UNIVERSIDAD POPULAR AUTÓNOMA DEL ESTADO DE PUEBLA

DECANATO DE CIENCIAS BIOLÓGICAS

Doctorado en Biotecnología

Evaluación de *Azotobacter vinelandii* ATCC 12837 inmovilizada en alginato como inoculante y su crecimiento *in vitro* en presencia de clorpirifós

VICTORIA CONDE AVILA

Directores:

DR. LUIS DANIEL ORTEGA MARTÍNEZ

DRA. BEATRIZ PÉREZ ARMENDÁRIZ

Asesores:

DR. OCTAVIO LOERA CORRAL

DR. CARLOS F. PEÑA MALACARA

UPAEP – Secretaría General
Dirección General de Apoyos Académicos
Dirección del Centro de Recursos para el Aprendizaje y la Investigación.
Biblioteca Central - **Karol Wojtyla**

Tesis Digitales Restricciones de uso:

DERECHOS RESERVADOS ©
PROHIBIDA SU REPRODUCCIÓN TOTAL O PARCIAL

Todo el material contenido en esta tesis está protegido por la Ley Federal del Derecho de Autor (LFDA) de los Estados Unidos Mexicanos (México).

El uso de textos, imágenes, gráficas, fragmentos de videos, y demás material que sea objeto de protección de los derechos de autor, será exclusivamente para fines educativos e informativos y deberá citar la fuente de donde la obtuvo mencionando el autor o autores involucrados en el documento.

Cualquier uso distinto como el lucro, reproducción, edición o modificación, será perseguido y sancionado por el respectivo titular de los Derechos de Autor.

Universidad Popular Autónoma del Estado de Puebla

DECANATO DE CIENCIAS BIOLÓGICAS
FACULTAD DE BIOTECNOLOGÍA

DOCTORADO EN BIOTECNOLOGÍA

SE APRUEBA LA TESIS:

Evaluación de *Azotobacter vinelandii* ATCC 12837 inmovilizada en alginato como inoculante y su crecimiento *in vitro* en presencia de clorpirifós

De la alumna:

VICTORIA CONDE AVILA

Dr. Luis Daniel Ortega Martínez
Director

Dra. Beatriz Pérez Armendáriz
Codirectora

Dr. Octavio Loera Corral
Asesor

Dr. Carlos F. Peña Malacara
Asesor

UPAEP
21 Sur 1103
Col. Barrio de Santiago
Puebla, Pue. México
C.P. 72410

Tel: 01 (222) 229 9400
Fax: 01 (222) 2 32 5251
01 800 224 2200
www.upaep.mx

ÍNDICE

RESUMEN ……..…….,….3

ABSTRACT ……..……...…5

CAPÍTULO I

1. INTRODUCCIÓN…..…….…….7

CAPÍTULO II

2. MARCO TÉORICO

Artículo de revisón:

DEGRADACIÓN DE PLAGUICIDAS POR MICROORGANISMOS INMOVILIZADOS…..…....10

2.1 Introducción ……..……..…….……..…..10
2.2 Plaguicidas, generalidades y residualidad ……..……..…..…12
2.3 Microorganismos implicados en la degradación de plaguicidas…….…....15
2.4 Inmovilización celular, técnicas y materiales ……..……..17
2.5 Ventajas y limitaciones de la inmovilización celular ……..….20
2.6 Degradación de plaguicidas con microorgansimos inmovilizados……..….22

CAPÍTULO III

3.1 PLANTEAMIENTO DEL PROBLEMA ..……...……..…45
3.2 JUSTIFICACIÓN…..……..……..…46
3.3 HIPÓTESIS…….…….……...……..…..46
3.4 OBJETIVOS…….....47
3.4.1 Objetivo general……..……..…….…….47
3.4.2 Objetivos específicos ……..……..……...……47
3.5 ESTRATEGÍA METODOLÓGICA……..48

CAPÍTULO IV

Artículo científico:

ENCAPSULATCIÓN DE *AZOTOBACTER VINELANDII* EN PERLAS DE ALGINATO-NA COMO INCOCULATE DE PLANTÚLAS DE TOMATE ……..……..…….49

CAPÍTULO V

Artículo científico:
CRECIMIENTO, DEGRADACIÓN DE CLORPIRIFÓS Y MICROENCAPSULACIÓN EMPLEANDO *AZOTOBACTER VINELANDI*I ATCC 12837 ……...……...……..……64

BIBLIOGRAFÍA ……102

RESUMEN

La inmovilización celular (IC) es un método que restringe a las células a un área delimitada preservando sus actividades metabólicas. La biodegradación de plaguicidas es una de las aplicaciones de la IC. En este trabajo se recopilan los aspectos más importantes de la IC microbiana como estrategia para la degradación de plaguicidas. Discutimos las principales sustancias empleadas, los microorganismos, materiales y técnicas utilizadas, así como sus ventajas y limitaciones. De acuerdo con la revisión de literatura, reportamos que la IC es una técnica en etapa experimental en la que generalmente aumentos en el porcentaje de degradación, mayor estabilidad, protección y tolerancia a plaguicidas se logran al implementar algún método de inmovilización, especialmente la encapsulación con polímeros. Sin embargo, identificamos la necesidad de evaluar la IC con organismos rizosféricos tolerantes; así como de generar alternativas con posibles aplicaciones *in situ* para la degradación de plaguicidas.

Bajo dicho contexto, se propuso evaluar el efecto de la rizobacteria *Azotobacter vinelandii* ATCC 12837 inmovilizada en cápsulas de alginato-Na como inoculante de plántulas de tomate (*Solanum Lycopersicum* L). Se realizaron dos tratamientos de inoculación: líquido y encapsulado, y el control sin microorganismos. Se determinaron variables de creciemiento vegetal, la viabilidad microbiana de *A. vinelandii* mediante métodos microbiológicos y moleculares. La inoculación con *A. vinelandii* en forma líquida e inmovilizada favoreció el crecimiento de las plántulas. Empleando el inóculo encapsulado aumentaron significativamente el porcentaje de germinación, el diámetro del tallo, la altura, longitud de las raíces, la concentración de NO_3 y Na, en comparación con el control. La viabilidad de la bacteria se mantuvo durante 28 días utilizando ambos métodos de inoculación. En este trabajo destacamos el uso del alginato-Na como material para la encapsulación de *A. vinelandii* y sugerimos el desarrollo de formulaciones de fácil manejo y escalamiento. Dichos resultados proporcionaron una base para optimizar el crecimiento de *A. vinelandii* y evaluar el efecto del plaguicida clorpirifós (CP) en su desarrollo *in vitro*. Al respecto, evaluamos el crecimiento, la tolerancia y biodegradación de CP; así como un proceso de microencapsulación con alginato-Na utilizando *A. vinelandii* ATCC 12837. Se desarrolló una estrategia basada en la modificación de los medios de cultivo (fuente de carbono y relación C:N) y de las condiciones de aireación, con el fin de aumentar la concentración celular de la bacteria y determinar su tolerancia y eficiencia en la degradación de CP. El cultivo en matraces agitados de la cepa ATCC 12837, utilizando sacarosa como fuente de carbono (C), mejoró significativamente el crecimiento en comparación con medios con manitol. Cuando la cepa se cultivó en condiciones limitadas de oxígeno (5,5, 11,25 mmol L^{-1} h^{-1}) y sin limitación de oxígeno (22 mmol L^{-1} h^{-1}), los parámetros de crecimiento no se vieron afectados. En los cultivos en medio líquido con CP, las bacterias

toleraron una alta concentración del plaguicida (500 ppm) y los parámetros de crecimiento (número de unidades formadoras de colonias, biomasa y proteína total) aumentaron incluso en condiciones con una fuente de C reducida (sacarosa 2 g L^{-1}). La cepa degradó el 99.6% de CP en 60 h en medio líquido con sacarosa y redujo el 50% de la concentración inicial (DT_{50}) en 6 h. Por otro lado, la microencapsulación con alginato-Na mediante secado por aspersión mantuvo una viabilidad de $4x10^9$ UFC g^{-1} de la formulación en polvo.

Nuestros resultados demostraron que el organismo modelo *A. vinelandii* ATTC 12837, identificada como promotora de crecimiento vegetal, es también una cepa tolerante y eficiente en la degradación de CP *in vitro*, y con el potencial de desarrollarse como una candidata prometedora para la biorremediación de CP con aplicaciones en la inoculación de microorganismos rizósfericos inmovilizados.

ABSTRACT

Cell immobilization (CI) is a method that restricts cells to a delimited area while preserving their metabolic activities. Pesticide biodegradation is one of the applications of CI. This study highlights the most important aspects of microbial CI as a strategy for pesticide degradation. We discuss the main substances employed, the microorganisms, materials and techniques used, as well as their advantages and limitations. According to the literature review, we report that CI is a technique in experimental stage in which generally increases in the percentage of degradation, a higher stability, protection and tolerance to pesticides are achieved by implementing some immobilization method, especially encapsulation with polymers. However, we identified the need to evaluate CI with tolerant rhizospheric organisms; as well as to generate alternatives with possible *in situ* applications for pesticide degradation.

In this context, we proposed to evaluate the effect of the rhizobacterium *Azotobacter vinelandii* ATCC 12837 immobilised in alginate-Na beads as an inoculant for tomato (*Solanum Lycopersicum* L) seedlings. Two inoculation treatments were carried out: liquid and encapsulated, and the control without microorganisms. Plant growth variables, microbial viability and the presence of *A. vinelandii* were determined by microbiological and molecular methods. Inoculation with *A. vinelandii* in liquid and encapsulated form favored seedling growth. Plants with the encapsulated inoculum significantly increased germination percentage, stem diameter, height, root length, NO_3 and Na concentration, compared to the control. Bacterial viability was maintained for 28 days using both inoculation methods. In this study, we highlight the use of alginate-Na as a material for encapsulation of *A. vinelandii* and suggest the development of easy to handle and scale-up formulations. These results provided a basis to optimize the *A. vinelandii* growth and to evaluate the effect of the pesticide chlorpyrifos (CP) on its *in vitro* development. In this regard, we evaluated the growth, tolerance and CP biodegradation, as well as a microencapsulation process with alginate-Na using *A. vinelandii* ATCC 12837. A strategy based on the modification of the culture media (carbon (C) source and C:N ratio) and aeration conditions was developed in order to increase the bacteria cell concentration and determine their tolerance or CP degradation. Shake flask culture of strain ATCC 12837, using sucrose as C source, significantly improved growth compared to media with mannitol. When the strain was grown under oxygen-limited (5.5, 11.25 mmol $L^{-1}h^{-1}$) and non-oxygen limited conditions (22 mmol L^{-1} h^{-1}), growth parameters were not affected. In cultures in liquid medium with CP, the bacteria tolerated a high concentration of the pesticide (500 ppm) and growth parameters increased even under conditions with a reduced C source (sucrose 2 g L^{-1}). The strain degraded 99.6 % of CP in 60 h in liquid medium in co-metabolism with sucrose and reduced by 50% the initial concentration (DT_{50}) in 6 h. On the other hand, microencapsulation with Na-alginate by spray drying maintained a viability of 4 $x10^9$ CFU g^{-1} in a powder formulation.

Our results demonstrated that the model organism *A. vinelandii* ATTC 12837, identified as a plant growth promoter, is also a highly tolerant and efficient strain in the degradation of CP *in vitro*, and with the potential to develop as a promising candidate for CP bioremediation and applications in the inoculation of immobilized rhizospheric microorganisms.

CAPÍTULO I

1. INTRODUCCIÓN

La producción agrícola se ha caracterizado por el uso intensivo de insumos tanto químicos como biológicos (Muñoz et al. 2008). Los insumos empleados con mayor frecuencia son los plaguicidas, principalmente el grupo de los organofosforados (POF´s) (Firozjaei et al. 2015). Después de la prohibición de la mayoría de los plaguicidas organoclorados, los POF´s comenazaron a sustituirlos y la tendencia de su uso ha crecido exponencialmente pese a ser agentes altamente tóxicos.

México es considerado uno de los países con mayor uso promedio de plaguicidas y en las últimas décadas su empleo ha incrementado de forma exponencial (Zhang 2018). En el caso particular del estado de Puebla, se ha reportado mayor uso de plaguicidas inhibidores de acetilcolinesterasa como los carbamatos (carbofuran), ditiocarbamatos (mancozeb) y POF´s como clorpirifós (CP), este último clasificado como moderadamente tóxico y altamente persistente; mismos que son aplicados bajo condiciones de amplia exposición e intervalos de aplicación frecuentes provocando mayor persistencia y residualidad en el ambiente (Ortega et al. 2014).

Los POF´s son derivados del ácido fosfórico, fosfónico y fosfortioico. Su mecanismo biocida es a través de la inhibición por fosforilación de la la enzima acetilcolinesterasa. Lo anterior provoca una acumulación del neurotrasmisor acetilcolina, lo que conlleva a una sobreestimulación del sistema nervioso, provocando la muerte de insectos y organismos no objetivo (Abraham y Silambarasan 2016).

Además, producen efectos adversos en la salud humana, desde nauseas hasta afectaciones musculares, daños en la regulación de la espermatogénesis, alteraciones en el sistema nervioso central y estrés oxidativo (Pasiani et al. 2012, Firouzajaei et al. 2015). A pesar de esto, debido a su eficacia y relativo bajo costo, los POF´s son constantemente empleados en diversos sistemas de producción agrícola (Costa et al. 2006).

Algunos de los problemas causados por el uso de plaguicidas aumentan gracias al manejo inadecuado, la mezcla de productos y carencia de criterio técnico (Ortega et al. 2014), mismos que llevan a intoxicación (Hernández-León et al. 2010), resistencia a plagas (Badii y Varela 2008), contaminación de aire, agua y suelo, así como a la pérdida de microbiota edáfica benéfica (Ipsilantis et al. 2012).

En este sentido, se han reportado serias afectaciones en la biología del suelo que involucran cambios cualitativos y cuantitativos en el microbioma, cambios en las actividades enzimáticas, alteraciones en los balances de nitrógeno y efectos adversos en los organismos simbióticos rizosféricos (Badii y Varela

2008, Hussain et al. 2009, Ipsilantis et al. 2012), por lo que resultan afectados directa o indirectamente los microorganismos y las propiedades del suelo requeridas para mantener la fertilidad, y por lo tanto, el óptimo crecimiento y producción de los cultivos (Ipsilantis et al. 2012; Das et al. 2016).

Aunque los plaguicidas pueden ser degradados por vía biótica y abiótica, la biorremediación representa una alternativa efectiva para transformarlos en compuestos más simples y menos tóxicos a través del potencial metabólico de los microorganismos para degradarlos o mineralizarlos completamente (Ortiz-Hernández et al 2013).

La biorremediación depende de diversos factores; sin embargo, dicho proceso se puede acelerar mediante el uso de diferentes tecnologías (Cycoń et al. 2017). La bioaumentación representa un método ventajoso de descontaminación y una de las áreas de mayor interés para el tratamiento de agua y suelos contaminados con POF´s (Cycoń et al. 2017).

No obstante, la selección de microorganismos para biorremediación de plaguicidas requiere de especial cuidado, ya que se deben considerar factores como el potencial de degradación, facilidad de cultivo microbiano, la capacidad de sobrevivir en altas concentraciones del plaguicida, su capacidad para adaptarse en suelos en competencia con microorganismos nativos, así como las posibles interacciones benéficas con plantas y otros microorganismos de la rizósfera (Mrozik y Piotrowska-Saget 2010).

En este sentido, se ha evaluado la tolerancia y la degradación de plaguicidas por rizobacterias promotoras de crecimiento vegetal (RPCV) en los géneros *Azospirillum* (Santos et al. 2020), *Bacillus* (Praveen Kumar et al. 2014), *Klebsiella* (Rani et al. 2019a), *Pseudomonas* (Giri y Rai 2012), *Serriata* (Cycón et al. 2013), *Ochrobactrum* (Abraham y Silambarasan 2016) y *Azotobacter* (Chennappa et al. 2018a).

Las *Azotobacter* spp. destacan en la fijación asimbiótica de N_2, solubilización de P (Sethi y Gupta, 2013), producción de hormonas vegetales (Chobotarov et al. 2017), sideróforos (Shahid et al. 2019), vitaminas (Revillas et al. 2000), síntesis de compuestos antimicrobianos (Nagaraja et al. 2016), producción de metabolitos de interés industrial como el alginato, el polihidroxibutirato (PHB) y alquilresorcinoles (Gurikar et al. 2016, Castillo et al. 2017, Suaza-García y Coy-Barrera 2014), así como en procesos de degradación de sustancias tóxicas (Chennappa et al. 2019).

Algunas especies de *Azotobacter* pueden degradar compuestos aromáticos como insecticidas, fungicidas y herbicidas (Castillo et al. 2011; Chennappa et al. 2016). Estas bacterias han mostrado particularmente tolerancia al endosulfán, forato, carbendazima, CP, entre otros (Castillo et al. 2011; Chennappa et al. 2014a; Gurikar et al.2016; Rani y Kumar et al. 2017).

Sin embargo, la incorporación de *Azotobacter* y otras RPCV directamente al suelo como inoculantes puede verse disminuida. Para hacer frente a esa limitante, se ha sugerido el uso de estrategias que brinden protección y prolonguen la viabilidad de los microorganismos en diferentes condiciones, incluido el estrés severo por plaguicidas (Lenart 2012; Barman et al. 2019; Kumar et al. 2019; Sarker et al. 2021).

Algunas investigaciones se han concentrado en el uso de microorganismos y consorcios inmovilizados en diversos materiales que les permitan mantener sus actividades metabólicas. En este sentido, la inmovilización celular (CI) tiene la finalidad de proteger a los microorganismos de las condiciones adversas del ambiente, prolongar su supervivencia y efectividad ante condiciones de estrés (Yañez-Ocampo et al. 2009; Colla et al 2014).

A continuación se describen algunos aspectos generales sobre plaguicidas, técnicas de IC, así como antecedentes particulares de la degradación de plaguicidas empleando microrganismos inmovilizados.

CAPÍTULO II

INTERNATIONAL JOURNAL OF ENVIRONMENTAL ANALYTICAL CHEMISTRY

Taylor & Francis
Taylor & Francis Group

https://doi.org/10.1080/03067319.2020.1715375

PESTICIDES DEGRADATION BY IMMOBILISED MICROORGANISMS

Check for updates

Victoria Conde-Avila[a], Luis Daniel Ortega-Martínez[a], Octavio Loera[b], Elie Girgis El Kassis[a], Jorge García Dávila[c], Carmen Martínez Valenzuela[d] and Beatriz Pérez Armendáriz[a]

[a]Facultad de Biotecnología, Universidad Popular Autónoma del Estado de Puebla, Puebla, México;
[b]Universidad Autónoma Metropolitana-Unidad Iztapalapa, Ciudad de México, CDMX, México;
[c]Universidad Politécnica de Tlaxcala, Tlaxcala, México;
[d]Unidad de Investigación en Ambiente y Salud, Universidad Autónoma de Occidente, Los Mochis, Sinaloa México

ABSTRACT

Cellular immobilisation (CI) is a method characterised by restricting the cells to a delimited area while preserving their metabolic, catabolic, and catalytic activities. Biodegradation of contaminants is one of the potential applications of CI. Immobilised cells can achieve the degradation of toxic substances with higher efficiency than cells in a free state. The aim of this work was to compile the most important aspects of CI as a strategy for the degradation of pesticides. We discuss the main targeted chemical substances, the used microorganisms, materials, and techniques, as well as their advantages, and limitations. We highlight increases in the percentage of degradation, greater stability, protection, and tolerance to pesticides when a CI strategy is implemented. Finally, the requirements for deepening our understanding of the involved kinetic, molecular, and transfer processes are discussed, particularly for their application *in situ*.

ARTICLE HISTORY

Received 1 October 2019

Accepted 27 December 2019

KEYWORDS

Biodegradation; cell immobilization; pesticides

CONTACT BeatrizPérez Armendáriz beatriz.perez@upaep.mx

1. INTRODUCTION

Pesticides are substances used to prevent, destroy, repel, or mitigate pests that represent a high economic risk in the agricultural sector [1,2]. They can be classified according to the organism in which they act, their mode of action, or their chemical composition [3]. Based on the latter, pesticides are classified into several groups notably organochlorines, organophosphates, carbamates, pyrethroids,

triazines, and neonicotinoids [4]. In recent years, the use of pesticides has increased alarmingly; according to Zhang [5], during the period from 2010 to 2014, the average cost/benefit was 0.645 g of total pesticides per kg of crop production and average annual use of 2,784 kg. ha^{-1}. The countries with the highest average consumption of pesticides during 2010–2014 (kg. ha^{-1}) were Japan (18.94), followed by China (10.45), Mexico (7.87), Brazil (6,166), Germany (5,123), France (4,859), United Kingdom (4,034), USA (3.886), and India (0.261). These quantities were used despite the fact that some of these substances have been restricted or banned in various countries. Pesticides are considered mutagenic and carcinogenic [6,7], as well as causative agents of intoxication [8], and loss of beneficial soil microbiota [9]. They pollute air, water and soil, and induce resistance in pests and microorganisms [10,11].

Pesticides can be degraded through biological and abiotic routes; however, bioremediation represents the most effective alternative to transform them into simpler and less toxic compounds, through the metabolic potential of microorganisms. However, this process can be affected by factors such as the microrganism's potential for degradation, ease of cultivation, inoculum size, its ability to survive in high concentrations of the pesticide; as well as its ability to adapt and compete with native microorganisms; and to survive possible interactions in the rhizosphere and other environmental factors [12]. To reduce such limitations, it has been sought to accelerate the decontamination process through the use of different bioremediation technologies [13,14]. Recent studies have focused on the use of microbial cellular immobilisation (CI) techniques in various materials and support to protect the microorganisms from adverse environmental conditions. This allows prolonging the survival of microbes introduced to contaminated sites [15–17], which has turned out to be an attractive method for the degradation of pesticides [18–20].

