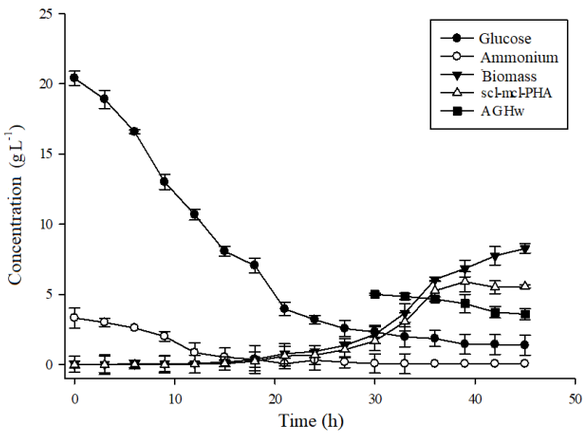

Figure 3 Consumption of glucose, ammonium, AG Hw, production of biomass and scl-mcl-PHA with C. necator

The second batch was fed (third stage of culture) with the fraction of OH Hw (5 g L^{-1}, C/N 200) in the presence of 0.43 g L^{-1} of Triton X-100, obtaining a consumption rate of 0.44 g L^{-1} h^{-1}. Shang *et al*., (2004); reported the same consumption rate when feeding glucose and valeric acid (< 1.20 g L^{-1}) to produce P (HBHV) with R. eutropha NCIMB 11599. The fraction of OH Hw decreased (from 5.0 to 2.77 g L^{-1}), Seeing an increase in biomass concentration from 4.02 to 23.13 g L^{-1} as well as the intracellular concentration of scl-mcl-PHA (from 3.32 g L^{-1} to 20.85 g L^{-1}) during the last 15 hours of cultivation (Khanna y Srivastava, 2007).

The accumulation of OH Hw is due to residual glucose as additional co-substrate, causing the waxy carbon source to become the second source of energy. The samples collected after 42 h of culture showed 89 to 90% w / w of scl-mcl-PHA. Lee et al., (2008); obtain the same values (90% w / w) by synthesizing P (3HB-co-3HV) with C. necator using kernel palm oil (5 g L^{-1}) and sodium propionate (5 g L^{-1}).

Synthesis of scl-mcl-PHA from fractions of AG Hw

When analyzing the incorporation of medium chain monomers from AG Hw fraction, it was observed that the concentration of biomass during the first 21 h of culture was 0.77 g L^{-1}. The lag phase was 12 hours with a □ of 0.18 h^{-1}. This concentration increased to 2.14 g L^{-1} at 30 h of culture, after the first feeding with glucose and ammonium at a C / N = 12 ratio. The fresh medium feed flow in this stage was 2.27 g h^{-1} with consumption rates of 2.40 g h^{-1} and 0.51 g h^{-1} for glucose and ammonium respectively.

After 30 h (residual ammonium consumption), the second feed was started with the AG fraction Hw. The amount of remaining residual glucose was 2.29 g L^{-1}, its consumption being observed up to a concentration of 1.36 g L^{-1} (Figure. 3). This behavior shows that the bioavailability and the molecular size of the carbon source are factors that favor the consumption of glucose preferably to an alternative carbon source. (Lee *et al*., 2008).

The maximum biomass concentration increased by supplying 5 g L-1 of the AG Hw fraction; while the fatty acid fraction of Hw decreased in the culture medium 1.41 g L^{-1} during the last 15 h of cultivation, with a consumption rate of 0.28 g h^{-1}. The final concentration of biomass produced during the synthesis of scl-mcl-PHA (8.27 g L^{-1}) from the fatty acid fraction of Hw, it was lower than that obtained with the waxy alcohol fraction of Hw (23.13 g L^{-1}).

When feeding the AG Hw fraction at 30 h of culture, an increase of 78% w / w to 86% w / w of scl-mcl-PHA of the generated biomass was observed, during the first 9 h of feeding of the co-substrate. Subsequent to 39 h of culture the intracellular decrease of scl-mcl-PHA was observed (Table 4). This behavior shows an adverse effect to that described by Lee et al., (2008) who assume that the absence of acetyl-CoA decreases the availability of CoASH and as a consequence the production of acetoacetyl-CoA by 3-ketothiolase.