The CI is a method characterised by the restriction to a delimited area, where the cells maintain their metabolic, catabolic, and catalytic activities [21]. The CI has been used mainly in the treatment of wastewater [22] although it is an area of study under development. To this use, it is required to improve and understand the processes associated with immobilisation methods, mainly when the sites to remedy are intended for the production of food.

The investigations oriented to the degradation of pesticides using CI have reported an increase in the efficiency of removal in comparison with the degradation by free cells. However, most of the work has been carried out on an *in vitro* scale. In this sense, information about the effect of different environmental factors is necessary to propose safe and economically acceptable applications for the removal of pesticides *in situ* [23].

While the use of CI for the degradation of xenobiotics have been previously reviewed [24]. the information regarding the use of this technology for the specific degradation of pesticides is scarce.

The aim of this work was to compile the most important aspects of CI as a strategy for the degradation of pesticides with a special emphasis on the main targeted compounds, the used microorganisms, materials, and techniques; as well as issues related to the advantages and limitations of their application *in situ*.

2. PESTICIDES, GENERALITIES AND THEIR RESIDUALITY

Pesticides are chemical substances, antagonistic biological agents or disinfectants used for the prevention and control of pests in crops of interest [25], which has been an indispensable tool to ensure agricultural production. Due to its effectiveness and relative low cost, compared to others of natural origin, the pesticides belonging to different chemical groups (e.g. organochlorines, organophosphates, carbamates, pyrethroids, tria- zines, neonicotinoids) have been used for decades in the agricultural sector to control the infestation of insects, fungi, nematodes, and herbs [26]. Several substances with biocidal activity are classified as toxic and persistent [21] and have been banned due to their harmful effects that intensify with constant and inappropriate use [27]. Despite the ban, the need to increase yields in food production has encouraged the demand for pesticides. They are still used annually in large quantities, and there is no tendency to stop using them [28]. It is estimated that about 85% of the pesticides used in the world are intended for agricultural purposes [29]. However, the continuous and irrational application has caused contamination and damage to unforeseen systems or organisms [30]. It is estimated that only 1 to 10% of pesticides reach their target organism [3], which means that the remaining contamination is a problem of global dimension [31] that impacts beneficial insects, mammals, and vertebrates [32]. Pesticides cause contamination of various systems such as water, air and soil [33], development of resistance caused by high levels of residuality [11,34], loss of the beneficial soil microbiota [9], phytotoxic effects, accumulation in the food web [35], alteration in nitrogen balance (N), and adverse effects in symbiotic organisms [9,36,37]. All those effects directly or indirectly impair the microbiota responsible for its degradation [38], the rhizospheric interactions and physical-chemical characteristics of the impacted sites.

Among the anthropogenic chemicals administered as pesticides that have been evaluated, we can list chlorpyrifos, endosulfan, glyphosate, atrazine, among many others. The disruptive effect of these pesticides on the endocrine system is known [39], as well as its carcinogenic effect in humans and other mammals [40,41]. Also, contamination by pesticides is aggravated by its persistence and residuality [42].

The routes through which these enter the environment can be by direct application, by spill spraying foliar treatments and concentrated by irrigation [43]. Moreover, they can pass through different

pathways after the entering to the environment and undergo transformation, degradation, biodegradation, adsorption, volatilisation, bioaccumulation, or runoff [44].

Given the diversity of chemical groups of pesticides, the factors that modify the presence, transport, and mobility of pesticides are complex and difficult to predict. Phenomena of adsorption-desorption, biodegradation, volatilisation, photodegradation, and decomposition are determined by both extrinsic and intrinsic factors and mediate the presence, transport and mobility of pesticides. Among the extrinsic factors are the environmental conditions at the time of application such as moisture content, amount and type of organic matter, pH, physical and chemical characteristics of the medium in which they are found. Furthermore, other factors are the type of soil, texture, acidity [45]; as well as the presence of fertilisers, bioavailability, and their access to underground or surface water and adsorption level [3]. Typically, soils with abundant organic matter decrease the availability of pesticides by adsorption in a greater proportion than sandy soils, presumably because of the amount of intraparticle surface and ionic interactions [46].

On the other hand, intrinsic factors include chemical composition, the strength of the chemical bonds, water solubility, dissociation constant (pKa) and half-life [47]. In this regard, the composition and chemical structure is a critical feature in the mobilisation and availability of pesticides because they determine their solubility, volatility, degradability or ability to become a compound of greater or lesser toxicity. In the case of one of the most used chemical groups in the world (organophosphates), it has been observed that its enzymatic degradation decreases as the molecular weight of the pesticide increases [48]. Notably, the dissociation of the phosphotriester bonds is affected, while, under aerobic conditions, the breaking of the aromatic rings is favored. However, some residues of these substances are practically present in all environments, especially in surface water, groundwater, and soils.

The persistence of pesticides in the soil is estimated by a parameter known as half-life (the time it takes a pesticide to be reduced to half its initial concentration) [49]. Table 1 shows the classification and average life of some pesticides. The longer the half-life, the more likely it is that these substances will contaminate and move to non-target sites. Because of this effect, the half-life is one of the main aspects to be considered during degradation. Due to the decay pattern of the original molecule, it is estimated that 50% reach to decompose at the half-life, the remaining 25% remains after two half-lives, and about 12% persists after three half-lives [49]. The classification of persistence is divided into persistent, non-persistent, moderately persistent, and permanent [50]. Therefore, recalcitrant contaminants can be classified as those that have values of half-life from months to years, depending on the edaphoclimatic conditions and the ecotoxicological damage that they cause both individually, or in combination with other agrochemicals.

Table 1. Toxicity, persistence and half-life of some pesticides.

Pesticide	Biocidal activity	Toxicity	Persistence	Half-life
2, 4-D	Herbicide	III	Shortly persistent	1 week
Aldicarb	Insecticide	Ia	Shortly persistent	2 weeks
Atrazine	Herbicide	IV	Shortly persistent	8 to 14 weeks
Carbaril	Insecticide	III	Shortly persistent	1 to 4 weeks
Carbendazim	Fungicide	IV	Shortly persistent	1 to 25 months
Carbofuran	Insecticide	II	Shortly persistent	4 to 8 weeks
Carbofuran phenol	Insecticide	ND	ND	ND
Cypermethrin	Insecticide	III	Slightly persistente	1 to 4 weeks
Chlorferon	ND	ND	ND	ND
Chlorpyrifos	Insecticide	III	Highly persistent	2 to 81 days
Coumaphos	Insecticide	II	Highly persistent	42 months
DDT	Insecticide	II	Highly persistent	5.3 years
Diethylthiophosphate DETP	ND	ND	ND	ND
Diazinon	Insecticide/acaricide	III	Shortly persistent	2 to 6 weeks
Difenoconazole	Fungicide	IV	Shortly persistent	3 weeks
Diuron	Herbicide	IV	Highly persistent	1 month to 1 year
Endosulfan	Insecticide/acaricide	II	Shortly persistent	7 weeks
Imidacloprid	Insecticide	III	Mediately persistent	7 to 27 weeks
Lindane	Insecticide	II	Highly persistent	15 months
Mesiotrione	Herbicide	IV	Shortly persistent	3 to 5 weeks
Methyl parathion	Insecticide	Ia	Slightly persistent	1 to 30 days
Methomyl	Insecticide	II	Slightly persistent	2 weeks
Pentachlorophenol	Funguicide-insecticide	II	Shortly persistent	7 weeks
Profenofos	Insecticide	III	Slightly persistent	2 to 3 days
Prometrina	Herbicide	IV	Shortly persistent	40 to 70 days
Propanil	Herbicide	IV	Slightly persistent	4 days
Tetrachlorvinphos	Insecticide	IV	Slightly persistent	2 to 3 weeks

Slightly persistent: less than 4 weeks. Shortly persistent: from 4 to 26 weeks. Mediately persistent: from 27 to 52 weeks. Highly persistent: more than 1 year and less than 20. Permanent: more than 20 years. ND: not determined. Ia: extremely toxic. Ib: highly toxic. II: mildly toxic. III: slightly toxic. IV: low probability of presenting acute danger.

Regarding the half-life, the concern for environmental impact has focused efforts to investigate and design strategies for pesticide degradation by methods that mitigate the pollution caused by its excessive use [51]. The selection of appropriate technologies for such purposes depends on the consideration of circumstances as the concentration, the type of pesticide, and the final use of the contaminated media.

However, pesticides can be degraded via biological and abiotic procedures. There are physicochemical methods such as oxidation with ozone, photolysis, photooxidation, ultrasonic degradation, incineration, and adsorption that allow the removal of the pesticides; but they are usually more expensive, less effective and less viable compared to biological methods [52]. Generally, its application in agricultural fields or soils destined for agricultural production is restricted due to the possibility of causing undesirable effects [14].

Although currently, most regulated pesticides are less persistent than their predecessors, they can remain as derivatives and exist even for longer periods to the parent compound [53]. Also, residual products are often even more toxic than the original components. For example, methyl parathion is susceptible to degradation by different routes; some of them result in the production of its intermediate paraoxon, a substance more toxic than its predecessor. In contrast, with biological

methods, it is possible that microorganisms adapt or are naturally adapted to grow and degrade polluting compounds using them as a source of carbon (C) and energy [32].

Microbial degradation is the primary mechanism for preventing the accumulation and persistence of pesticides in the environment. In this sense, bioremediation represents an effective and innovative alternative by reducing the concentrations or toxicity of the compounds, transforming them into simpler and less toxic compounds, and even restoring the initial conditions through the metabolic potential of the microorganisms [21]. Bioremediation is currently considered the most appropriate and the best-accepted method for decontaminating matrices contaminaded with pesticides because it is efficient and affordable [54,55]. Different authors affirm that it is an ideal and flexible technique for the complete degradation of pesticides such as carbamates [32]. Bioremediation technologies are classified basically *in situ* and *ex situ*; the latter is the most attractive although challenging to implement because they depend on the present microbiota and its activity to achieve complete mineralisation [56].

Compared to the list of compounds used as pesticides, there are few microorganisms, consortiums and their metabolic pathways characterised to ensure the degradation *in situ* to fewer pollutant compounds [21]. While some claim that the main reason for the persistence of pesticides in the environment is the lack of suitable microorganisms or enzymes for complete degradation since pesticides are mostly substances synthesised by man [57].

3. MICROORGANISMS INVOLVED IN THE DEGRADATION OF PESTICIDES

Microbial degradation uses living organisms to eliminate contaminants in soil, water, and waste. The degradation of pesticides using microorganisms has been extensively reported [58]. It has been also reported how the complexity of polluting substances are transformed by the microbiota when they are used as the only energy source [32,59]. In this regard, multiple microbial strains are capable of using pesticides as a carbon (C), nitrogen (N) source, and other elements such as phosphorus (P). Many of these strains were identified and characterised to expand the knowledge of the processes required to reduce the ecological footprint of existing biocidal compounds [36,60]. The use of bacteria is highlighted as the main microorganisms evaluated for degradation, description of some of the metabolic routes, as well as the conditions in which the rupture and mineralisation processes are carried out [61].

Among the main microorganisms studied for this purpose are bacteria belonging to the genera *Arthrobacter, Flavobacterium, Micrococcus, Pseudomonas, Rhizobium* and *Sphingomonas* [62], which have demonstrated their capacity to consume pesticides as the only C and N source [32]. On the other hand, the use of fungi for these purposes is scarce. Among the fungi recently evaluated for the degradation of

pesticides are *Phanerochaete chrysosporium, Pleurotus ostreatus*, and *Trametes versicolor* [63], as well as *Penicilillium* that uses reactive oxygen species as the primary mechanism [64].

The efficiency of some microorganisms has been demonstrated to transform pesticides into less toxic substances or reduce them to non-toxic components has been demonstrated. Also, the complexity of the interactions to which they are exposed make the strains with degradation activity and catabolically competent rarely survive and proliferate in the soil or they fail to express their required catabolic potential in bioremediation *in situ* [65,66].

Interestingly, the activity of the microbiota is regulated by various conditions such as temperature, pH, humidity, concentration, and bioavailability of the contaminant. These conditions intervene with the degradation efficiency, the metabolic potential, the size of the inoculum, the ability to survive in high concentrations of the pesticide, its ability to adapt in competition with autochthonous microorganisms, as well as possible interactions, and other environmental factors [12]. Based on this, the techniques used should consider such conditions for the optimal establishment of the microbial growth, since sites contaminated with pesticides generally contain complex mixtures of different compounds instead of a single contaminant [32]. Therefore, the degradation of pesticides can be a complicated process for pure cultures. In this sense, microbial consortiums have a higher potential in the degradation of pesticides [22]. A significantly higher percentage of degradation has been reported with microbial consortia compared to isolated microorganisms [67]. This is due to life in a consortium contributes to a better survival and, in turn, increases the number of catabolic pathways available for the degradation of pesticides by adding up their metabolic potentials (metabolic synergism) [68]. In nature, microbiomes interact in this way [69], in comparison with the limits of the metabolism of a single species [59]. However, when using free cells for the degradation of toxic substances there have been problems such as difficulty in handling, decrease in cell density, competition and reduction of adaptation and infiltration rates [66].

To reduce the limitations of the metabolism of a single species, the decontamination process has been accelerated by using additional treatments to potentiate the degradation and to ensure the survival and viability of the microorganisms [13,70]. In this sense, some works are focused on the use of CI techniques as a potential alternative in areas such as bioremediation, since it provides protection against adverse environmental conditions and prolongs the survival of microorganisms introduced to contaminated sites [15–17]. CI is an attractive method for the degradation of pesticides [18,20]. In recent years, it has increased the number of researches dedicated to the study for degrading microorganisms of a great variety of pesticides, which respond efficiently when some method of immobilisation is applied (see Table 2).

It has been mainly reported the use of bacteria of the genera *Agrobacterium, Arthrobacter, Bacillus, Brevibacterium, Burkholderia, Enterobacter, Flavobacterium, Flavimonas, Micrococcus, Pseudomonas,*

Sphingomonas and Streptomyces [17,20,32,66,70,75–79]. Additionally, some fungi species such as *Trichoderma longibrachiatum, Aspergillus oryzae* [80] and *Trametes versicolor* [42] have been used. All of them have shown the ability to degrade different pesticides according to the specific chemical groups that distinguish them, using extracellular and intracellular enzymes that allow them to use the residues of pesticides as a source of C and their conversion into simpler products, or even to arrive at its complete mineralisation [81].

4. CELLULAR IMMOBILISATION, TECHNIQUES AND MATERIALS

To carry out the CI, different techniques are used according to the objectives such as flocculation, adsorption to a porous surface or matrix, covalent bonding, entrapment, natural aggregation and encapsulation (the most used technique) [17].

The main uses of CI have been applied in industrial and medical processes whose purpose is the efficient procurement of products (amino acids, organic acids, antibiotics, and enzymes) [82–84]. In recent years, the interest in the use of CI has returned, especially in areas such as bioremediation, biological control, and application of agrochemicals, among others. On the other hand, the biodegradation of pollutants is one of the most recent and potential applications of the CI oriented to favour the processes of degradation of diverse toxic substances [17], because in the environmental area is common to find limitations for the incorporation of free microorganisms in the sites to be remedied. That is why there was a renewed interest for the use of CI techniques to increase the productivity and viability of cells, as well as maintain their catalytic activity for extended periods [85]. The use of this technology should provide desirable, appropriate, and prolonged conditions for cell development, zero toxicity or potential contaminant and constant properties to be used in water and soil.

The CI technique has emerged as an environmental, technological alternative due to CI of degrading microorganisms is used in the processes of removal and or degradation of pollutants; thus, higher yields and percentages of removal are commonly achieved by incorporating them under the protection of immobilisation. However, one limitation of the use of CI *in situ* for remediation has been the survival of the microorganisms in confinement conditions, their interaction with the support or entrapment materials and the mechanical stability of the materials used in the immobilisation.

To date, only some reports has focused on the search for alternatives for the optimisation of biological treatments in the degradation of pesticides using CI methods. Mainly two types of processes are used, those based on physical retention (such as entrapment in porous matrices or membrane encapsulation) and those in which a chemical bond is used (e.g. biofilm formation).

The encapsulation is the most studied method for the practical application of CI. This is a simple and effective method [86], in which different materials, mostly polymeric gels that keep the cells protected

inside a membrane are used. However, some restrictions related to the stability of the matrix that is formed can occur, as well as the mass transfer through function as a slow-release system [87]. In contrast, techniques that employ the formation of biofilms or cellular entrapment take advantage of the natural aggregation and the ionic interactions that bind them to improve degradative pathways [88].

In this sense, because the structure of the materials for CI is deeply related to adherence, microbial degradation activity and conservation of this activity, the selection and search of suitable supports is fundamental [23,89]. Different matrices and supports have been evaluated to enhance the degradation efficiency to adequate to the conditions in which they will be required [23,68]. It is expected that CI materials have characteristics like null toxicity, mechanical stability, efficiency, practicality, and low cost, as well as the ability to form porous surfaces to provide ideal immobilisation conditions [20].

The materials of support or matrices that have been used for the encapsulation and trapping of cells in the degradation of pesticides are very varied. Some examples of inorganic materials are clays, silicates, crystals, ceramics, diatomaceous earth, polyurethane foam, sol-gel, polyacrylamide, agarose, polyvinyl alcohol, porous volcanic stones, and nylon fibre. Whereas materials of organic origin are cellulose, agar, alginates, k-carrageenan, chitin, collagen, activated charcoal, sponge of *Loffa cylindrica*, biochar, fibres of vegetable debris, and grains (see Table 2).

In most studies, the use of polymeric gels increases the capacity of degradation due to factors related to diffusion phenomena [15,90] and allows the cells an adequate exchange to support high pollutants concentrations. Mathematical models have been used to establish the optimum diameter for the intermembrane diffusion of the contaminants (with an optimum of between 2–3 mm) for the entrapment in gels (polyacrylamide gel-chitosan and sol-gel) in the formation of encapsulating matrices [91].

Immobilisation by Ca-alginate entrapment (D-acid-mannuronic and L-guluronic acid copolymer) is the most common and efficient non-toxic natural polymer, successfully used to immobilise microbial cultures *in vitro* [22]. It is characterised by its physical properties, ease of formation of the cover for the encapsulation, and capacity of modification of the polymeric matrix. Furthermore, under this system, the microorganisms are released slowly by dissolving the gel formed once the contact with calcium chelating agents such as sodium citrate and potassium phosphate is reached [92]. The use of chelating agents is beneficial under certain circumstances since slow release is convenient in degradation processes in continuous systems, in which to preserve metabolic activity for prolonged periods is necessary [89,93].

However, despite the effectiveness of the use of encapsulation in algae for the removal of pesticides in liquid media, its use has not been recommended for application in soils since the susceptibility to degradation and the low mechanical stability of this material [67]. Alternatively, it has been suggested increasing the percentage of alginate in the preparation of the capsules or supplementing the matrix with the addition of other polymers or particles such as polyvinyl alcohol, polysulphones, bentonite clay, agar, or polyacrylamide [32,64,77,89,94,95]. These modifications would also alter other physical properties related to degradation. For example, phosphorylated polyvinyl alcohol (PVA) in combination with Ca-alginate forms a more durable matrix that can interact to improve the properties of the matrix, without negative risks to the microorganisms or to the environment [70].

Conversely, in terms of CI due to entrapment or biofilm formation, matrices or natural supports have been evaluated more frequently [23]. proposed the use of fibres as a support for the aggregation of cells capable of degrading contaminants, and since then other materials, vegetable or synthetic fibres have been evaluated [17].

Among the synthetic fibres, polyurethane foam stands out, whose removal efficiency of carbofuran phenol and cypermethrin reached 100% when used as a support [87,93]. Regarding natural supports, the lignocellulosic residues are the most frequently used [17,42,80], which offer advantages for their biostructural characteristics such as reusability, mechanical stability, and non-toxicity. *Luffa cylindrica* L. (loofa) has been used as natural support of a cross-linked fibre network for CI of microorganisms, which is optimal for cell adhesion. The loofa sponge has been considered the best support for its characteristics of renewability, biodegradability, and simple use in the degradation of organophosphates [28].

In the same way, it has been proposed the use of coconut fibre [23], microniches of green coffee beans [76], sugarcane bagasse [66], biolayers of straw, peat, and soil [42], fibres of *Opuntia spp*., and *Agave sp* [17]., to adsorb and facilitate the dissipation of pesticides such as carbendazim, DDT, 2,4-D, mesotrione, atrazine, and MP. These biostructures allow the enzymatic activities required for microbial degradation [42] and offer advantages as reusability, zero toxicity, mechanical stability, and interstructural space that allows the growth of microorganisms without problems of rupture or loss of diffusion, which could occur in capsules formed by polymers.

Organic amendments are immobilisation strategies that reduce the bioavailability of pesticides by chemical interaction [96], which provide organic matter for the sorption of the contaminant and stimulation of plant growth. Thus, for example [66], tested the use of sugar cane bagasse as a support for the immobilisation of *Bacillus pulmilus* in the degradation of mesotrione. The use of this biomaterial promoted the rapid adaptability of the bacterium and its growth in soil. On the other hand, biochar increases the sorption of hydrophobic organic compounds by entrapment in their micropores

and condensed structures [97]; furthermore, it reduces the bioavailability of pesticides [98]. Biochar is the result of a process called pyrolysis in which organic matter undergoes thermal decom-position in the absence of air [99]. It is differentiated from charcoal by its use as a soil amendment [99]. Biochar indirectly increases the content of organic matter, total C, total N, and C/N ratio, causing changes in the composition and abundance of the biological community and creating higher soil fertility. Organic amendments such as biochar create interactions between microorganisms and plants that allow the effective removal of soil contaminants such as difenoconazole [78].