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Statistical analysis of scl-mcl-PHA synthesis treatments

The contrast of equality of population variances of the scl-mcl-PHA concentrations, determined that there is a statistical difference between the synthesis yields obtained between experimental treatments. Table 1 shows that the level of significance is greater than 0.05 in the experiments carried out with the fractions of AG Hw and OH Hw.

The significance values obtained show that C. necator produces different amounts of scl-mcl-PHA as a function of the fraction of Hw fed as a carbon source. The treatment with OH Hw, presented the highest value of mcl-PHA, while the concentrations of polymer obtained with AG Hw were lower.

	Statistic of Levene	gl1	gl2	Next
PHA OH Hw	2.768	5	12	0.069
PHA AG Hw	2.703	5	12	0.073

Table 1 Variance homogeneity test for the quantification of scl-mcl-PHA synthesized from Hw fractions
PHA OH Hw: Treatment with waxy alcohols of Hw.
PHA AG Hw: Treatment with fatty acids of Hw.
gl1: degrees of freedom between groups
gl2: intra-group degrees of freedom
Sig .: significance

The ANOVA, determined that the levels of significance between classes were less than 0.05, for which the hypothesis of equality of means is rejected (H0: □1, OH Hw = □ 2, AG Hw). In Table 3 (Dunnett's chart), the comparison of means between intergroup concentrations of scl-mcl-PHA obtained with the fractions of Hw fed is made, determining that there is statistical difference, when comparing the intervals between 30 to 39 h against the 45 h of cultivation (considered as reference for obtaining the highest concentration of biomass).

This suggests the production of scl-mcl-PHA is ascending in the experiment with the fraction of OH Hw fed in the third stage of culture.

	Sum of squares	gl	Half quadratic	F	Next
PHA OH Hw					
Inter-groups	268.944	5	53.789	328.56	0.000
Intra-groups	1.964	12	0.164		
Total	270.908	17			
PHA AG Hw					
Inter-groups	44.164	5	8.833	432.84	0.000
Intra-groups	0.245	12	0.020		
Total	44.409	17			

Table 2 One-way ANOVA for the quantification of scl-mcl-PHA synthesized with C. necator from fractions of Hw
PHA OH Hw: Treatment with waxy alcohols of Hw.
PHA AG Hw: Treatment with fatty acids of Hw.
gl: degrees of freedom
Sig: significance

The statistical analysis allowed us to infer that the fraction of AG Hw is less assimilated compared to that of OH Hw during the synthesis of the polymer. The highest value of difference of means between experiments was obtained at 39 h of culture with AG Hw. This shows that the concentration of scl-mcl-PHA decreases, as a consequence of the poor assimilation of the waxy carbon source after 39 h of culture.

Dependent variable	(I) Time	(J) Time	Difference of means (I-J)	Typical error	95% confidence interval Lower limit	Upper limit
PHA OH Hw	30	45	-11.16000*	0.330	-12.1184	-10.2015
	33	45	-8.91366*	0.330	-9.8721	-7.9552
	36	45	-8.21933*	0.330	-9.1778	-7.2608
	39	45	-5.41766*	0.330	-6.3761	-4.4592
	42	45	-2.35733*	0.330	-3.3158	-1.3988
PHA AG Hw	30	45	-3.88266*	0.116	-4.2210	-3.5442
	33	45	-2.51366*	0.116	-2.8520	-2.1752
	36	45	-.30500	0.116	-.6434	0.0334
	39	45	.33466	0.116	-0.0037	0.6730
	42	45	-.05866	0.116	-0.3970	0.2797

Table 3 Multiple comparisons: Dunnett's test for the quantification of scl-mcl-PHA synthesized from fractions of Hw
PHA OH Hw: Treatment with waxy alcohols of Hw.
PHA AG Hw: Treatment with fatty acids of Hw.
*. The difference in means is significant at the 0.05 level

When comparing the values of difference of intergroup means, it is concluded that the treatment with the highest intracellular concentration of scl-mcl-PHA, was the fermentation of OH Hw at 45 h of culture (Table 3).