Moorman et al. [100] used organic amendments to improve the degradation of herbicides in contaminated soils. The use of available organic amendments, such as compost, plant residues, and green manure, can be an effective way to improve the degradation and removal of pesticides if they are used in the CI of degrading microorganisms.

It has been reported the metabolism and sorption for pesticides such as atrazine, carbofuran, carbaryl, among others, by communities in aggregates in biofilms in rivers [99]. In these environments, the time of exposition of the biofilm to the contaminant (methomyl) increases the modification of the bacterial community, due to changes induced by natural selection [101]. Interestingly, it has been demonstrated that microorganisms added in the form of biofilms are less susceptible to environmental changes such as pH, temperature, the presence of toxic substances and its derivatives [66,102,103]. Thus, biofilms are useful in the treatment of wastewater due to its simplicity of use and stability [104]. [85] used brick particles as an inert material, whose characteristics allow the formation of biofilm. This is a low-cost material with desirable mechanical properties compared to alginate, which is characterised by low robustness, low durability, and apparent loss of biological activity. Based on this, it has been proposed the use of alginate with PVA (2 – 4%) to improve its physical properties and promote the stability of the material in the removal of atrazine.

5. ADVANTAGES AND LIMITATIONS OF THE MICROORGANISM CI

Different CI techniques have emerged as an alternative to improve some difficulties in the degradation of pollutants by free cells, as the low yields of these cells. Despite this, some of the effects caused by immobilisation on physiology, microbial metabolism, and their interaction in different environments and conditions are still unknown [105].

The use of immobilisation confers advantages such as the facilitated separation of the cells from the medium since the cells are confined to a specific space. Also, the immobilisation allows the repeated or continuous use of cells without loss of biological activity [67]. After reuse, the cells have adapted better to the conditions of the reaction compared to free cells, which increases the operational lifetime, stability, and survival of microorganisms. However, some authors have suggested that cells could suffer

the effect of restricting the diffusion of the substrate to the interior of the membrane (diffuse resistance), thereby significantly reducing the degradation rate [86].

Furthermore, immobilisation increases cell density, which could lead to an increase of the metabolic activity, productivity, stability, protection against acidification, and other environmental factors such as nutritional deficiency, water stress, variations in pH, and temperature [86].

In contrast, few reports indicate that immobilisation reduces microbial activity due to the low intraparticular diffusion of contaminants [106]. Most publications do not report adverse alteration [67]. In addition, it has been argued that the permeability is sufficient for the transfer of substrate to the interior of the cell [18], which provides resistance to heavy metals and greater tolerance to high concentrations of pollutants. Additionally, the permeability leads to prolonging the stability and decreasing the washes in continuous processes by retaining metabolic activity for longer (cycles) [105]. In addition, immobilisation allows operating at higher flow rates in bioreactors [93] increasing removal rates with the establishment of microsites for microorganisms [86], cellular protection at low cost, and decreasing loss of microorganisms by leaching [70].

Therefore, immobilised cells have the potential to degrade pesticides at a higher concentration compared to free cells [67], although its use could be limited by mass transfer, adsorption, and properties of the supports, as well as the characteristics of the microorganism, and its adaptation to the matrix and environmental conditions [77].

Regarding cell changes, some authors have suggested that immobilisation of cells in Ca-alginate, alters the physiology by increasing the permeability of the membrane by a rise in 1,2-epoxypropane production [70]. Others indicate that the impurities present in commercial alginate are responsible for the physiological changes in the cells, their tolerance to toxic compounds [107], and protection against inhibitory factors [67]. In addition, greater degradation rates have been obtained with the immobilisation even in comparison with those obtained by using immobilised enzymes despite their specific activity for the degradation of pesticides [75]. On the other hand, one of the limitations in the use of Ca-alginate is the ease of dissolution by polyphosphates and citric acid, as well as by competition with other ions such as Na^+. These limitations do not guarantee the conservation of its properties, which leads to no-degradation of the pollutant during the active phase of bioremediation [91]. Additionally, the fact of oxygen restriction for microorganisms in the entrapment with alginate beads could diminish or slow down the activity and proliferation of the degrading microorganisms [22].

In this sense, amendments such as biochar have been used for carbon sequestration with applications in remediation. With the use of this material, pesticides such as difenoconazole have been

degraded by 99% in soil with or without cellular culture. Also, biochar altered the composition of the bacterial community in the soil and reduced the bioavailability of pollutants [78], suggesting its use as a method that decreases the concentration of persistent pesticides in arable soil [108].

The above, promotes the use of agricultural waste in remediation processes, as they can provide both nutrients and support to microorganisms; coffee residues and other crops can be reused, whose porosity and composition are efficient because have been added with microbiota in the form of microniches [76]. However [109], consider that the materials used as supports interfere in the diffusion of oxygen in the process.

It has been reported that some pesticides adsorb more than others in the same support, this suggests that the supports can interact with the contaminant either by decreasing its concentration or limiting its bioavailability depending on chemical characteristics [15].

Regarding the environmental context, the methods of encapsulation and biofilm formation have been mainly suggested, but it is necessary to mention that the majority of studies and characterisation of these have been carried out on a laboratory scale and designed for use in the remediation of wastewater.

6. DEGRADATION OF PESTICIDES USING CI

The first works on the use of CI with environmental applications focused on the degradation of phenolic compounds and their derivatives [110,111]. Since then, this technique has been tested in the degradation of a large number of compounds, including pesticides, achieving the complete degradation of many of the most used and persistent substances [47]. In this regard, the vast majority of studies agree that there is an increase in the percentage of removal and efficiency when CI is used compared to free cells. For example, it has been reported the degradation of pesticides such as atrazine [22,42,94], carbofuran, carbaryl [32,86], cypermethrin [93], chlorpyrifos [110; 68], difenoconazole [111], endosulfan [84], mesotrione [66], parathion-methyl [17], among others. The advantages of using CI are independently corroborated of the method or material used for the immobilisation.

On the other hand [67], used cells immobilised in Ca-alginate for the detoxification and biodegradation of coumaphos and their hydrolysis products, chloroform and diethylthio-phosphate (DETP). The degradation rate of the immobilised consortium of degrading bacteria was five times higher respect to freely suspended cells. The authors stated that the enhanced degradation was due to the protection provided by the immobilisation against inhibitory substances present in the culture medium.

Bazot and Lebeau [70] developed a mixed microbial consortium immobilised in PVA phosphorylated in a sand column at *in vitro* scale. The results suggested that both immobilised and free cells maintained their ability to degrade atrazine in a similar way (45-65%). However, the free cells were leached more than the immobilised cells. The latter provided a constant elimination efficiency of atrazine in a long-term experiment and higher viability, while in the treatment with free cells, the degradation decreased due to the gradual loss of bacterial cells. In this sense, in the remediation of residual contaminated water, the rate of degradation increases and allows the reuse of cells without decreasing degradative activity.

Yañez-Ocampo et al. [15] used an immobilised bacterial consortium for the removal of methyl parathion (MP) and Tetrachlorvinphos (TCV). The authors analysed the kinetics of removal in comparison with a liquid medium, supplemented with MP (25 mg L^{-1}) and glucose (1 g L^{-1}) as a co-substrate. The results showed that both the viability of the consortium and the percentage of removal of MP and TCV were significantly higher when using alginate beads, and especially with the formation of biofilm in particles of tezontle (72% and 65%), in relation with the cells in suspension.

Fernández-López et al. [17] tested different methods of immobilisation to increase the degradation of MP by the bacterium *Burkholderia cenocepacia* in three different supports: zeolite powder, *Opuntia sp*., and fibres of *Agave sp*. The results revealed a significant increase in the hydrolysis of MP and the degradation of p-nitrophenol (PNP) with immobilised cells compared to free cell cultures. Additionally, the immobilised cells were able to resist and degrade higher concentrations of PNP compared to cell suspension cultures. Viability in free cultures, as well as degradation of PNP, were affected at concentrations higher than 25 mg L^{-1}. Nevertheless, the immobilisation of bacterial cells in *Opuntia sp*. and in *Agave sp*. fibres lead to the complete degradation of 100 mg L^{-1} of MP and PNP. However, the sorption percentage of the immobilisation materials (natural fibres) as control treatments was 50 to 70%. According to this, the conclusion is that the adsorption of the pesticide was a determining factor in the decontamination process, without involving precisely the microorganisms, although collectively these factors make the degradation more efficient.

Other techniques of immobilisation were evaluated by [94]. Entrapment and biofilm formation were tested to determine the toxicity of the effluent, product of the degradation of atrazine by germination of rice plants (*Oryza sativa*). Removal of atrazine using colonised cells in biofilm on brick particles was higher than the removal achieved by cells trapped in a Na-alginate-PVA matrix (initial concentrations of 1000 mg L^{-1}). This result indicates that despite the irregularities of the matrices for entrapment, this method may be even more effective than encapsulation in polymeric gels.

In contrast [112], reported that the degradation of atrazine using alginate beads was ineffective due to their low shelf life. *Rhodococcus erythropolis* encapsulated in Na-alginate beads was able to

degrade atrazine in liquid and soil, but due to decreased cell viability during storage, wet alginate beads were not convenient. On the other hand, the encapsulation with bentonite and 1% skimmed milk powder improved cell viability, maintaining its ability to degrade the compound. Other results suggest that both immobilised and free systems have the same removal efficiency, although the immobilised ones have higher stability and consistency in long-term use [53].

Izcapa-Treviño et al. [63] evaluated the degradation of atrazine in mineral medium, soil, and wastewater. The immobilisation of a consortium in Na-alginate was effective in the degradation of atrazine, allowing its survival for more than 90 days based on the count of colony forming units (CFU). The alginate was stable and effective in the degradation due to the prolongation of its activity, for that reason, they recommend its use on free cells in remediation studies.

The results of scientific works indicate that the degradation is determined not only by the activity and efficiency of the microorganisms but also depends on the stability and conservation of the activity under different conditions. Regarding this, some authors have suggested that the CI efficiency also corresponds to cellular and genetic variations induced by the immobilisation method [113]. Presumably, the processes of biodegradation in immobilised systems are determined by intraparticular diffusion and biochemical reactions [89], while the degradation capacity improves when adsorbent matrices are used. This result is explained by the reversible sorption of the pollutant [28] that modifies its diffusion, availability, and reduces toxicity by minimising saturation; which allow microorganisms (either alone or in metabolic synergy) to be less exposed to pollutant and be able to act in degradation.

Partovinia and B. Rasekh [23] were one of the first authors who proposed to study physical medium to enhance the degradation and the factors that affect the capacity of the consortium immobilised in them. Also, he included the study of changes in the microbial population in response to environmental variations, and concentrations of N and P. He recommended materials such as loofa sponge and coconut fibre as natural alternatives, as well as the reuse of activated carbon, and polyesters from non-textile factories as accessible alternatives. With these alternatives for immobilisation, it was reported a higher cell concentration that increased up to an order of magnitude in comparison with free cultures, in addition to a higher and more rapid degradation, mainly when loofa sponge was used, standing out as one of the most suitable supports for biodegradation of pesticides such as carbendazim and 2,4-D.

Regarding the effect of different factors such as pH, temperature, as well as the concentrations of $NH4^{+}$ -N and PO_4^{3-} that produce changes in the population of microbiota degrading the pesticide; it has been observed that pH values in the range 6–9 favour degradation without losing effectiveness for several cycles [93]. In contrast, the degradation ability of immobilised consortiums decreases

considerably at low pH, as well as low concentrations of PO_4^3. The effect of pH is probably related to the formation of intermediate products that at low pH values, can strongly inhibit growth or cause cell death, while incubation at different temperatures does not have a significant effect. Changes in P and N concentrations can be modified in response to the amount of fertiliser applied; due to P and N are essential nutrients, changes in their concentrations affect the degradation capacity. According to [23], low levels of P affect the ability to degrade pesticides, while low concentrations of N have no significant effects; however, when both elements are at low levels, pesticides such as carbendazim are degraded considerably.

Concerning the degradation efficiency, increases in the percentage of removal are generally reported when any method of immobilisation is used, regardless of the type of pesticide. The effect of the immobilisation can result from an increase in reaction rates due to the high cellular density, as well as by adsorption processes. Thus, the natural aggregation methods are the most efficient due to the ability of bacteria to form biofilms naturally as

a capacity for survival [114]. The biofilm is formed for the production of exopolysaccharides, lipids, proteins, and surfactant agents. These molecules allow them to be held together by mechanisms that are regulated by the genotype, the growth phase of the microorganism, the nutrients, and their availability, as well as by environmental factors, which together confer to microorganisms adaptative and metabolic advantages [22,115].

Several studies have shown that biofilms formed on substrates or supports are less sensitive to environmental changes compared to suspension cultures [15,53,94,111,116]. They describe how the supports provide the conditions for the formation of microbial aggregates, and they also function as efficient methods of contaminant sorption on its own, thereby contributing to the adsorption and removal of pesticides. This suggests that the abiotic loss of pesticides can be attributed to the adsorption in the support material rather than to the microbial degradation [17].

The most successful cases have been tested primarily in systems restricted to *in vitro* conditions, either in flasks or in small bioreactors with controlled conditions that favour the survival and prolong the biological activity of the degrading microorganisms [117]. In this sense, the current methods for decontamination that require the removal or processing of a large amount of soil are expensive, slow, or not viable [21]. Generally, these methods are not applicable in agricultural fields because they can lead to problems that affect the necessary conditions for the establishment of crops such as soil erosion, loss of fertility, or nutrient leaching. Therefore, commercial remediation approaches are still being studied to reduce risks and to accomplish the requirements to protect human and ecological health.

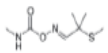
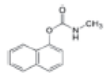
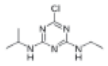
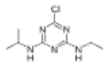
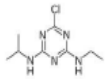
The *in situ* application of CI in soils is a new alternative that could be profitable for the remediation of sites contaminated with pesticides. However, as mentioned, works with this approach are scarce [53] and do not elucidate the mechanisms that are carried out in the immobilisations, as well as the effect that the CI could have on microorganisms and their bioaugmentation under conditions of the real systems.

It is considered that CI effects may vary for different reasons, such as the taxonomic and genetic differences of the microorganisms, as well as the variety of techniques, culture conditions, and activity between interfaces. There are no proposals that satisfy the demand, and some promising alternatives do not have conclusive or homogeneous results.

In the efforts to reduce these limitations, the work of [53] is highlighted due to the study about the survival of *Pseudomonas sp*. in sterile soil and its ability to retain atrazine degradation functionality. The evaluation was carried out with free bacteria and immobilised in the zeolite incorporated in the soil. Their results showed an increase in the number of cells when both free and immobilised bacteria were inoculated in soil. Only those subjected to CI in zeolite, retained full functionality to degrade atrazine after ten weeks. This activity of degradation was observed regardless of the medium used to the culture of the strain when they were recultivated in plates with the contaminant. However, they did not determine the percentage of degradation.

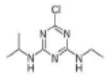
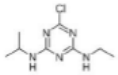
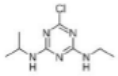
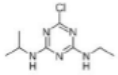
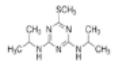
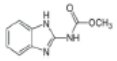
The following is a summary of some papers that report different CI techniques for the degradation of pesticides, their main characteristics and most important considerations in terms of degradation.

Table 2. Selected studies on the biodegradation of pesticides using immobilised cells.

Degrading microorganisms	Nomenclature IUPAC	Generic name	Structural formula	Class	Biocidal action
Micrococcus sp. SMN-1	1-methyl-2-nitrobenzene	2- nirotoluen		Intermediary in the synthesis of pesticides	NA
Enterobacter cloacae TA7	2-Methyl-2-(methylthio)propanal O-(N-methylcarbamoyl)oxime 2,2-Dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate metilcarbamato de 1-naftilo	Aldicarb Carbofuran Carbaril	  	N-methylated carbamates	Insecticide
Pseudomonas sp. ADP	2-cloro-4-(etilamina)-6-(isopropilamina)-1,3,5-triazina	Atrazine		Triazine	Herbicide
Pichia kudriavzevii Atz-EN-01	2-cloro-4-(etilamina)-6-(isopropilamina)-1,3,5-triazina	Atrazine		Triazine	Herbicide
Microbial consortium (*Massilia, Stenotrophomonas, Klebsiella, Sphingomonas, Ochrobactrum, Arthrobacter, Microbacteriu, Xanthomonas* y *Ornithinimicrobium*)	2-cloro-4-(etilamina)-6-(isopropilamina)-1,3,5-triazina	Atrazine		Triazine	Herbicide

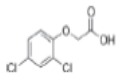
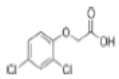
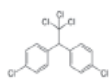
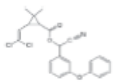
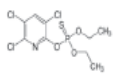
(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Nomenclature IUPAC	Generic name	Structural formula	Class	Biocidal action
Microbial consortium (*Bacillus*, *Pseudomonas* y *Burkholderia*)	2-cloro-4-(etilamina)-6-(isopropilamina)-1,3,5-triazina	Atrazine		Triazine	Herbicide
Stereum hirsutum Ru-104, *Inonotus* sp. SP2, *Trametes versicolor* HL01	2-cloro-4-(etilamina)-6-(isopropilamina)-1,3,5-triazina	Atrazine		Triazine	Herbicide
Pseudomonas sp.	2-cloro-4-(etilamina)-6-(isopropilamina)-1,3,5-triazina	Atrazine		Triazine	Herbicide
Agrobacterium radiobacter J14a	2-cloro-4-(etilamina)-6-(isopropilamina)-1,3,5-triazina	Atrazine		Triazine	Herbicide
Leucobacter sp. JW-1	N2,N4-di-isopropil-6-metiltio-1,3,5-triazina-2,4-diamina,	Prometryn y s-triazines		S-triazines	Systemic herbicide
Bacterial consortium	Methyl-2-benzimisadole carbamato 2,4-dichlorophenoxyacetic acid	Carbendazim 2,4-D		Carbamate Organochlorine	Fungicide Herbicide

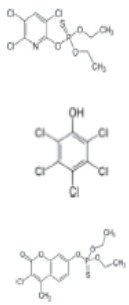
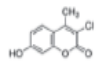
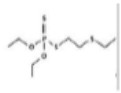
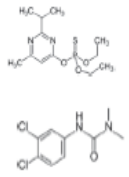
(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Nomenclature IUPAC	Generic name	Structural formula	Class	Biocidal action
Pseudomonas fluorescens	2,4-dichlorophenoxyacetic acid 1,1 -(2,2,2-Tricloroetano-1,1-diyl)bis(4-clorobenceno)	2,4-D DDT Dichloro diphenyl trichloroethane		Organochlorine	Herbicide
Klebsiella pneumoniae ATCC13883T	2,2-Dimethyl-2,3-dihydro-1-benzofuran-7-ol	Carbofuran phenol		Carbamate	Insecticide
Micrococcus sp. strain CPN 1	(1RS)-cis,trans-3-(2,2-diclorovinil)-2,2-dimetilciclopropano carboxilato de (RS)-ciano-3-Fenoxibencilo	Cypermethrin		Pyrethroid	Insecticide
Bacterial consortium	O, O-dietil O-3,5,6-trichloropyridin-2-il fosforotioato	Chlorpyrifos		Organophosphorus	Insecticide
Streptomycess sps.	O, O-dietil O-3,5,6-trichloropyridin-2-il fosforotioato 2,3,4,5,6-pentaclorofenol	Chlorpyrifos Pentachlorophenol	 	Organophosphorus Organochlorine	Insecticide insecticide, herbicide, contact fungicide

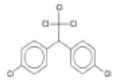
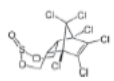
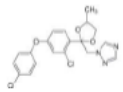
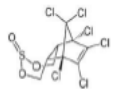
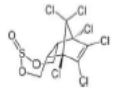
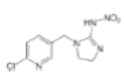
(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Nomenclature IUPAC	Generic name	Structural formula	Class	Biocidal action
Microbial consortium	3-cloro-7-dietoxifosfinotioiloxi-4-metil-2-cromenona 3-Chloro-7-hydroxy-4-methylcoumarin O,O-Dietil-O-4-nitrofenil-tiofosfato	Coumaphos Chlorferon Dietylthiophosfate (DETP)		Organophosphorus Coumaphos by-product Coumaphos by-product	Insecticide
Streptomyces sp. AC1-6	O,O-Diethyl O-[4-methyl-6-(propan-2-yl) pyrimidin-2-yl] phosphorothioate	Diazinon		Organophosphorus	Isecticide
Delftia acidovorans WDL34 (WDL34) *y Arthrobacter* sp. N4 (N4)	$C_9H_{10}Cl_2N_2O$, 3-(3,4-diclorofenil)-1,1-dimetilurea	Diuron		Urea, chlorinated	Herbicide
Pseudomonas aeruginosa y Flavimonas oryzihabitans,	1,1 -(2,2,2-Tricloroetano-1,1-diyl)bis(4-clorobenceno) 6,7,8,9,10,10-hexacloro-1,5,5a,6,9,9a-hexahidro-6,9-metano-2,4,3-benzodioxatiepina-3-óxido	Dichloro diphenyl trichloroethane (DDT) Endosulfan		Organochlorine Organochlorine	Insecticide Insecticide-acaricide

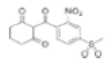
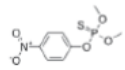
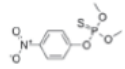
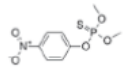
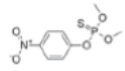
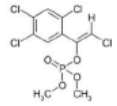
(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Nomenclature IUPAC	Generic name	Structural formula	Class	Biocidal action
Sphingomonadaceae *Pseudomonadaceae*	1-[2-[4-(4-clorofenoxi)-2-clorofenil]-4-metil-1,3-dioxan-2-ilmetil]-1H-1,2,4-triazol,	Difenoconazole		Triazole	Fungicide
Pseudomonas aureaginosa	6,7,8,9,10,10-hexacloro-1,5,5a,6,9,9a-hexahidro-6,9-metano-2,4,3-benzodioxatiepina-3-óxido	Endosulfan		Organochlorine	Insecticide-acaricide
Pseudomonas fluorescens	6,7,8,9,10,10-hexacloro-1,5,5a,6,9,9a-hexahidro-6,9-metano-2,4,3-benzodioxatiepina-3-óxido	Endosulfan		Organochlorine	Insecticide-acaricide
Microbial consortium *Trichoderma longibrachiatum* y *Aspergillus oryzae*	N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide	Imidacloprid		Neonicotiniod	Systemic insecticide by contact and ingestion
Microbial consortium *Streptomyces* sp. A2, A5, A11 and M7	1,2,3,4,5,6-hexaclorociclohexano	Lindane		Organochlorine	Insecticide
Bacillus pumilus HZ-2	2-[4-(Methylsulfonyl)-2-nitrobenzoyl] cyclohexane-1,3-dione	Mesotrione		diona or triketon (or tri-acetone) HPPD inhibitor	Selective herbicide (pre and post emergency)

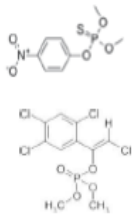
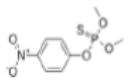
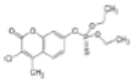
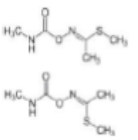
(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Nomenclature IUPAC	Generic name	Structural formula	Class	Biocidal action
Burkholderia sp.	O,O-Dimethyl O-(4-nitrophenyl) phosphorothioate	Methyl parathion		Organophosphorus	Contact Insecticide (stomach, respiratory, non-systemic)
Flavobacterium ATCC 27,551, *E.coli*RAZEK	O,O-Dimethyl O-(4-nitrophenyl) phosphorothioate	Methyl parathion		Organophosphorus	Contact Insecticide (stomach, respiratory, non-systemic)
Microbial consortium	O,O-Dimethyl O-(4-nitrophenyl) phosphorothioate	Methyl parathion		Organophosphorus	Contact Insecticide (stomach, respiratory, non-systemic)
Bacterial consortium	O,O-Dimethyl O-(4-nitrophenyl) phosphorothioate	Methyl parathion Tetrachlorvinphos (TCV)	 	Organophosphorus	Contact Insecticide (stomach, respiratory, non-systemic)
Bacterial consortium	O,O-Dimethyl O-(4-nitrophenyl) phosphorothioate (Z)-2-Chloro-1-(2,4,5-trichlorophenyl) vinyl dimethyl phosphate	Methyl parathion Tetrachlorvinphos (TCV)	 	Organophosphorus	Contact Insecticide (stomach, respiratory, non-systemic)

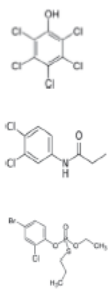
(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Nomenclature IUPAC	Generic name	Structural formula	Class	Biocidal action
Bacterial consortium	O,O-Dimethyl O-(4-nitrophenyl) phosphorothioate 3-cloro-7-dietoxifosfinotioiloxi-4-metil-2-cromenona	Methyl parathion Coumaphos		Organophosphorus	Contact Insecticide (stomach, respiratory, non-systemic)
Bacterial consortium (*Sphingomonas* sp.)	S-metil (EZ)-N-(metilcarbamoiloxi) tioacetimidato	Methomyl		Carbamate	Insecticide
Rhizopus oryzae ENH	2,3,4,5,6-pentaclorofenol	Pentachlorophenol		Organochlorine	insecticide, herbicide, contact fungicide
Microbial consortium *Acidovorax sp., Luteibacter (rhizovicinus), Xanthomonas sp., Flavobacte- rium sp., Variovorax sp., Acinetobacter (calcoaceticus), Pseudomonas sp., Rhodococcus sp., and Kocuria sp*	3′ ,4′ dicloropropionanilida	Propanil		Anilide, chlorinated	post-emergent contact herbicide

(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Nomenclature IUPAC	Generic name	Structural formula	Class	Biocidal action
Pseudozantomonas suwonensis strain HNM	O-(4-Bromo-2-clorfenil) O- etil S – propil fosforotioato)	Profenofos		Organophosphorus	Broad spectrum non-systemic insecticide

Degrading microorganisms	Method and/or immobilisation material	Initial concentration of pesticide	Removal efficiency	Growing conditions and/or scale	Monitoring/quantification technique	Reference
Micrococcus sp. SMN-1	**Entrapment polyurethane foam** (PUF), Na alginate (SA), Ca alginate, polyvinyl alcohol (SA-PVA), agar and polyacrylamide.	15-30 mM	90% Cl	*In vitro* Cultivation in batch and batch-fed	HPLC	[77].
Enterobacter cloacae TA7	Entrapment on agar (cubes)	50 mg L^{-1} 50 ppm	100% Cl 85% free cells (FC)	*In vitro* liquid medium	HPLC UV-detector	[32].
Pseudomonas sp. ADP	Entrapment in zeolite	1 g L^{-1}	NA	Sterile soil	NA	[53].
Pichia kudriavzevii Atz-EN-01	Encapsulation in Na-alginate and PVA	500 mg L^{-1} 1000 mg L^{-1}	> 99%	*In vitro* Cultivation in batch and batch fed	HPLC	[93].
	Matrix and biofilm formation in brick particles					
Microbial consortium (*Massilia, Stenotrophomonas, Klebsiella, Sphingomonas, Ochrobactrum, Arthrobacter, Microbacteriu, Xanthomonas* y *Ornithinimicrobium*)	Packed bed biofilm	100 mg L^{-1}	99.9%	*In vitro* Biofilm bioreactor packed bed	HPLC UV-detector	[71].
Microbial consortium (*Bacillus, Pseudomonas* y *Burkholderia*)	Entrapment in Na-alginate	4 mg L^{-1}	100%	MM, soil and wastewater	HPLC UV-detector	[63].
Stereum hirsutum Ru-104, *Inonotus* sp. SP2, *Trametes versicolor* HL01	biobed	80 mg Kg^{-1}	93%	*In vitro* biobed	HPLC	[42].
Pseudomonas sp.	Zeolite	100 mg L^{-1} 100 ppm	Nd ¿? Revisar	*In vitro* (liquid medium)	Colorimetry	[65].

(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Method and/or immobilisation material	Initial concentration of pesticide	Removal efficiency	Growing conditions and/or scale	Monitoring/quantification technique	Reference
Agrobacterium radiobacter J14a	Phosphorylated polyvinyl alcohol	1.5 mg L^{-1}	45-65%	*In vitro* (sand column)	HPLC	[70].
Leucobacter sp. JW-1	Na alginate-PVA	50 mg L^{-1}	99.9% atrazine, 99.9% ametryn, 97.8% propazine, 100.0% simetryn, 77.9% simazine, 98.9% terbuthylazine 95.2% prometon, 98.9% atraton, 31.6% terbumeton	*In vitro* Pure crops and residual water		[72]
Bacterial consortium	Adsorption in *Luffa cylindrica*, activated carbon, coconut fibre, polyester fibre and Fabios®	20 µM MBC 2mM 2,4-D	MBC 80- 95% en CI and 12% en FC 2,4-D 100% CI 24h, 100% FC 48h	*In vitro* (biorreactor)	HPLC	[23].
Pseudomonas fluorescens	Tezontle particles	100-500 mg L^{-1} 150 mg L^{-1}	99% 55–99%		GC-MS	[73].
Klebsiella pneumoniae ATCC13883T	Polyurethane foam (PUF), polyacrylamide, agar, alginate and alginate-bentonite clay (PAC).	20 mM 30 mM	100% PUF	*In vitro* (Batch and semi-continuous bioreactor)	TLC	[86].
Micrococcus sp. strain CPN 1	Polyurethane foam (PUF), polyacrylamide, agar, Na-alginate	10 mM 20 mM	100% PUF	*In vitro* Batch and semi-continuous bioreactor)	HPLC	[92].
Bacterial consortium	Ca-alginate + polysulfon microsphere entrapment	100- 600 mg L^{-1}	> 96%	*In vitro*	GC–MS and GC-ECD	[68].
Streptomycess sps.	Entrapment in Na-alginate beads	1.66 mg L^{-1}	0.17% and 71.05% 5.24% and 14.72%,	*In vitro*	HPLC	[110].
Microbial consortium	Entrapment in Ca-alginate beads	600 mg L^{-1} 50 mg L^{-1} 250 mg L^{-1}	100%	*In vitro* Continuous bioreactor	HPLC	[67].
Streptomyces sp. AC1-6	Entrapment in alginate beads		60% > CI			
Delftia acidovorans WDL34 (WDL34) *y Arthrobacter* sp. N4 (N4)	Entrapment in Ca-alginate beads	20 mg L^{-1}	53%	*In vitro* Conical liquid flasks	HPLC	[75].
Pseudomonas aeruginosa y Flavimonas oryzihabitans,	Microniches in coffee beans	50 mg L^{-1}	32-37% *Aureaginosa* 51% 30% with glucose	*In vitro* Conical liquid flasks	GC/MS	[76].

(*Continued*)

Table 2. (Continued).

Degrading microorganisms	Method and/or immobilisation material	Initial concentration of pesticide	Removal efficiency	Growing conditions and/or scale	Monitoring/quantification technique	Reference
Microbial consortium *Acidovorax sp., Luteibacter (rhizovicinus), Xanthomonas sp., Flavobacterium sp., Variovorax sp., Acinetobacter (calcoaceticus), Pseudomonas sp., Rhodococcus sp., and Kocuria sp*	Biofilm in volcanic stones	24.9 mg L^{-1}	100%	*In vitro* Continuous bioreactor	Spectrophotometrically HPLC	[116].
Pseudozantomonas suwonensis strain HNM	Na-alginate, Na-alginate- alcoholpolyvinyl alcohol, Na-alginate-bentonite clay	3-6 mM	95% CI 85% FC at 25ºC	*In vitro* Continuous and semi-continuous bioreactor	HPLC-UV detector GC-MS	[88].

NA: Not applicable; ND: not described; HPLC: High Performance Liquid Chromatography; GC-MS: Gas Chromatography–Mass Spectrometry; TLC: Thin-Layer Chromatography; GC-EDC: Gas Chromatography – Electron Capture Detector; LC-MS: Liquid chromatography–mass spectrometry.

Conclusion

The immobilisation gives characteristics to the cells that favour the degradation capacity of pesticides through the increase of their metabolic activity, their rate of growth, stability, and protection against toxicity or environmental conditions. However, additional studies are needed to specify the mechanisms responsible for the particular physiology of immobilised cells in different methods and conditions. This will allow for implementing the use of immobilisation techniques in the biodegradation of pesticides *in situ* on a large scale. Notably, it is necessary to emphasise the description of degradation processes, soil diffusibility, elucidation of the mechanisms by which pesticides suffer sorption or desorption in matrices or materials for entrapment, or support; as well as if they are degraded or retained in the supports and to what extent, as well as the kinetic, molecular and transfer phenomena. The resolution therefore of these questions would, therefore, make it possible to adapt their use, technical, and economic feasibility, to formulate recommendations for the degradation of pesticides in target sites, and not only under liquid culture conditions. Therefore, we agree with the idea that it is still an area of opportunity for future research.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

[1] J. Cortes, R. Sánchez, E. Díaz-Plaza, J. Villen and A. Vázquez, J. Agric. Food Chem. 54, 1997 (2006). doi:10.1021/jf0524675.

[2] FAO 2019. Organización de las Naciones Unidas para la Alimentación y la Agricultura. Definiciones para los fines del codex alimentarius. Consultado el 18 de febrero de 2019. Disponible en: http://www.fao.3/W5975S/w5975s08.htm

[3] M. Arias-Estevéz, E. López-Periago, E. Martínez-Carballo, J. Simal-Gándara, J.C. Mejuto and L. García-Río, Agric. Ecosyst. Environ. 123, 247 (2008). doi:10.1016/j.agee.2007.07.011.

[4] M. Calderón, S. Gómez, R. Villalobos, C. Martínez, Y. Carbajal, C. Calderón, J. Cortés, R. García, D. Flores, M. Rodríguez, P. Méndez and R. Bañuelos, J Toxicol 2012, 612647. doi:10.1155/2012/612647

[5] W.-J. Zhang, Proc. Int. Acad. Ecol. Environ. Sci. 8 (1), 1 (2018). https://www.researchgate.net/publication/323302056_Global_pesticide_use_Profile_trend_cost_benefit_and_more.

[6] A. Mehta, R.S. Verma and N. Srivastava, Pestic. Biochem. Physiol. 94, 55 (2009). doi:10.1016/j.pestbp.2009.04.001.

[7] O. Arellano-Aguilar and J. Rendón von Osten. 2016. La huella de los plaguicidas en México. Greenpeace México A.C. Disponible en: https://www.greenpeace.archive-mexico/Global/mexico/Graficos/2016/comida-sana/Plaguicidas_en_agua_ok_EM.pdf

[8] G. Briceño, M.S. Fuentes, G. Palma, M.A. Jorquera, M.J. Amoroso and M.C. Diez, Int. Biodeterior. Biodegrad. 73 (9), 1 (2012). doi:10.1016/j.ibiod.2012.06.002.

[9] I. Ipsilantis, C. Samourelis and D.G. Karpouzas, Soil Biol. Biochem. 45, 147 (2012). doi:10.1016/j.soilbio.2011.08.007.

[10] B. MH and S. Varela, Culcyt/Toxicol. Insecticidas 28, 5 (2008). http://erevistas.uacj.mx/ojs/index.php/culcyt/article/view/375.

[11] B. Ramakrishnan, K. Venkateswarlu, N. Sethunathan and M. Megharaj, Sci. Total Environ. 654, 177 (2019). doi:10.1016/j.scitotenv.2018.11.041.

[12] A. Mrozik and Z. Piotrowska-Seget, Microbiol. Res. (Pavia) 165, 363 (2010). doi:10.1016/j.micres.2009.08.001.

[13] M. Cycoń, A. Mrozik and S. Piotrowska-Seget, Chemosphere 172, 57 (2017). doi:10.1016/j.chemosphere.2016.12.129.

[14] E. Morillo and J. Villaverde, Sci. Total Environ. 586, 576 (2017). doi:10.1016/j. scitotenv.2017.02.020.

[15] G. Yañez-Ocampo, E. Sánchez-Salina, G.A. Jimenez-Tobon, M. Penninckx and M.L. Ortiz- Hernández, J. Hazard. Mater. 168, 1554 (2009). doi:10.1016/j.jhazmat.2009.03.047.

[16] T.S. Colla, R. Andreazza, F. Bucker, M.M. de Souza, L. Tramontini, G.R. Prado, A.P.G. Frazzon, F.A. D.O. Camargo and F.M. Bento, Environ. Sci. Pollut. Res. 21, 2592 (2014). doi:10.1007/s11356- 013-2139-2.

[17] M.G. Fernández-López, C. Popoca-Ursino, E. Sánchez-Salinas, R. Tinoco-Valencia, J.L. Folch- Mallol, E. Dantán-González and M.L. Ortiz-Hernández, Microbiol. Open 6, e00507 (2017). doi:10.1002/mbo3.507.

[18] J.W. Kim, E.I. Rainina, W.W. Mulbry, C.R. Engler and J.R. Wild, Biotechnol. Prog. 18, 429 (2002). doi:10.1021/bp0200346.

[19] V. Pradeep and U.M. Subbaiah, 3Biotech 6, 124 (2016). doi:10.1007/s13205-016-0438-2.

[20] M.U. Mustapha, N. Halimoon, W. Lutfi, W. Johari and M.Y. Abdul Shukol, J. Agric. Sci. 44 (2), 39 (2018). doi:10.21608/jsas.2018.3465.1061.

[21] A. Kumar, L. Nain and N. Singh, J. Environ. Sci. Health Part B 52, 229.236 (2017). doi:10.1080/03601234.2016.1270680.

[22] A. Pattanasupong, H. Nagase, E. Sugimoto, Y. Hori, K. Hirata, K. Tani, et al. J. Biosci. Bioeng. 98, 28 (2004). DOI:(04)70238-8",1,0,0>10.1016/S1389-1723(04)70238-8.

[23] A. Partovinia and B. Rasekh, Crit. Rev. Environ. Sci. Technol. 48 (1), 1 (2018). doi:10.1080/10643389.2018.1439652.

[24] S.S.S. Saravi and M. Shokrzadeh, Role of Pesticides in Human Life in the Modern Age: A Review, in *Pesticides in the Modern World Risks and Benefits* edited by M Stoytcheva (IntechOpen, 2011). DOI:10.5772/18827.

[25] L.G. Costa, Clin. Chim. Acta 366 (1–2), 1 (2006). doi:10.1016/j.cca.2005.10.008.

[26] C. Boutin, B. Strandberg, D. Carpenter, S.K. Mathiassen and P.J. Thomas, Environ. Pollut. 185, 295 (2014). doi:10.1016/j.envpol.2013.10.009.

[27] D.A. Moreno-Medina, E. Sánchez-Salinas and M.L. Ortiz-Hernández, Rev. Int. Contam. Ambie. 30, 51 (2014). http://scielo.unam.mx/pdf/rica/v30n1/v30n1a5.pdf.

[28] M.R. Cervantes, Rev. Virtual REDESMA 4, 1 (2010). [en línea] http://cebem.cmsfiles/articulos/REDESMA_09_art02.pdf.

[29] M.L. Ortiz-Hernández and E. Sánchez-Salinas, Rev. Int. Contam. Ambie. 26 (1), 27 (2010). https://www.redalyc.html/370/37014382003/.

[30] S. Akbar, S. Sultan and M. Kertesz, J. Basic Microbiol. 55 (7), 819 (2015). doi:10.1002/jobm.201400805.

[31] A. Fareed, H. Zaffar, A. Rashid, M.M. Shah and A. NaqviT., Bioremediat. J. 21 (3–4), 119 (2017). doi:10.1080/10889868.2017.1404964.

[32] J.A. Gutiérrez, M.I. Pinzón, A. Londoño, D. Blach and A.M. Rojas, Agrosur J. 38 (3), 199 (2010). doi:10.4206/agrosur.2010.v38n3-03.

[33] A.J. Bruggeman, D. Kuehler and D.P. Weeks, Plant Biotechnol. J. 12, 894 (2014). doi:10.1111/pbi.12192.

[34] X. Li, Y. Gan, X. Yang, J. Zhou, J. Dai and M. Xu, Food Chem 109, 348 (2008). doi:10.1016/j.foodchem.2007.12.047.

[35] S. Hussain, T. Siddique, M. Saleem, M. Arshad and A. Khalid, Adv. Agron. 102, 159 (2009). doi:(09)01005-0",1,0,0>10.1016/S0065-2113(09)01005-0.

[36] C.S. Jacobsen and M.H. Hjelmso, Curr. Opin. Biotechnol. 27, 15 (2014). doi:10.1016/j.copbio.2013.09.003.

[37] W.R. Brogan 3rd and R.A. Relyea, Environ. Pollut. 220 (A), 688 (2017). doi:10.1016/j.envpol.2016.10.030.

[38] T.B. Hayes, V. Khoury, A. Narayan, M. Nazir, A. Park, T. Brown, L. Adam, E. Chan, D. Buchholz, T. Stueve and S. Gallipeau, Proc. Nat. Acad. Sci. 107, 4612e4617 (2010). doi:10.1073/pnas.0909519107.

[39] J.A. VanLeeuwen, D. Waltner-Toews, T. Abernathy, B. Smit and M. Shoukri, Int. J. Epidemiol. 28, 836e840 (1999). doi:10.1093/ije/28.5.836.

[40] J. Doe and P. Botham, Chapter 3.3 - Chemicals and Pesticides: A Long Way to Go, in *History of Toxicology and Environmental Health* (2019), pp. 177. DOI:12-813697-3.00021-

4",1,0,0>10.1016/B978-0-12-813697-3.00021-4.

[41] M.C. Diez, S. Elgueta, C. Santos and N. Lima, Arch. Agron. Soil Sci. 2016. doi:10.1080/03650340.2016.1155699

[42] G.C. López-Pérez, M. Arias-Estevéz, E. López-Periago, B. Soto-González, B. Cancho-Grande and J. Simal-Gándara, J. Agric. Food Chem. 54 (5), 1797 (2006). doi:10.1021/jf0525737.

[43] S.A.A. Firozjaei, A.M. Latifi, S. Khodi, S. Abolmaali and A. Choopani, J. Appl. Biotechnol. Rep. 2(2), 215 (2015). http://irisweb.ir/files/site1/rds_journals/1802/article-1802-276969.pdf.

[44] [44] C. Ye, Bull Environ Contam Toxicol 71 (5), 919 (2003). doi:10.1007/s00128-001-0217-8.

[45] T. Alekseeva, V. Prevot, M. Sancelme, C. Forano and P. Besse-Hoggan, J. Hazard. Mater. 191 (1–3), 126 (2011). doi:10.1016/j.jhazmat.2011.04.050.