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Characterization by GC of scl-mcl-PHA monomers synthesized with Hw fractions

The GC analysis of scl-mcl-PHA hydrolysates synthesized with OH Hw (Table 4) confirmed the incorporation of a 12 C monomer (determined as 3 HDD by 1 H NMR and 13 C by 400 MHz in a previous work (Quintanar-Gómez et al., 2017; data not shown) with a retention time of 12.5 min. The% mol of 3HDD was 1.7 in relation to that of 3HB (98.3% mol). For scl-mcl-PHA obtained from AG Hw, the retention time was 12.38 min; close to the oleic acid standard (13.0 min).

The percentage composition of each monomer was 97 mol% of 3HB and 3 mol% of 3HD (Table 4). The% mol of mcl-PHA was attributed to the previous accumulation of PHB in the second stage of cultivation, generating a metabolic deceleration effect, which determines the low assimilation of the Hw fractions (Khanna and Sirvastava, 2007). The concentration of mcl-PHA obtained was higher than that reported by Rathinasabapathy et al., (2013); who synthesized a scl-mcl-PHA, with 99.81% mol of 3HB, 0.06% mol of 3HV, 0.09 mol% of 3HHx, 0.04 mol% of 3HO using canola oil. These results confirmed that C. necator incorporates mcl-PHA from fractions of Hw.

Fatty acid	Holding time		
	Reference	scl-mcl-PHA	
		OH Hw	AG Hw
Butyric acid	2.5	2.5	2.5
Valeric acid	6.25	0	0
Octanoic acid	9.4	0	0
Oleic acid	13.0	12.5	12.38
Palmitic acid	13.2	0	0

Table 4. Butyric acid
Valeric acid
Octanoic acid
Oleic acid
Palmitic acid.

Acknowledgement

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Conclusions

Cupriavidus necator assimilated the Hw fractions separately as carbon sources added during the third stage of cultivation of the batch fed. The low production of mcl-PHA was due to the metabolic deceleration caused by the residual glucose of the culture medium.

Both Hw fractions allowed to incorporate medium chain monomers, during the synthesis of scl-mcl-PHA, confirmed from the CG analysis. At the end of the synthesis processes, both residual fractions of Hw, could be separated by flocculation, to be reused again in later fermentations. These materials promise expectations when used in the biomedical field of tissue regeneration or controlled release of drugs, due to their biocompatible potential and the improvement of their chemical properties in relation to crystalline materials such as PHB.

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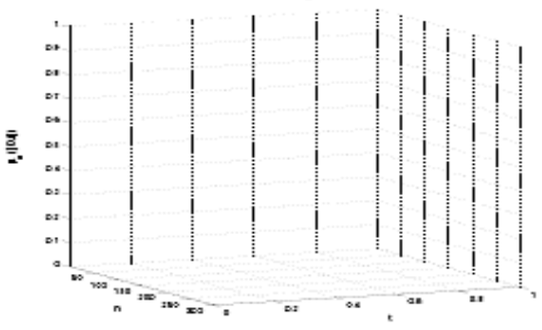

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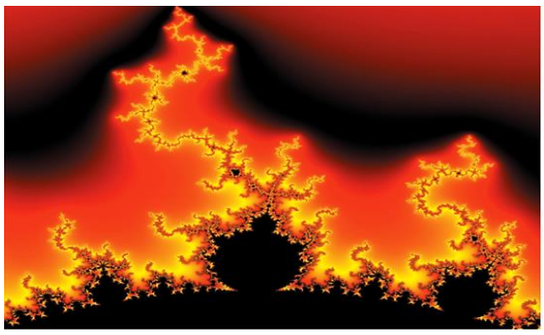

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