[46] G. Yañez-Ocampo, A. Wong-Villarreal, P. Del Águila-Juárez, J. Lugo-de la Fuente and R. Vaca- Paulín, JSM Environ. Sci. Ecol. 4 (3), 1032 (2016). https://www.researchgate.net/publication/308699959_Central_Bringing_Excellence_in_Open_Access_Composting_of_Soils_Polluted_with_Pesticides_A_Microbial_Approach_and_Methods_for_Monitorin.

[47] M.L. Ortiz-Hernández, R. Quintero-Ramírez, A. Nava-Ocampo and A.M. Bello-Ramírez, Fundam Clin Pharmacol 17, 717 (2003). doi:10.1046/j.1472-8206.2003.00202.x.

[48] R.E. Jacobsen, P. Fantke and S. Trapp, SAR QSAR Environ. Res. 26 (4), 325 (2015). doi:10.1080/1062936X.2015.1034772.

[49] G.D. Bending, M. Friloux and A. Walker, FEMS Microbiol. Lett. 212 (1), 59 (2002). doi:10.1111/j.1574-6968.2002.tb11245.x.

[50] D.S. Powlson, P.J. Gregory, W.R. Whalley, J.N. Quinton, D.W. Hopkins, A.P. Whitmore, P. R. Hirsch and K.W.T. Goulding, Food Policy 36, S72–S87 (2011). doi:10.1016/j. foodpol.2010.11.025.

[51] L. Yang, S.H. Chen, M. Hu, W. Hao, P. Geng and Y. Zhang, Biol. Fertil. Soils 47, 917 (2011). doi:10.1007/s00374-011-0602-0.

[52] S.A. Stelting, R.G. Burns, A. Sunna and C.R. Bunt, Bioremediat. J. 18 (4), 309 (2014). doi:10.1080/10889868.2014.938723.

[53] C.C. Azubuike, C.B. Chikere and G.C. Okpokwasili, World J. Microbiol. Biotechnol. 32 (11), 180 (2016). doi:10.1007/s11274-016-2137-x.

[54] S. Khan, H. Zaffar, U. Irshad, R. Ahmad, A.R. Khan, M.M. Shah, M. Bilal, M. Iqbal and T. Naqvi, Arch. Biol. Sci. 68 (1), 51 (2016). doi:10.2298/ABS141218007K.

[55] C. Vogt and H.H. Richnow, Bioremediation via in Situ Microbial Degradation of Organic Pollutants, in *Geobiotechnology II. Advances in Biochemical Engineering/Biotechnology*, edited by A. Schippers, F. Glombitza and W. Sand (Springer, Berlin, Heidelberg, 2014), Vol. 142, pp. 123. DOI:10.1007/10_2013_266.

[56] J.S. Van Dyk and B. Pletschke, Chemosphere 82 (3), 291 (2011). doi:10.1016/j.

chemosphere.2010.10.033.

[57] S.G. Parte, A.D. Mohekar and A.S. Kharat, Afr. J. Microbiol. Res. 11 (24), 992 (2017). doi:10.5897/AJMR2016.8402.

[58] M.L. Ortiz-Hernández, E. Sánchez-Salinas, E. Dantan-González and M.L. Castrejón-Godínez, Pesticide Biodegradation: Mechanisms, Genetics and Strategies to Enhance the Process, in *Biodegradation-Life of Science*, edited by R. Chamy and F. Rosenkranz edited volume (Intech, 2013), pp. 251. DOI:10.5772/52777.

[59] D.G. Karpouzas and B.K. Singh, Adv. Microb. Physiol. 51, (2006). DOI:(06)51003-3",1,0,0>10.1016/s0065-2911(06)51003-3. doi: 10.1016/S0065-2911(06)51003-3.

[60] X. Ye, F. Dong and X. Lei, Nat. Resour. Conserv. Res. 1, (2018). DOI:10.24294/nrcr.v1i1.242.

[61] T. Naqvi, N. Kanhar, A.H. Shar, M. Hussain and S. Ahmed, Pak. J. Bot. 43, 1079 (2011). http://www.pakbs.pjbot/PDFs/43(2)/PJB43(2)1079.pdf.

[62] A. Kumar and N. Singh, Environ Monit Assess 188, 142 (2016). doi:10.1007/s10661-016-5144-3.

[63] C. Izcapa-Treviño, O. Loera, A. Tomasini-Campocosio, F. Esparza-García, J.A. Salazar-Montoya, M.A. Díaz-Cervantes and R. Rodríguez-Vázquez, J. Environ. Sci. Health Part B 44 (8), 798 (2009). doi:10.1080/03601230903238368.

[64] S. Stelting, R.G. Burns, A. Sunna, G. Visnovsky and C.R. Bunt, Appl. Clay Sci. 64, 90 (2012). doi:10.1016/j.clay.2011.12.006.

[65] J. Liu, S. Chen, J. Ding, Y. Xiao, H. Han and G. Zhong, Appl. Microbiol. Biotechnol. 99, 10839–10851 (2015). doi:10.1007/s00253-015-6935-0.

[66] J. Ha, C.R. Engler and J.R. Wild, Bioresour. Technol. 100 (3), 1138 (2009). doi:10.1016/j.biortech.2008.08.022.

[67] S. Khalid, J.-I. Han, I. Hashmi, G. Hasnain, M.A. Ahmed, S.J. Khan and M. Arshad, Sci. Total Environ. 631–632, 1046 (2018). doi:10.1016/j.scitotenv.2018.03.101.

[68] B. Bhadbhade, S. Sarnaik and P. Kanekar, J. Appl. Microbiol. 93, 224 (2002). doi:10.1046/j.1365-2672.2002.01680.x.

[69] S. Siripattanakul, W. Wirojanagud, J. McEvot, F.X.M. Caset and E. Khan, J. Hazard. Mater. 168, 1373 (2009). doi:10.1016/j.jhazmat.2009.03.025.

[70] S. Bazot and T. Lebeau, Bioresour. Technol. 100, 4257 (2009). doi:10.1016/j. biortech.2009.03.067.

[71] A. Macías-Flores, A. Tafoya-Garnica, N. Ruiz-Ordaz, A. Salmeron-Alcocer, C. Juarez-Ramírez, D. Ahuatzi-Chacon, M.E. Mondragón-Parada and J. Galíndez-Mayer, World J. Microbiol. Biotechnol. 25, 2195e2204 (2009). doi:10.1007/s11274-009-0125-0.

[72] J. Liu, D. Pan, X. Wu, H. Chen, H. Cao, Q.X. Li and R. Hua, Sci. Total Environ. 615, 78 (2018). doi:10.1016/j.scitotenv.2017.09.208.

[73] H. León-Santiestebán, M. Meraz, K. Wrobel and A. Tomasini, J. Hazard. Mater. 190, 707 (2011).

doi:10.1016/j.jhazmat.2011.03.101.

[74] R. Hernández-León, I. Velázquez-Sepúlveda, M.C. Orozco-Mosqueda and G. Santoyo, FYTON Rev. Int. Bot. Exp. 79, 133 (2010). http://www.revistaphyton.fund-romuloraggio.org.ar/vol79/ Hernandez-Leon.pdf.

[75] M. Abdel-Razek, S. Abdel-Razek, J.L. Folch-Mallol, L. Perezgasga-Ciscomani, E. Sánchez-Salinas, M.L. Castrejón-Godínez and M.L. Ortiz-Hernández, J. Environ. Sci. Health Part B 48 (6), 449 (2013). doi:10.1080/03601234.2013.761863.

[76] B. Barragán-Huerta, C. Costa-Pérez, J. Peralta-Cruz, J. Barrera-Cortés, F. Esparza-García and R. Rodríguez-Vázquez, Int. Biodeterior. Biodegrad. 59, 239 (2007). doi:10.1016/j. ibiod.2006.11.001.

[77] S.I. Mulla, M.P. Talwar, Z.K. Bagewadi, R.S. Hoskeri and H.Z. Ninnekar, Chemosphere 90, 1920 (2012). doi:10.1016/j.chemosphere.2012.10.030.

[78] J. Cheng, X. Lee, W. Gao, Y. Chen, W. Pan and Y. Tang, Appl. Soil Ecol. 121, 185 (2017). doi:10.1016/j.apsoil.2017.10.009.

[79] J.M. Saez, C.S. Benimeli and M.J. Amoroso, Chemosphere 89, 982 (2012). doi:10.1016/j. chemosphere.2012.06.057.

[80] S. Gangola, K.P. Pankaj and A. Sharma, J. Biorem. Biodegrad. 6, 310 (2015). doi:10.4172/2155-6199.1000310.[81] M. Ramadas and P. Thiagarajan, IOP Conf. Ser. 2017, 232636022050. doi:10.1088/1757-899X/ 263/2/022050

[82] R.G. Najafpour, *Biochemical Engineering and Biotechnology* (Elsevier, Holanda, 2007). ISBN: 9780080468020. https://www.elsevier.com/books/biochemical-engineering-and-biotechnology/najafpour/978-0-444-52845-2.

[83] P. Zuca and E. Sanjust, Molecules 19, 14139 (2014). doi:10.3390/molecules190914139.

[84] P.M. Gotovtsev, E. Yu, K.V. Yuzbasheva, V.V. Gorin, G.U. Butylin, N.I. Badranova, E. B. Perkovskaya, Z.B. Mostova, N.I. Namsaraev, A.V. Rudneva, R.G. Komova and S.S.P. Vasilov, J. Appl. Biochem. Microbiol. 51 (8), 792 (2015). doi:10.1134/S0003683815080025.

[85] K. Jesitha, K.M. Nimisha, C.M. Manjusha and P.S. Harikumar, Environ. Processes 2 (1), 225 (2015). doi:10.1007/s40710-015-0059-5.

[86] K. Adinarayana, B. Jyothi and P. Ellaiah, AAPS Pharm. Sci. Technol. 6, E391–E397 (2005). doi:10.1208/pt060348.

[87] J.C. Kadakol, C.M. Kamanavalli and Y. Shouche, World J. Microbiol. Biotechnol. 27, 25 (2011). doi:10.1007/s11274-010-0422-7.

[88] E.J. Stwart, M. Ganesan and M.J. Solomon, Sci Rep 5, 1 (2015). doi:10.1038/srep13081.

[89] M.P. Tlwar and H.Z. Ninnekar, J. Basic Microbiol. 55, 1 (2015). doi:10.1002/jobm.201400978.

[90] K. Cheng, J. Wu, W. Yang and S. Hwang, Biotechnol. Bioeng. 83, 821 (2003). doi:10.1002/ bit.10730.

[91] M.A. Martínez-Trujillo and M. García-Rivero, Rev. Mex. Ing. Quím. 11 (1), 55 (2012). https://

www.redalyc.articulo.oa?id=62024415005.

[92] B.T. Mhammad and M.T. Bustard, J. Ind. Microbiol. Biotechnol. 35(7), 677 (2008). doi:10.1007/s10295-008-0325-x.

[93] P.N. Tallur, S.I. Mulla, V.B. Megadi, M.P. Talwar and H.Z. Ninnekar, Braz. J. Microbiol. 46 (3), 667 (2015). doi:10.1590/S1517-838246320130557.

[94] A.E. Abigail and N. Das, Int. Biodeterior. Biodegrad. 104, 53 (2015). doi:10.1016/j.ibiod.2015.05.014.

[95] M. Idrees, S.A.M.A.S. Eqani and H. Bokhari, Chem. Ecol. 29 (8), 33 (2013). doi:10.1080/02757540.2013.820714.

[96] M.S. Khorram, Q. Zhang, D. Lin, Y. Zheng, H. Fang and Y. Yu, J. Environ. Sci. 44, 269 (2016). doi:10.1016/j.jes.2015.12.027.

[97] X.Y. Yu, C.L. Mu, C. Gu, C. Liu and X.J. Liu, Chemosphere 85, 1284 (2011). doi:10.1016/j.chemosphere.2011.07.031.

[98] S. Mukherjee, L. Weihermüller, W. Tappe, D. Hofmann, S. Köppchen, V. Laabs, H. Vereecken and P. Burauel, Sci. Total Environ. 559, 63 (2016). doi:10.1016/j.scitotenv.2016.03.145.

[99] J. Lehmman, M.C. Rilling, J. Thies, C.A. Massiello, W.C. Hockaday and D. Crowley, Soil Biol. Biochem. 43, 1812 (2011). doi:10.1016/j.soilbio.2011.04.022.

[100] T.B. Moorman, K.C. Jennifer, E.L. Arthur and J.R. Coats, Biol. Fertil. Soils 33 (6), 541 (2001). doi:10.1007/s003740100367.

[101] C.-J. Tien, M.-C. Lin, W.-H. Chiu and C.S. Chen, Environ. Pollut. 179, 95 (2013). doi:10.1016/j.envpol.2013.04.009.

[102] D.G. Ohandja and D.C. Stuckey, J. Chem. Technol. Biotechnol. 81 (11), 1736 (2006). doi:10.1002/jctb.1596.

[103] M. Toyofuku, T. Inaba, T. Kiyokawa, N. Obana, Y. Yawata and N. Nomura, Biosci. Biotechnol. Biochem. 80, 7 (2016). doi:10.1080/09168451.2015.1058701.

[104] S.M. Zhu and S.L. Chen, Aquacult. Eng. 26, 221e237 (2002). doi:(02)00022-5",1,0,0>10.1016/S0144-8609(02)00022-5.

[105] J. Zur, D. Wojcieszynsk and U. Guzik, Molecules 1, 958 (2016). doi:10.3390/ molecules21070958.

[106] S. Klen, J. Khun, R. Avrahami, S. Tarre, M. Beliavski, M. Grenn and E. Zussman, Biomacreomolecules 10, 1752 (2009). doi:10.1021/bm900168v.

[107] H. Keweloh, R. Diefenbach and H.J. Rehm, Arch. Microbiol. 157, 49 (1991). doi:10.1007/BF00245334.

[108] U.L. Chitiva and J. Dussán, Rev. Colomb. Biotecnol. 5 (2), 5 (2003). http://www.redalyc.articulo.oa?id=77650201.

[109] P.R. Waghmare, A.A. Kadam, G.D. Saratale and S.P. Govindwar, Bioresour. Technol. 168, 136 (2014).

doi:10.1016/j.biortech.2014.02.099.

[110] M.S. Fuentes, G.E. Briceño, J.M. Saez, C.S. Benimeli, M.C. Diez and M.J. Amoroso, Biomed. Res. Int. 2013, 392573. doi:10.1155/2013/392573

[111] C.S. Chen, T.W. Wu, H.L. Wang, S.H. Wu and C.J. Tien, Int. J. Environ. Sci. Technol. 2014. doi:10.1007/s13762-014-0675-z

[112] T. Vancov, K. Jury, N. Rice, L. Van Zwieten and S. Morris, J. Appl. Microbiol. 102, 212 (2007). doi:10.1111/j.1365-2672.2006.03047.x.

[113] W. Wang and Y. Wu, Appl. Microbiol. Biotechnol. 101 (2), 783 (2017). doi:10.1007/s00253-016-7933-6.

[114] O. Auguet, M. Pijuan, J. Batista, C.M. Borrego and O. Gutierrez, Appl. Environ. Microbiol. 81, 7271 (2015). doi:10.1128/AEM.01538-15.

[115] B. Kolwzan, Ochrona Srodowiska 33 (4), 3 (2011). http://yadda.icm.edu.pl/yadda/element/bwmeta1.element.baztech-article-BPOB-0044-0001.

[116] V.E. Herrera-González, N. Ruiz-Ordaz, J. Galíndez-Mayer, C. Juarez-Ramírez, F. Santoyo-Tepole and E.M. Montiel, World J. Microbiol. Biotechnol. 29, 467e474 (2013). doi:10.1007/s11274-012- 1200-5.

[117] S. El Fantroussi and S.N. Agathos, Curr. Opin. Microbiol. 8 (3), 268 (2005). doi:10.1016/j.mib.2005.04.011.

CAPÍTULO III

3.1 PLANTEAMIENTO DEL PROBLEMA

El uso continuo de POF´s, especialmente clorpirifós (CP) ha provocado problemas de contaminación ambiental, efectos nocivos a la salud humana, afectaciones en las propiedades del suelo y pérdida de microbiota edafica benéfica (Walvekar et al. 2017). Pese a que CP es suceptible a degradación microbiana, usualmente altas concentraciones del compuesto inhiben el crecimiento bacteriano, reduciendo la cantidad de organismos que pueden tolerarlo y degradarlo bajo diferentes condiciones (Abraham y Silambarasan 2016), especialmente en suelos donde la adición de microorganismos suele verse afectada por factores edafoclimáticos y exclusión competitiva.

La encapsulación de bacterias en polímeros no tóxicos como el alginato, es una alternativa a las formulaciones de inoculantes con potenciales aplicaciones en la degradación de plaguicidas (Talwar y Ninnekar 2015; Nagaraja et al. 2016). La encapsulación suele aumentar la tolerancia y las tasas de degradación (Krell et al. 2017). Sin embargo, dicha tecnología es generalmente experimental y aún presenta inconvenientes para su aplicación *in situ*.

En este contexto, la búsqueda de microorganismos tolerantes y degradadores; asi como estrategias que favorezcan su inoculación son reelevantes para que plaguicidas como CP se eliminen por biorremediación (Bose et al. 2021). Al respeto, la evaluación de la tolerancia y degradación de plaguicidas por rizobacterias promotoras de crecimiento vegetal (RPCV), particularmente empleando organismos modelo como *A. vinelandii*, ha cobrado interés ya que son bacterias naturalmente adaptadas al suelo y producen multiples metabolitos que favorecen el desarrollo de cultivos (Chennappa et al 2019). Sin embargo, existen escasos reportes que empleen rizobacterias para tal propósito; y se desconocen muchos de los efectos de CP sobre las principales RPCV o del uso de técnicas de inmovilización para su inoculación.

3.2 JUSTIFICACIÓN

Pese a que existen algunos estudios centrados en el papel de las RPCV en la tolerancia y degradación de plaguicidas, particularmente especies de *Azotobacter*, la información aun es limitada respecto a la inmovilización y el efecto de los POF´s como el CP sobre organismos modelo como *Azotobacter vinelandii*, incluidos los métodos de encapsulación para favorecer su inoculación o establecimiento. En tal sentido, el presente trabajo pretende aportar información sobre la evaluación de la rizobacteria *A. vinelandii* ATCC 12837 inmovilizada; asi como su desepmeño en la tolerancia y degradación de CP como estrategia multipropósito para la implementación de prácticas agrícolas sostenibles que favorezcan la eliminación de contaminantes; ya que cepas tolerantes mantendrían su capacidad de promover el crecimiento, reducir la aplicación de fertilizantes, establecerse en sitios contaminados, e incluso utilizarse como potenciales agentes descontaminantes.

3.3 HIPÓTESIS

- La inoculación de *A. vinelandii* ATCC 12837 inmovilizada en alginato-Na mantiene su viabilidad y mejora el desarrollo de plántulas de tomate.

- *A. vinelandii* ATCC 12837 tolera y degrada clorpirifós (CP) en condiciones de cultivo *in vitro*.

3.4 OBJETIVOS

3.4.1. OBJETIVO GENERAL

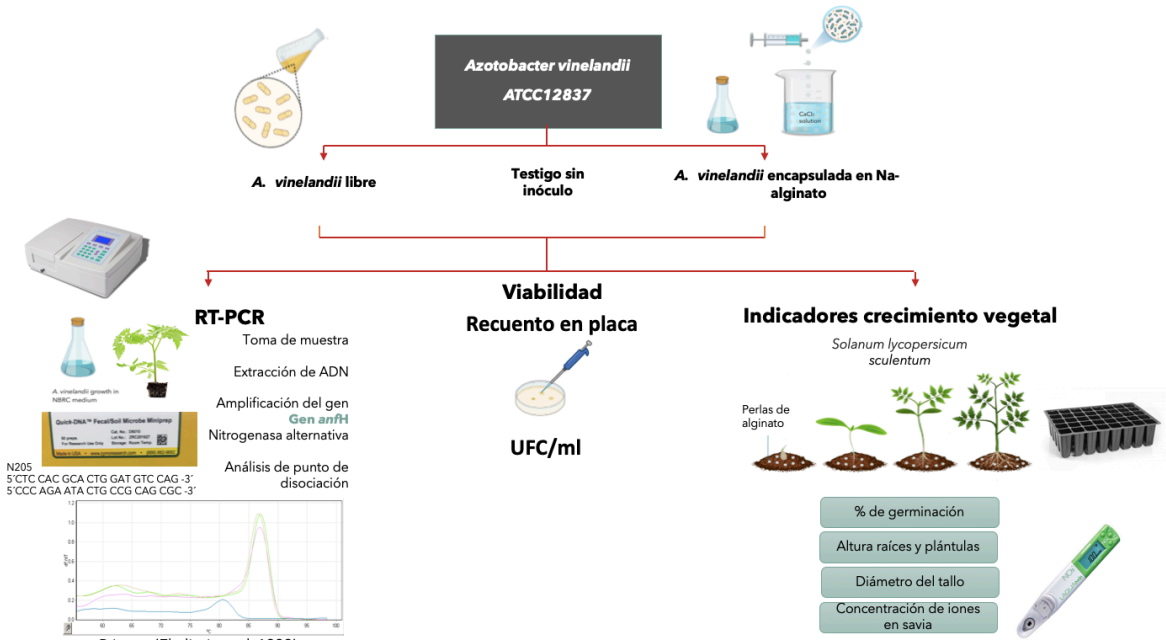
Evaluar el efecto de *Azotobacter vinelandii* ATCC 12837 inmovilizada en alginato sobre el desarrollo de plántulas de tomate, así como su crecimiento *in vitro* en presencia de clorpirifós, mediante técnicas microbiológicas, moleculares, respirométricas y análiticas.

3.4.2 OBJETIVOS ESPECÍFICOS

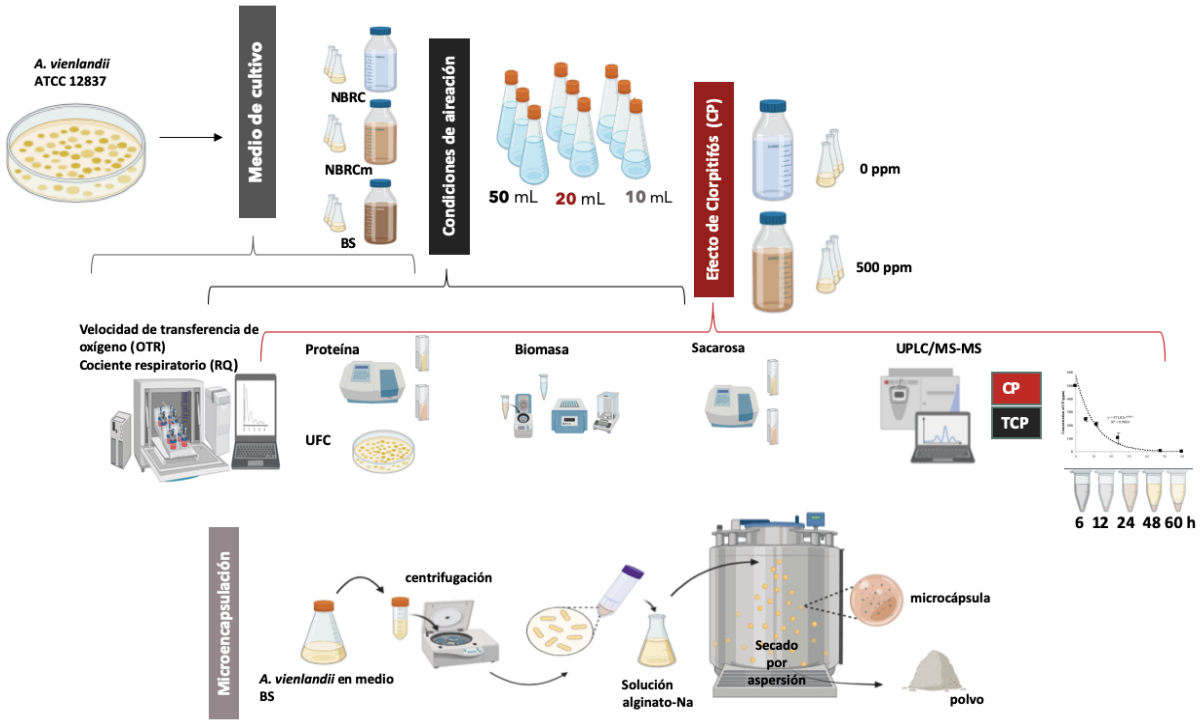
1. Determinar el efecto de *A. vinelandii* inmovilizada en alginato-Na como inoculante sobre el desarrollo de plántulas de tomate.
2. Comparar la presencia y viabilidad de *A. vinelandii* inoculada en suelo de forma libre e inmovilizada en alginato-Na.
3. Determinar condiciones de cultivo para el crecimiento y microencapsulación de *A. vinelandii*.
4. Identificar el efecto de CP en el desarrollo *in vitro* de *A. vinelandii*.
5. Cuantificar la degradación de CP *in vitro* por *A. vinelandii*.

3.5 ESTRATÉGIA METODOLÓGICA

Objetivos 1 y 2



Objetivos 3, 4 y 5



CAPÍTULO IV

ENCAPSULATION OF *AZOTOBACTER VINELANDII* IN ALGINATE-NA BEADS AS A TOMATO SEEDLING INOCULANT

Victoria Conde-Avila[1], Luis Daniel Ortega-Martínez[1*], Octavio Loera[2], Beatriz Pérez-Armendáriz[1], Carmen Martínez Valenzuela[3], Belisario Leyva[4]

[1]Facultad de Biotecnología, Universidad Popular Autónoma del Estado de Puebla, México. 13 Poniente No. 1927 Col. Barrio de Santiago, C.P.72410, Puebla, Pue. México. [2]Metropolitana - Unidad Iztapalapa. Av. San Rafael Atlixco 186, Leyes de Reforma 1ra Secc. 09340 Ciudad de México, CDMX.[3]Unidad de Investigación en Ambiente y Salud, Universidad Autónoma de Occidente, Los Mochis, Sinaloa, México.[4]Universidad Autónoma de Occidente, Unidad Guasave, Sinaloa México. Avenida Universidad S/N, Fraccionamiento Villa Universidad, C.P. 81048, Guasave, Sinaloa, México.

**Corresponding autor* luisdaniel.ortega@upaep.mx

Victoria Conde Avila: victoria.conde@upaep.mx ORCID: 0000-0003-2700-8603
Octavio Loera: loera@xanum.uam.mx ORCID 0000-0002-8194-1608
Beatriz Pérez Armendáriz: beatriz.perez@upaep.mx ORCID: 0000-0002-2287-8338
Carmen Martínez Valenzuela: camava9@gmail.com ORCID: 0000-0003-1784-9986
Belisario Leyva: NEO1702@gmail.com ORCID:0000-0001-6149-7506
Luis Daniel Ortega Martínez: luisdaniel.ortega@upaep.mx ORCID:0000-0003-4672-8809

ABSTRACT

Encapsulation is an immobilization method characterized by restricting microbial cells to a delimited area while preserving their metabolic viability. This technique represents an alternative to improve the adaptive capacity of bacteria in the face of interactions with native microorganisms and environmental factors that limit their inoculation. This work aimed to evaluate the effect of *Azotobacter vinelandii* encapsulated in alginate-Na beads as an inoculant of tomato (*Solanum Lycopersicum* L) seedlings. Two inoculation treatments were carried out: liquid and encapsulated, and the control without microorganisms. Physiological variables, microbial viability, and presence of *A. vinelandii* were determined by qPCR. Inoculation with *A. vinelandii* in liquid and encapsulated form favored seedling growth. Plants with the encapsulated inoculum significantly increased germination percentage (20%), stem diameter (38%), seedling height (34%), root length (69%), NO_3 concentration (41%), and Na (30%); compared to the control. Encapsulation of *A. vinelandii* in alginate-Na macrocapsules allowed its establishment in the rhizosphere and was corroborated by viable count and molecular methods. The viability of the bacteria was maintained for 28 days using both inoculation methods.

Key words: Alginate, Immobilization, Inoculation, PGPR, Viability

INTRODUCTION

Inoculation of plants with bacteria and other symbionts to improve crop yield is a frequently employed technology [below], highlighting *Azotobacter* species belonging to the plant growth-promoting rhizobacteria (PGPR) group [2]. The genus *Azotobacter* has the ability to produce different types of secondary metabolites, such as amino acids, polymers, phytohormones, antibiotics, antifungals, and siderophores [3].

Azotobacter vinelandii, is a bacterium noted for its efficiency in asymbiotic nitrogen (N_2) fixation, production of growth and biological control substances, as well as generation of products of industrial interest [4]. It produces B vitamins (niacin, riboflavin, and biotin) and amino acids (aspartic acid, glutamic acid, glycine, histidine, arginine, proline, etc.) that are key in the cellular processes of plant metabolism of fats, carbohydrates, and proteins [5]. It also releases auxins such as indoleacetic acid (IAA), responsible for plant cell division, differentiation, and growth [6], as well as hormone-acting growth regulators such as gibberellic acid (GA) [7]. It also excretes, antifungal compounds (azotobacin, azotochelin, aminochelin, protochelin, and 2,3-dihydroxyacidbenzoic acid [8], as well as volatile substances such as hydrogen cyanide (HCN) that inhibit the growth of phytopathogenic microorganisms, especially fungi of the genera *Aspergillus* and *Fusarium* [2].

Despite the potential beneficial effects, the incorporation of PGPRs, such as *A. vinelandii*, directly into the soil can present difficulties related to their survival, colonization, and long-term effectiveness [9,10]. In general, when PGPR suspensions are inoculated without a suitable carrier, the population declines rapidly[1].This phenomenon, combined with the low production of bacterial biomass, the difficulty to maintain their metabolic activities in the face of abiotic stress, and the physiological state at the time of application, limit their stabilization in the rhizosphere soil. A determining factor in the survival of these microorganisms is the competition with the native microbiome, often better adapted, and to withstand predation by soil microfauna. Therefore, inoculation of PGPR in the form of liquid cell dilutions in the soil is not recommended [11].

Consequently, an important function to consider in the formulation of microbial inoculants is to provide a suitable microenvironment, combined with physical protection for a prolonged period to prevent a rapid decline in their viability [12]. Cell immobilization is a technique that aims to favor the colonization of beneficial microorganisms and increase their viability by providing protection to external factors, tolerance to contaminants, as well as improving the properties of formulations to be applied either in water or soil [13,14].

Microbial encapsulation (the most common of the immobilization methods), is characterized by trapping alive and metabolically active microorganisms in a polymeric matrix [15]. Such a technique employs different materials intending to ensure the establishment of microorganisms in the soil and maintain their

metabolic activity prolonged [16,17]. For agricultural and environmental uses, encapsulation of microorganisms has been tested with materials such as agar, λ, and κ carrageenan, pectin, chitosan, and alginate, the latter being one of the most widely used [18,19,20,21] .

The advantages of alginate formulations are their non-toxic nature, biodegradability, affordability, slow release of microorganisms trapped by the polymeric structure [22], and their ability to generate both wet and dry formulations [23,24]. When alginate is employed as an encapsulation material, higher yields and colonization percentages are commonly achieved, suggesting it to be an alternative with potential applications [8], although its use is generally experimental [25,3] .

Some limitations in the evaluation of encapsulation as an inoculation technology for agricultural purposes have been the variability of use conditions, soil heterogeneity, survival of microorganisms in confined conditions, difficult scaling methods or drying processes, interaction with support and trapping materials, the mechanical stability of the materials used, effectiveness times, among others.

Finally, few investigations evaluate the survival of encapsulated PGPR using microbiological and molecular techniques to monitor the viability of microorganisms inoculated in soil and not only their effect on response variables in selected crops [10].

This study aimed to evaluate the viability in the soil of *A. vinelandii* encapsulated in alginate-Na beads using confirmatory molecular methods and its effect as an inoculant on tomato (*Solanum Lycopersicum* L) seedling development.

MATERIALS AND METHODS

Microorganism

The experiments were carried out using the strain *A. vinelandii* ATCC12837. Cells were cryopreserved at -70 C using a 4% glycerol solution. For propagation in both plate and liquid culture, NBRC medium was used (composition in gL-1: yeast extract, 1; D-mannitol, 5; K2HPO4, 0.7; KH2PO4 ,0.1; MgSO47H2O, 1; bacteriological agar (for plates), 15). The initial pH was adjusted to 7.2 using a 2N NaOH solution.

Obtaining the inoculum

A. vinelandii cells were grown at 28°C in 500 mL Erlenmeyer flasks with 100 mL of NBRC medium at 150 rpm. The flasks were incubated until a concentration of 1.61×10^9 CFU mL^{-1} was reached (72h). The biomass was collected by centrifugation at 1000 × g for 10 min and the supernatant was discarded. The recovered biomass was mixed with sterile saline (NaCl 0.09%) and used as liquid inoculum for subsequent experiments.

Encapsulation in alginate-Na

For encapsulation of *A. vinelandii*, the biomass obtained by centrifugation, after culturing as described above, was mixed in a sterile alginate-Na solution at a final concentration of 2% (w/v) [26]. An aliquot of the alginate-Na and *A. vinelandii* suspension was added by drip to a sterile 0.1 M calcium chloride ($CaCl_2$) solution (Fig.1), from which an average of 20 beads mL^{-1} of 3-5 mm in diameter was obtained.

Location and conditions of the experiment

The experiment was conducted at the Center for Technological Innovation in Protected Agriculture - UPAEP located at a latitude of 20°50', south 17°52' north latitude; east 96°43', west 99°04' west longitude. Under greenhouse conditions, special polyethylene trays were used for seedling production with 50 cavities (2.5x2.5x7cm), which were disinfected for 24 h with a 5% NaClO solution and then washed with water and filled with the commercial substrate of Dutch peat Sunshine® sun agro Horticulture Inc., agrolite and vermiculite in a 50:35:15 ratio, respectively.

Soil inoculation

Two inoculation treatments were prepared, the first with *A. vinelandii* in liquid solution and the second with *A. vinelandii* encapsulated in alginate-Na as described above. A cell density of $1.61x10^9$ CFU mL^{-1} was used for both treatments. Inoculum density was estimated by serial dilution of base 10 with saline by placing 100 µl on NBRC agar plates in triplicate, incubated for 48 h at 28 °C and the colony-forming units (CFU mL^{-1}) developed were quantified.

Substrate inoculation was performed with 2 mL of *A. vinelandii* inoculum in liquid solution and its equivalent encapsulated in alginate-Na beads per seedling, with ten replicates per treatment. A control treatment without inoculum was added to which 2 mL of distilled water was applied. In the end, irrigation was added until the field capacity of the substrate was reached in all treatments.

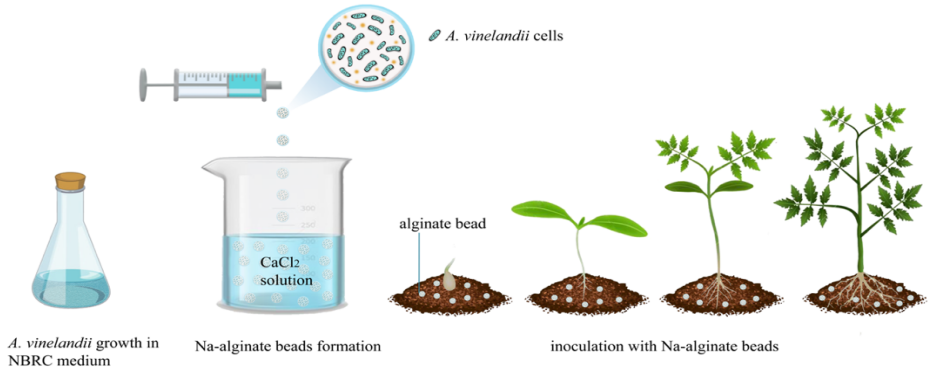


Figure 1. Representation of encapsulation and inoculation with *A. vinelandii* in soil.

Sowing

After inoculation with the corresponding treatments, tomato seeds (*S. Lycopersicum* L.) variety Sun 7705 were sown at 5 mm depth, placing one seed per cavity in seedbeds with 10 replicates per treatment. Finally, irrigations were applied every 24 h by sprinkling manually (10 mL per cavity).

Determination of plant morphological parameters

Independent measurements were taken from 10 seedlings per treatment. To estimate the germination percentage, radicle emergence at 3 and 4 days after sowing (das) was evaluated as an indicator. Seedling emergence was evaluated from day 4 to 15 days. For this purpose, the number of seedlings whose cotyledons were seen crossing the substrate surface was quantified daily. At 28 das, seedling height, roots, and stem diameter were evaluated using a millimeter tape (with a reading error of 0.05 cm). The reference points for height were the base of the stem and the apical bud from the base of the stem to the coping. Stem diameter measurements were taken at 5 cm from the base of the stem with a vernier (6¨/150 mm®).

Determination of NO3, Na, K, Ca

The sample consisted of 25 petioles per experimental unit. The cell extract was obtained by maceration until four to six drops were obtained, which were placed on ion sensors using the ion-selective electrode method [27], using portable Horiba LAQUA twin® (Horiba Europe, Leichlingen, Germany) Ions NO_3, Na, K and Ca.

Azotbacter vinelandii cell count.

To determine the number of CFU mL^{-1}, a plate count was performed as described above, starting from 1 g soil samples for each treatment. Subsequently, cultures were made with the bacteria recovered from the soil from the plates and the samples were analyzed by real-time PCR (qPCR) to verify the presence of *A. vinelandii*.

DNA extraction and qPCR for the detection of A. vinelandii

DNA extraction from soil samples was performed at 7, 14, and 28 days post-inoculation (dpi) using the Quick-DNATM Fecal/Soil Microbe Miniprep kit (Zymo Research) according to the manufacturer's instructions. Genomic DNA extract was quantified by UV-Visible spectrophotometry using a NanoDrop 2000 kit (Thermo Scientific™).

Real-time PCR amplification of *A. vinelandii* DNA was carried out on a Rotor-Gene Q thermal cycler using the Rotor-Gene SYBR® Green PCR Kit (Qiagen) and the *A. vinelandii*-specific primers N205F (5′-CCCAGAATACTGCCGCAGCGCGC-3′) and N205R (5′-CTCCACACGCACTGGATGTCCAG-3′) [28].

The reaction mixture contained: 12.5 µL 2x Rotor-Gene SYBR Green PCR Master Mix, 2.5 µL of the N205F and N205R 10 µM primer mix, 1 µL (5 ng) of genomic DNA extracted from soil samples, and 9 µL of molecular biology grade water (Millipore) to obtain a final reaction volume of 25 µL. The cycling program was as follows: 1) 95°C for 5 min, 2) 95°C for 10 s, 3) 60°C for 10 s, 4) repeat steps 2 and 3 45 times. To verify the specificity of the amplicons, a dissociation curve was generated by applying an increasing temperature gradient from 55°C to 99°C.

Design and statistical analysis

A randomized design with 10 replicates per treatment was used, the data obtained were examined for normality and homogeneity of variance using the Anderson-Darling and Levene test respectively. A one-way ANOVA and Tukey's multiple comparisons test were applied with the Graphpad prism program. alpha <0.05 was considered. Data are presented as mean values and standard deviations.

RESULTS

Plant morphological parameters

The germination percentage of tomato seeds increased by 20% when using the encapsulated inoculum with respect to the treatment without *A. vinelandii* and 10% with the liquid inoculum. Seedling height was greater in the treatments with *A. vinelandii* with liquid inoculum (27 cm) and encapsulated in alginate-Na beads (26.67 cm) compared to the treatment without inoculum (19.87 cm). Similarly, greater stem diameter was obtained, as well as a higher concentration of NO_3 and Na in the seedlings. There were no significant statistical differences in the rest of the variables (Table 1).

Table 1. Germination percentage and morphological variables of tomato seedlings inoculated with *A. vinelandii*.

Treatments	Germination	Stem diameter	Seedling height	Root size	NO_3^-	K^+	Na	Ca^+
	%	mm	cm		ppm			
No inoculum	**70**^{c}	**2.1**^{c} ± 0.15	**19.8**^{b} ± 1.1	**10.4**^{c} ± 0.82	**816.8**^{b} ±-46	**114.6**^{a} ± 4	**124**^{b} ± 10	**4075.6**^{a} ± 65
Liquid inoculum	**80**^{b}	**2.8**^{b} ± 0.5	**27**^{a} ± 3.0	**13.8**^{b}± 0.57	**985**^{b} ± 11	**122.6**^{a} ± 12	**129.8**^{b} ± 11	**4136**^{a} ± 54
Alginate-Na beads inoculum	**90**^{a}	**2.9**^{a} ± 0.10	**26.6**^{a} ± 3.4	**17.6**^{a} ± 1.1	**1153**^{a} ± 45	**126.2**^{a} ± 11	**161.8**^{a} ± 5	**4144.4**^{a} ± 11

The mean is presented in bold ± standard deviation. Different letters in the same columns indicate significant statistical differences, according to Tukey's test ($p \leq 0.05$).

Viability in the soil of encapsulated A. vinelandii

Screening of *A. vinelandii* by microbiology showed the presence of the bacterium at different sampling times after inoculation for both liquid and encapsulated inoculum. Particularly, the number of CFU was higher in the treatment with alginate-Na beads at 7, 14, and 28 dpi (Table 2). With both free and encapsulated inoculum, CFU decreased over time.

Table 2. Colony-forming units (CFU mL-1) of A. vinelandii recovered from soil samples.

Treatments	**CFU mL^{-1}**		
	7 dpi	**14 dpi**	**28 dpi**
No inoculum	0	0	0
Liquid inoculum	**1.3** x10^{6}±0.03 ^{b}	**0.9** x10^{6}±0.05 ^{b}	**0.5** x10^{6} ±0.1^{b}
Alginate-Na beads inoculum	**2.1** x10^{6}±0.1 ^{a}	**1.6** x10^{6} ±0.07^{a}	**1.1** x10^{6} ±0.09^{a}

The mean is presented. Different letters in the same columns indicate significant statistical differences according to Tukey's test (p≤0.05).

Real-time PCR

The qPCR result was analyzed using the amplification and dissociation curves of samples extracted at 7, 14, and 28 dpi. At 7 dpi, the presence of *A. vinelandii* DNA was detected. Amplification curves were observed for both the *A. vinelandii* positive control and the liquid-inoculated and encapsulated inoculated soil samples (Fig. 2a). Analysis of the dissociation curve to verify the specificity of the amplicons (Fig. 2b) presented a narrow and defined dissociation peak corresponding to *A. vinelandii* control, and one for each sample extracted from soil inoculated with liquid *A. vinelandii* and encapsulated in alginate-Na beads. The dissociation curves of both the positive control and the *A. vinelandii* soil extraction samples showed a peak between 89°C and 91 °C, thus indicating the specificity of the reaction corresponding to the dissociation temperature (Tm) of the PCR products expected for the bacterium [28]. On the other hand, at 14 dpi, amplification of the positive control and soil extraction samples of *A. vinelandii* was observed (Fig. 3a), in the treatments with the liquid and encapsulated inoculum the Ct was reached after 30 cycles, indicating a possible decrease in the amount of DNA in the sample. In the dissociation point analysis (Fig. 3b), the Tm of the peak of the control and soil samples were slightly different and showed unspecificities for detecting *A. vinelandii* in soil. Finally, at 28 dpi (Fig. 4a and 4b), amplification was observed but was not specific for *A. vinelandii*, so the bacterium was undetectable in soil regardless of the inoculation treatment using this technique.

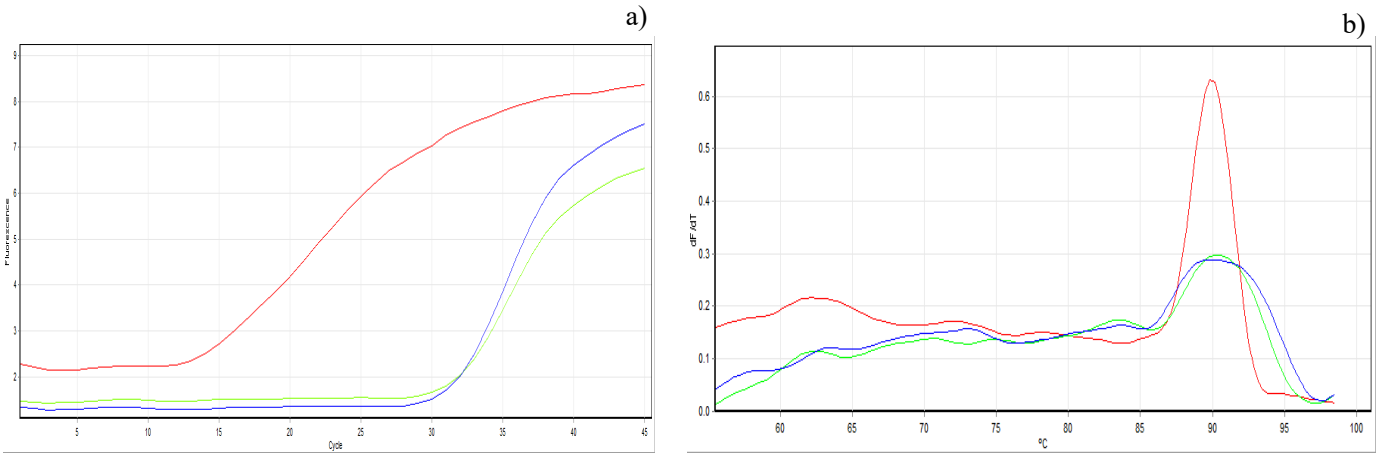


Fig. 2. a) Amplification curves of soil samples at 7 dpi. *A. vinelandii* control (-), soil inoculated with liquid *A. vinelandii* (-) and soil inoculated with *A. vinelandii* in alginate-Na beads (-). b) Dissociation curves of samples with *A. vinelandii* in axenic culture (control) (-) and soil inoculated with liquid *A. vinelandii* (-) and encapsulated in alginate-Na beads (-).

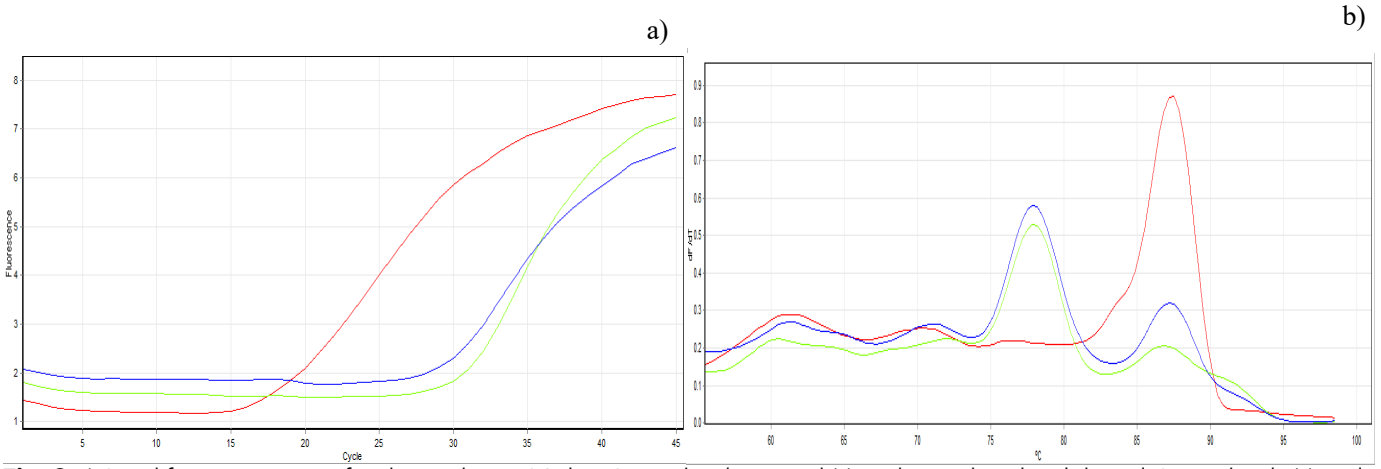


Fig. 3 a) Amplification curves of soil samples at 14 dpi. *A. vinelandii* control (-), soil inoculated with liquid *A. vinelandii* (-) and soil inoculated with *A. vinelandii* in alginate-Na beads (-). b) Dissociation curves of samples with *A. vinelandii* in axenic culture (control) (-) and soil inoculated with liquid *A. vinelandii* (-) and encapsulated in alginate-Na beads (-).

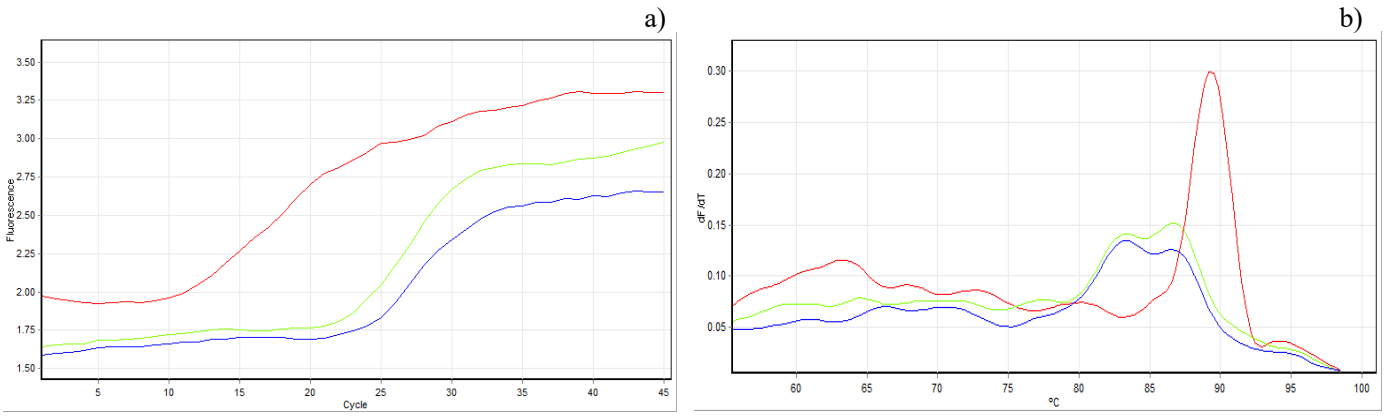


Fig. 4 a) Amplification curves of soil samples at 28 dpi. *A. vinelandii* control (-), soil inoculated with liquid *A. vinelandii* (-) and soil inoculated with *A. vinelandii* in alginate-Na beads (-). b) Dissociation curves of samples with *A. vinelandii* in axenic culture (control) (-) and soil inoculated with liquid *A. vinelandii* (-) and encapsulated in alginate-Na beads (-).

DISCUSSION

Inoculation with encapsulated microorganisms can improve their performance due to protection from external factors. It has been demonstrated that PGPRs produce different types of secondary metabolites under optimal conditions of development. These substances directly influence the overall plant growth of different crops, which explains their effectiveness as inoculants [29,2,30].

In this regard, as in our work, benefits have been obtained with both free and encapsulated inoculation for tomato plants. Ahmad et al. [29] reported increases in fruit N content (0.32%) when using treatments with *Azotobacter* and 3 solubilizing strains of K. On the other hand, Saima et al. [31] concluded that among different inoculants with mycorrhizae and N- and K-solubilizing bacteria; *Azotobacter* sp. showed to be superior in increasing tomato growth, yield, and quality. Similarly, Vassilev et al. [32] demonstrated that the inoculation of the fungus *Glomus deserticola* and the phosphorus (P)-solubilizing yeast *Yarrowia lipolytica* encapsulated in alginate were effective for the establishment and growth of tomato plants in nutrient-deficient soils. In our case, the NO_3 content in tomato seedlings was 20 and 40% higher with *A. vinelandii* free and encapsulated in alginate-Na, respectively, in relation to the control without bacteria. This is related to the capacity of *A. vinelandii* to fix N_2 by the nitrogenase complex under diazotrophic conditions since no additional N sources or chemical fertilization were added.

About germination, Ayala-Villegas et al. [33] found that seeds that germinate in a shorter time are more homogeneous and vigorous, while those that germinate slowly produce heterogeneous and small seedlings. In this sense, our results are similar to those shown by Delgado et al. [34] who concluded that using *Azotobacter paspali* accelerated the germination percentage between 61.7 and 55 % in *Coffea arabica* L. For their part, Marquina et al. [35] mention that, in paprika plants, inoculation with rhizospheric isolates increased the germination percentage of seeds up to 23 %, besides, they induced more homogeneous and resistant seedlings. In our case, the percentage of germination increased by 20% with encapsulated *A. vienalandii* and 10% with the free bacterium. Some authors attribute this effect to the GA produced by bacteria of the genus *Azotobacter* [7]. *A vinelandii* is capable of producing GA and has been associated with increases in germination, new cell growth, and participation in flowering and cell division processes [36]. In our work, the speed and efficiency in germination with *A. vinelandii* inoculation were enhanced by adding the polymeric protection of alginate-Na.

Regarding vegetative growth and plant morphology, our results coincide with those obtained by Escobar et al. [37], who indicate that four native *Azotobacter* spp. strains, AIA-producing, N-fixing, and rock phosphate solubilizing, positively influenced tomato vegetative development. Some authors have mentioned that the agrobiological effectiveness of PGPR is related to the growth stimulus exerted by a high population level in the rhizosphere of inoculated plants [38]. However, it has also been reported that, under inoculation

with microorganisms encapsulated in alginate, plant growth parameters were the same in treatments inoculated with free microorganisms [32]. Such is the case of *G. deserticola* free and encapsulated in alginate, which required double inoculation to obtain significant statistical differences in all the variables analyzed. The highest growth rates were obtained in co-immobilization, that is when two fungal microorganisms were trapped on a carrier increasing mycorrhization of tomato plant roots [32].

In our results, root size increased 32% with the addition of free *A. vinelandii* and the roots were 69% larger when the encapsulated inoculum was added. This was possibly related to auxins such as AIA, characteristic of *A. vinelandii*, which increase root length by stimulating the division and differentiation of root cells.

On the other hand, the viability of the inoculated microorganisms is affected by the method and material of encapsulation. Particularly by employing alginate beads, encapsulation of the fungus *Metarhizium brunneum* promoted selective mycelium formation by a combination of osmotic pressure and viscosity, improvements in drying survival of encapsulated mycelium, increased endophytism in tomato stem tissue after mycelium application to roots, as well as colonization, stabilization in the rhizosphere, and symbiotic effect [39]. Also, some changes in formulations (such as drying techniques in the formation of alginate beads for encapsulation) allowed, the growth of the fungus in various soil types. Sabaratnam and Traquair [40] used alginate beads (1-5 mm in diameter) to encapsulate PGPR and mycorrhizal fungi such as *Streptomycetes* sp. Their wettable dry powder formulation improved fungal survival and showed efficacy in biological control of the fungal pathogen *Rhizoctonia* in tomato. While encapsulation of the PGPR *Bacillus subtilis* in alginate beads enriched with humic acid increases viability, with minimal cell loss for five months, constant cell release, and beneficial effects on lettuce production [41].

Friel and McLoughlin [42] suggested that the wet alginate encapsulated inoculum using *Agaricus bisporus* had a shorter adaptation period and higher growth rate, relative to liquid medium and conventional inoculum. The improvements were attributed to the high biomass carrying capacity of the beads, mycelial protection in the microenvironment of the capsules, and spatial distribution on the substrate. On the other hand, alginate formulations (macro beads with and without skim milk and seed coating) of *B. subtilis* and *Pseudomonas corrugata* were found to be superior to liquid inoculants or carbon-based inoculants for maize plants at low temperatures, improving their permanence in extreme environments [43]. Similarly, Rekha et al. [44] found that encapsulation in dry alginate beads prolonged the survival of PGPR *Azospirillum brasilense* and *Pseudomonas fluroescens*, immobilized in two types of alginate-bead inoculants (with and without skim milk supplementation).

Finally, although there are few works in which, in addition to the effect on plant variables, the viability of the inoculated microorganisms with any immobilization method was evaluated by microbiological and

molecular methods, Krell et al.[39] demonstrated by microscopy and qPCR that at 21 dpi the fungus *M. brunneum* encapsulated in alginate-Ca remained in roots of tomato plants in different types of soil; 64% of the plants inoculated with the encapsulate presented higher endophytism particularly when using wet beads. In our case, *A. vinelandii* showed a different behavior during the sampling times, since 7 dpi qPCR results showed its establishment in the rhizosphere with both liquid and encapsulated inoculum. However, at 14 dpi its presence in the soil decreases, to finally not be detected in both treatments by molecular methods after 28 dpi despite the significant observable effects on agronomic and microbiological variables during the seedling stage.

CONCLUSIONS

In this work we highlight the use of alginate-Na as a material for the encapsulation of the PGPR *A. vinelandii* and that its performance in the inoculation of tomato seedlings improved important agronomic variables, such as germination, stem diameter, seedling, and root size, as well as NO_3 content. The encapsulation of *A. vinelandii* in alginate-Na beads, although spatially restricting its distribution in the soil, does not limit its metabolic activities, allowing its release and establishment during the first phenological stage of the crop. Finally, we show evidence on the encapsulation of *A. vinelandii* in tomato crops as an inoculant for growth promotion and chemical fertilization reduction purposes. These findings provide a basis for testing the encapsulation of this and other PGPRs as inoculants. The development of formulations that enhance colonization in the rhizosphere soil and maintain viability during the complete production cycle is recommended, since in this work the viability of the bacterium was maintained for 28 days, regardless of the inoculation method employed. It is suggested to consider different ways to evaluate the viability of the inoculated microorganisms to corroborate the permanence times and effectiveness in the crops.

REFERENCES

1. Bashan, Y., de-Bashan, L., Prabhu, S. & Hernandez, J. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil* **378**, 1-33; 10.1007/s11104-013-1956-x (2013).
2. Chennappa, G., Naik, M., Amaresh, Y., Nagaraja, H. & Sreenivasa, M. *Azotobacter*: A Potential Biofertilizer and Bioinoculants for Sustainable Agriculture. *Microorganisms for Sustainability* 78-87; 10.1007/978-981-10-6241-4_5 (2017).
3. Vejan, P., Abdullah, R., Khadiran, T., Ismail, S. & Nasrulhaq Boyce, A. Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability—A Review. *Molecules* **21**, 573; 10.3390/molecules21050573 (2016).

4. Gurikar, C., Naik, M. & Sreenivasa, M. *Azotobacter*: PGPR Activities with Special Reference to Effect of Pesticides and Biodegradation. *Microbial Inoculants in Sustainable Agricultural Productivity* 229-244; 10.1007/978-81-322-2647-5_13 (2016).
5. Revillas, J., Rodelas, B., Pozo, C., Martínez-Toledo, M. & González-López, J. Production of B-group vitamins by two *Azotobacte*r strains with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions. *Journal of Applied Microbiology* **89**, 486-493; 10.1046/j.1365-2672.2000.01139.x (2000).
6. Reshma, P. et al. Induced systemic resistance by 2,4-diacetylphloroglucinol positive fluorescent *Pseudomonas* strains against rice sheath blight. *Indian J Exp Biol.* **56**, 207–212 (2018).
7. Upadhyay, S., Singh, D. & Saikia, R. Genetic Diversity of Plant Growth Promoting Rhizobacteria Isolated from Rhizospheric Soil of Wheat Under Saline Condition. *Current Microbiology* **59**, 489-496; 10.1007/s00284-009-9464-1 (2009).
8. Nagaraja, H. et al. Antifungal activity of *Azotobacter nigricans* against trichothecene-producing *Fusarium* species associated with cereals. *Food Science and Biotechnology* **25**, 1197-1204; 10.1007/s10068-016-0190-8 (2016).
9. Mrozik, A. & Piotrowska-Seget, Z. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. *Microbiological Research* **165**, 363-375; 10.1016/j.micres.2009.08.001 (2010).
10. Strobel, S. et al. Industrially-Scalable Microencapsulation of Plant Beneficial Bacteria in Dry Cross-Linked Alginate Matrix. *Industrial Biotechnology* **14**, 138-147; 10.1089/ind.2017.0032 (2018).
11. Nakkeeran, S., Fernando, W. & Siddiqui, Z. Plant Growth Promoting Rhizobacteria Formulations and its Scope in Commercialization for the Management of Pests and Diseases. *PGPR: Biocontrol and Biofertilization* 257-296; 10.1007/1-4020-4152-7_10 (2005).
12. Bashan, Y., Hernandez, J., Leyva, L. & Bacilio, M. Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. *Biology and Fertility of Soils* **35**, 359-368; 10.1007/s00374-002-0481-5 (2002).
13. Covarrubias, S., de-Bashan, L., Moreno, M. & Bashan, Y. Alginate beads provide a beneficial physical barrier against native microorganisms in wastewater treated with immobilized bacteria and microalgae. *Applied Microbiology and Biotechnology* 93, 2669-2680; 10.1007/s00253-011-3585-8 (2012).
14. Nussinovitch, A. *Polymer Macro- and Micro-Gel Beads: Fundamentals and Applications*. 232-253 (Springer, 2010).
15. Mustapha, M., Halimoon, N., Wan Johari, W. & Abd Shakur, M. Soil Microorganisms and Their Potential in Pesticide Biodegradation; A Review Article. *Journal of Sustainable Agricultural Sciences* **44**, 39-61; 10.21608/jsas.2018.3465.1061 (2018).

16. Jesitha, K., Nimisha, K., Manjusha, C. & Harikumar, P. Biodegradation of Endosulfan by *Pseudomonas fluorescens*. *Environmental Processes* **2**, 225-240; 10.1007/s40710-015-0059-5 (2015).
17. Zohar-Perez, C., Ritte, E., Chernin, L., Chet, I. & Nussinovitch, A. Preservation of Chitinolytic *Pantoae agglomerans* in a Viable Form by Cellular Dried Alginate-Based Carriers. *Biotechnology Progress* **18**, 1133-1140; 10.1021/bp025532t (2002).
18. Conde-Avila, V. et al. Pesticides degradation by immobilised microorganisms. *International Journal of Environmental Analytical Chemistry* 1-31; 10.1080/03067319.2020.1715375 (2020).
19. John, R., Tyagi, R., Brar, S., Surampalli, R. & Prévost, D. Bio-encapsulation of microbial cells for targeted agricultural delivery. *Critical Reviews in Biotechnology* **31**, 211-226; 10.3109/07388551.2010.513327 (2011).
20. Ruiz, J. & Salas, M. Evaluation of Organic Substrates and Microorganisms as Bio-Fertilisation Tool in Container Crop Production. *Agronomy* **9**, 705; 10.3390/agronomy9110705 (2019).
21. Talwar, M. & Ninnekar, H. Biodegradation of pesticide profenofos by the free and immobilized cells of *Pseudoxanthomonas suwonensis* strain HNM. *Journal of Basic Microbiology* **55**, 1094-1103; 10.1002/jobm.201400978 (2015).
22. Kadakol, J., Kamanavalli, C. & Shouche, Y. Biodegradation of Carbofuran phenol by free and immobilized cells of *Klebsiella pneumoniae* ATCC13883T. *World Journal of Microbiology and Biotechnology* **27**, 25-29; 10.1007/s11274-010-0422-7 (2011).
23. Bashan, Y. & Gonzalez, L. Long-term survival of the plant-growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant. *Applied Microbiology and Biotechnology* **51**, 262-266; 10.1007/s002530051391 (1999).
24. Campos, D. et al. Microencapsulation by spray drying of nitrogen-fixing bacteria associated with lupin nodules. *World Journal of Microbiology and Biotechnology* **30**, 2371-2378; 10.1007/s11274-014-1662-8 (2014).
25. Joe, M., Saravanan, V., Islam, M. & Sa, T. Development of alginate-based aggregate inoculants of *Methylobacterium sp.* and *Azospirillum brasilense* tested under in vitro conditions to promote plant growth. *Journal of Applied Microbiology* **116**, 408-423; 10.1111/jam.12384 (2014).
26. Yañez-Ocampo, G., Sanchez-Salinas, E., Jimenez-Tobon, G., Penninckx, M. & Ortiz-Hernández, M. Removal of two organophosphate pesticides by a bacterial consortium immobilized in alginate or tezontle. *Journal of Hazardous Materials* **168**, 1554-1561; 10.1016/j.jhazmat.2009.03.047 (2009).
27. Carson, L., Ozores-Hampton, M. & Morgan, K. Correlation of petiole sap nitrate-nitrogen concentration measured by ion selective electrode, leaf tissue nitrogen concentration, and tomato yield in Florida. *Journal of Plant Nutrition* **39**, 1809-1819; 10.1080/01904167.2016.1187743 (2016).

28. Ehaliotis, C., Papadopoulou, K., Kotsou, M., Mari, I. & Balis, C. Adaptation and population dynamics of *Azotobacter vinelandii* during aerobic biological treatment of olive-mill wastewater. *FEMS Microbiology Ecology* **30**, 301-311; 10.1111/j.1574-6941.1999.tb00658.x (1999).
29. Ahmad, Z. et al. Impact of Azotobacter and Inorganic Fertilizers on Yield Attributes of Tomato. *International Journal of Current Microbiology and Applied Sciences* **7**, 3803-3809; 10.20546/ijcmas.2018.702.450 (2018).
30. Myresiotis, C., Vryzas, Z. & Papadopoulou-Mourkidou, E. Biodegradation of soil-applied pesticides by selected strains of plant growth-promoting rhizobacteria (PGPR) and their effects on bacterial growth. *Biodegradation* **23**, 297-310; 10.1007/s10532-011-9509-6 (2011).
31. Saima, S. et al. Impact of microbial inoculants on growth and yield of tomato *(Solanum lycopersicon L.)* under temperate conditions. *Journal of Pharmacognosy and Phytochemistry* **8**, 1261-1264 (2019).
32. Vassilev, N., Vassileva, M., Azcon, R. & Medina, A. Application of free and Ca-alginate-entrapped *Glomus deserticola* and *Yarowia lipolytica* in a soil–plant system. *Journal of Biotechnology* **91**, 237-242; 10.1016/s0168-1656(01)00341-8 (2001).
33. Ayala-Villegas, M., Ayala-Garay, Ó., Aguilar-Rincón, V. & Corona-Torres, T. Evolución de la calidad de semilla de *Capsicum annuum L.* durante su desarrollo en el fruto. *Revista Fitotecnia Mexicana* **37**, 79-87; 10.35196/rfm.2014.1.79 (2014).
34. Delgado, Y., Cupull, R., Pérez, C., Sánchez, A. & Vilchez, M. Efecto de *Azotobacter spp.* en la estimularción de la germinación y el desarrollo de posturas de *Coffea arabica L. Centro Agrícola* **30**, 26-31 (2003).
35. Marquina, M., Ramírez, Y. & Castro, Y. Efecto de bacterias rizosféricas en la germinación y crecimiento del pimentón *Capsicum annuum L.* var. Cacique Gigante. *Bioagro* **30**, 3-16 (2018).
36. Chennappa, G. et al. *Azotobacter*—A Natural Resource for Bioremediation of Toxic Pesticides in Soil Ecosystems. *New and Future Developments in Microbial Biotechnology and Bioengineering* 267-279; 10.1016/b978-0-444-64191-5.00019-5 (2019).
37. Escobar, C., Horna, Y., Carreño, C. & Mendoza, G. Characterization of native strains of *Azotobacter spp.* and its effect on growth of *Lycopersicon esculentum* Mill. "tomato" in Lambayeque. *Scientia agropecuaria* **2**, 39-49; 10.17268/sci.agropecu.2011.01.05 (2011).
38. Kumar, A., Singh, V., Tripathi, V., Singh, P. & Singh, A. Plant Growth-Promoting Rhizobacteria (PGPR): Perspective in Agriculture Under Biotic and Abiotic Stress. *Crop Improvement Through Microbial Biotechnology* 333-342; 10.1016/b978-0-444-63987-5.00016-5 (2018).
39. Krell, V., Jakobs-Schoenwandt, D., Vidal, S. & Patel, A. Encapsulation of *Metarhizium brunneum* enhances endophytism in tomato plants. *Biological Control* **116**, 62-73; 10.1016/j.biocontrol.2017.05.004 (2018).

40. Sabaratnam, S. & Traquair, J. Formulation of a Streptomyces Biocontrol Agent for the Suppression of *Rhizoctonia* Damping-off in Tomato Transplants. *Biological Control* **23**, 245-253; 10.1006/bcon.2001.1014 (2002).
41. Young, C., Rekha, P., Lai, W. & Arun, A. Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. *Biotechnology and Bioengineering* **95**, 76-83; 10.1002/bit.20957 (2006).
42. Friel, M. & McLoughlin, A. Immobilisation as a strategy to increase the ecological competence of liquid cultures of *Agaricus bisporus* in pasteurised compost. *FEMS Microbiology Ecology* **30**, 39-46; 10.1111/j.1574-6941.1999.tb00633.x (1999).
43. Trivedi, P., Pandey, A. & Palni, L. Carrier-based Preparations of Plant Growth-promoting Bacterial Inoculants Suitable for use in Cooler Regions. *World Journal of Microbiology and Biotechnology* **21**, 941-945; 10.1007/s11274-004-6820-y (2005).
44. Rekha, P., Lai, W., Arun, A. & Young, C. Effect of free and encapsulated *Pseudomonas putida* CC-FR2-4 and *Bacillus subtilis* CC-pg104 on plant growth under gnotobiotic conditions. *Bioresource Technology* 98, 447-451; 10.1016/j.biortech.2006.01.009 (2007).

AUTHOR CONTRIBUTION STATEMENT

All authors designed, read, and approved the final version of the paper.

C V. contributed to data curation, investigation, formal analysis, visualization, and writing the original draft.

O L. contributed to designing this study, methodology, result visualization, interpretation, and draft review.

L O. contributed to conceptualization, methodology, interpretation, and draft review.

P B. project administration, fund acquisition, supervision, revising, and editing

M C. performed statistical analyses, draft reviewing

L B. edited the paper, making of the figures

COMPETING INTERESTS

There's no financial/personal interest or belief that could affect the objectivity of the study.

CAPÍTULO V

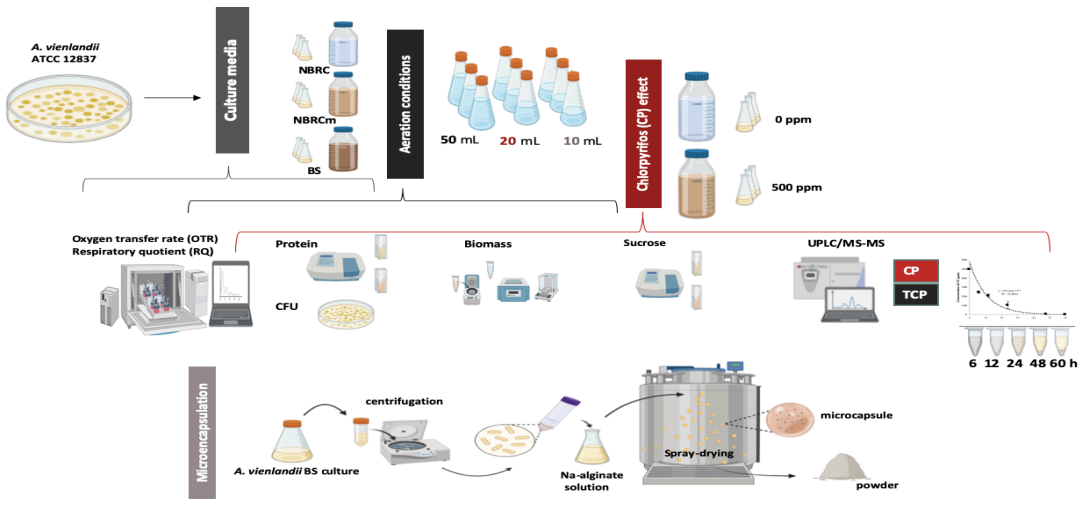
GROWTH, CHLORPYRIFOS BIODEGRADATION AND MICROENCAPSULATION USING *AZOTOBACTER VINELANDII* ATCC 12837

Victoria Conde-Avila[1], Carlos Peña[2], Beatriz Pérez-Armendáriz[1], Octavio Loera[3], Carmen Martínez Valenzuela[4], Belisario Leyva[4], Pedro de Jesús Bastidas Bastidas[5], Holjes Salgado-Lugo[2], Luis Daniel Ortega-Martínez[1*]

[1] Facultad de Biotecnología, Universidad Popular Autónoma del Estado de Puebla, México. 13 Poniente No. 1927 Col. Barrio de Santiago, C.P.72410, Puebla, Pue. México. [2]Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Apdo. Post. 510-3 Cuernavaca, 62250 Morelos, México.[3] Universidad Autónoma Metropolitana-Unidad Iztapalapa. Av. San Rafael Atlixco 186., 09340 Ciudad de México, CDMX. [4]Doctorado en Sustentabilidad, Universidad Autónoma de Occidente, Unidad Guasave. Avenida Universidad S/N, Fraccionamiento Villa Universidad, C.P. 81048, Guasave, Sinaloa, México. [5]Centro de Investigación en Alimentación y Desarrollo, A.C. (Unidad Culiacán). Carretera a El dorado km. 5.5, Campo el Diez, Culiacán, Sinaloa, México, C.P. 80129.

[1] Victoria Conde Avila: 0000-0003-2700-8603
[2] Carlos Peña: 0000-0002-0381-6529
[1] Beatriz Pérez Armendáriz: 0000-0002-2287-8338
[3] Octavio Loera: 0000-0002-8194-1608
[4] José Belisario Leyva Morales: 0000-0001-6149-7506
[4] Carmen Martínez Valenzuela: 0000-0003-1784-9986
[5] Pedro de Jesús Bastidas Bastidas: 0000-0003-0419-0481
[2]Holjes Salgado-Lugo: 0000-0003-2878-2959
[1] Luis Daniel Ortega Martínez: 0000-0003-4672-8809
*Corresponding author: luisdaniel.ortega@upaep.mx

GRAPHICAL ABSTRACT



ABSTRACT

The aim of this study was to evaluate the growth, biodegradation of chlorpyrifos and microencapsulation using *Azotobacter vinelandii* ATCC 12837. This strategy was based on the modification of culture media and aeration conditions to increase the cellular concentration of *A. vinelandii* and determine its pesticide tolerance and degradation ability. The culture in shaken flasks, using sucrose as a carbon source, significantly improved the growth compared to media with mannitol. When the strain was cultivated under oxygen-limited (5.5, 11.25 mmol L^{-1} h^{-1}) and no-oxygen-limited conditions (22 mmol L^{-1} h^{-1}), the growth parameters were not affected. In cultures in a liquid medium with chlorpyrifos, the bacteria tolerated a high pesticide concentration (500 ppm) and the growth parameters were improved even under conditions with a reduced carbon source (sucrose 2 g L^{-1}). The strain degraded 99.6 % of chlorpyrifos at 60 h of cultivation, in co-metabolism with sucrose; notably, *A. vinelandii* ATCC 12837 reduced by 50% the initial concentration (DT_{50}) in only 6 h. On the other hand, microencapsulation using Na-alginate by spray drying maintained the viability of 4 x10^9 UFC g^{-1} in a powder formulation.

KEYWORDS

Microencapsulation, Oxygen consumption rate, Pesticide degradation, Rhizobacteria

KEY POINTS

- *Azotobacter vinelandii* ATCC 12837 tolerates, grows, and degrades high concentrations of chlorpyrifos *in vitro*.
- Respirometric parameters of *Azotobacter vinelandii* ATCC 12837 were not adversely affected by chlorpyrifos.
- Spray drying with a protective polymer promotes microencapsulation.

1. INTRODUCTION

One of the ecotoxicological problems caused by the intensive use of organophosphate pesticides (OP) is damage to non-target organisms. Pesticides can inhibit the growth of beneficial microorganisms, such as plant growth-promoting rhizobacteria (PGPR) (Walvekar et al. 2017), or reduce metabolic capacities related to their efficacy as inoculants (Sethi and Gupta 2013; Abo-amer et al. 2014; Muttawar and Wadhai 2014).

The evaluation of the effects of the most widely used OP worldwide on PGPR has gained interest because tolerant organisms could maintain their promoting activities, establish in contaminated sites, even used as potential decontaminating agents (Abraham and Silambarasan 2016; Shahid et al. 2019; Sumbul et al. 2020; Chitara et al. 2021). Tolerance and degradation to various pesticides by PGPR have been evaluated in the

genera *Azospirillum* (Santos et al. 2020), *Bacillus* (Myresiotis et al. 2012; Praveen Kumar et al. 2014), *Klebsiella* (Rani et al. 2019a), *Pseudomonas* (Giri and Rai 2012), *Serriata* (Cycón et al. 2013), *Ochrobactrum* (Abraham and Silambarasan 2016) and *Azotobacter* (Chennappa et al. 2018a), the latter being one of the most important for agricultural proposes.

Azotobacter spp. are efficient in asymbiotic N_2 fixation, P solubilization (Sethi and Gupta, 2013), production of phytohormones (Chobotarov et al. 2017), siderophores (Shahid et al. 2019), vitamins (Revillas et al. 2000), synthesis of antimicrobial compounds (Nagaraja et al. 2016), production of metabolites of industrial interest such as the alginate and polyhydroxybutyrate (PHB) (Gurikar et al. 2016), as well as in the synthesis of enzymes involved in degradation processes of toxic substances (Chennappa et al. 2019).

Some *Azotobacter* species degrade aromatic compounds such as insecticides, fungicides, and herbicides (Castillo et al. 2011; Chennappa et al. 2016). These bacteria have particularly shown tolerance to endosulfan, phorate, carbendazim, chlorpyrifos (CP), pendimethalin, among others (Castillo et al. 2011; Chennappa et al. 2014a; Gurikar et al.2016; Rani and Kumar et al. 2017), without showing growth inhibition (Chennappa et al. 2016). Also, there are reports describing the degradation of lindane (Andupama and Paul, 2009), phorate (Moneke et al. 2010), endosulfan (Castillo et al. 2011), pendimethalin (Chennappa et al. 2014b; Chennappa et al.2018a), glyphosate (Mousal et al. 2021), and CP by *Azotobacter* isolates (Chennappa et al. 2019).

In contrast, other authors have reported adverse effects for *Azotobacter* spp. (Askar and Khudhur 2013; Chennappa et al. 2013; Walvekar et al. 2017; Kumar et al. 2019); e.g. reduced growth rate in the presence of CP (Menon et al. 2004), glyphosate (Moneke et al. 2010) and endosulfan (Castillo et al. 2011), inhibition of diazotrophic activity (Menon et al. 2004; Chennappa et al. 2019), reduced respiration rate with glyphosate, pendimethalin and fomesafen (Chennappa et al. 2013, Wu et al. 2014; Chennappa et al. 2014b), cell damage and loss of viability after exposure to different concentrations of glyphosate and atrazine (Shahid et al. 2019)

The genus *Azotobacter* can exhibit varied behaviors depending on the species and strains, growth conditions, type of pesticide, and contaminant concentrations; therefore, it is useful to evaluate the effect of these factors on model organisms such as *Azotobacter vinelandii* (Noar and Bruno-Bárcena, 2018); *A. vinelandii* is a strictly aerobic free-living bacterium with growth and metabolite production, both *in vitro* and in soil, closely related to physicochemical parameters (Lenart 2012; Plunkett et al. 2020), nutrient concentration and availability (essentially carbon and nitrogen sources) (Tejera et al. 2005; Then et al. 2016), microbial interactions (Bhosale et al. 2013), exposure to toxic substances (Chennappa et al. 2019) and oxygenation levels (Peña et al. 2007; Castillo et al. 2013), the latter being one of the critical parameters because of the high oxygen rate consumption of *Azotobacter* spp. On this regard, some aspects of the respiration in *A. vinelandii* have been evaluated widely concerning its growth and polymers synthesis (Lozano et al. 2011; Castillo et al. 2020). Culture factors such as the oxygen transfer rate (OTR) and respiratory quotient

(RQ) are crucial in describing the physiological state under different growth conditions. They are related to parameters such as the specific growth rate and metabolite production (Gómez-Pazarín et al. 2015). Additionally, it can be useful for monitoring degradation processes (Kahraman and Altin 2020). However, information on the effects of pesticides on the growth and respirometric profile of *A. vinelandii* is scarce.

On the other hand, the use of *A. vinelandii* and other PGPR as inoculants requires the evaluation of growth conditions, standardization of formulations, increases in their viability (Nagaraja et al. 2016; Strobel et al. 2018), and the validation of the production and inoculation methods for possible large-scale implementation (Rani et al. 2019b). The use of strategies that provide protection and prolong the viability of microorganisms under varying conditions, including severe pesticide stress, has been suggested (Lenart 2012; Barman et al. 2019; Kumar et al. 2019; Sarker et al. 2021).

Currently, inoculation of microorganisms, either for agricultural or environmental purposes, employs liquid formulations that have limited lifetimes, low effectiveness, and high transportation costs (Nakkeeran et al. 2005; Bashan et al. 2014; Aasfar et al. 2021). To reduce those drawbacks, methods like encapsulation, which is characterized by trapping live, metabolically active microorganisms in a polymeric matrix have been proposed (Mustapha et al. 2018). Encapsulation of bacteria in non-toxic polymers such as alginate, naturally produced by *A. vinelandii*, is an alternative to inoculant formulations with potential applications in pesticide degradation (Talwar and Ninnekar 2015; Nagaraja et al. 2016).

Although its use is generally experimental, encapsulation of bacteria in alginate wet macro-alginate beads (diameter >1mm) has shown to provide protection, increase in the viability and improve plant growth, compared to free organism inoculums (Young et al. 2006). In the presence of pesticides, encapsulation increases tolerance and removal rates by degrading bacteria (Krell et al. 2017; Conde-Avila et al. 2020). However, such technology still presents drawbacks for large-scale production and application.

Spray drying is a technique well established and scalable for obtaining dry formulations of viable microorganisms. It has been mainly employed for the microencapsulation of enzymes, lipids, and microorganisms in the industry (Strobel et al. 2018). However, in agri-environmental applications, it is necessary to optimize process conditions to improve yields and decrease the mortality of microorganisms due to heating and dehydration conditions during the process.

Although there are some studies focused on the role of *Azotobacter* species in tolerance and degradation of pesticides, the information about the effect of OP like CP in the *A. vinelandii* growth and respiratory activity is scarce, including the encapsulation methods. Therefore, the objective of this study was to evaluate the *in vitro* growth and chlorpyrifos-tolerance and degradation of *A. vinelandii* ATCC 12837, for its use as a microencapsulated inoculant. It was developed a strategy based on the modification of culture media and

aeration conditions to evaluate and increase the cellular concentration of *A. vinelandii* and also determine its CP tolerance and degradation; in addition, the treatment of microencapsulation by spray drying was evaluated.

2. MATERIALS AND METHODS

2.1 Microorganism

Experiments were carried out using *A. vinelandii* ATCC 12837. Cells were cryopreserved at −70°C in 40 % (w/w) glycerol solution and maintained by monthly subculture on Burk´s-sucrose (BS) agar slopes and stored at 4° C (Peña et al. 2011).

2.2 Preparation of inoculum

The inoculum was prepared as follows: *A. vinelandii* cells were grown at 29 °C in 250 mL Erlenmeyer flasks, containing 50 mL of BS medium for 24 h at 200 rpm. Flasks were incubated until they reached a biomass concentration of 1 g L^{-1} (measured by dry weight). The liquid culture was diluted at 10 % with a fresh BS liquid medium. This suspension was used as inoculum. Each flask was inoculated with 0.1 g L^{-1} of biomass.

2.3 Culture media

Four different media were used for *A. vinelandii* culture with the following composition (g L^{-1}): 1) BS: sucrose 20, yeast extract (Difco) 3, K_2HPO_4 0.66, KH_2PO_4 0.16, NaCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, $CaSO_4$ 0.05, $Na_2MoO_4 \cdot 2H_2O$ 0.0029, $FeSO_4 \cdot 7H_2O$ 0.027, MOPS [50 mmol]. 2) BS2: the same BS composition except by sucrose (2 g L^{-1}). 3) NBRC: Mannitol 5, yeast extract (Difco) 3, K_2HPO_4 0.7, KH_2PO_4 0.1, MgSO4·$7H_2O$ 1, MOPS [50 mmol]. 4) NBRCm: the same composition except by mannitol (21.3 g L^{-1}). The initial pH was adjusted to 7.2 using NaOH 2N solution. To avoid precipitation during autoclaving, the $FeSO_4 \cdot 7H2O$ and $Na_2MoO_4 \cdot 2H_2O$ solutions were separated from the other medium components during sterilization (121 °C, 35 min). The C: N ratio (g mol/g mol) of the BS, BS2; NBRCm, and NBRC media were 29, 5.9, 29, and 21, respectively.

2.4 Culture conditions

Cultures were carried out in 250 mL Erlenmeyer flasks at 200 rpm and maintained at 29 °C for 72 h in an orbital incubator with a shaking diameter of 2.5 cm. In addition to the flasks used for online measurements of respiration activity, cultures were developed in some parallel flasks, three of which were regularly withdrawn (every 6, 12, or 24 h) and submitted to off-line analytical measurements. Cells of *A. vinelandii* were grown in 250 mL Erlenmeyer flasks containing 50 mL of BS, NBRC, and NBRCm media and the culture conditions previously described. The effect of different aeration conditions was evaluated by growing the cells of *A. vinelandii* in 250 mL Erlenmeyer flasks at different filling volumes, containing 10, 20, and 50 mL of BS medium

and cultivated as previously described. In order to evaluate the CP effect, cultures were carried out in 250 mL Erlenmeyer flasks containing 50 mL of BS and BS2 culture media with 0, and 500 ppm of technical grade CP (Clorver® 480 EC from Versa Agrochemicals) and cultivated under the conditions previously described. Uninoculated media with the same concentration of CP were used as a control.

2.5 Measurements of respiration activity

Oxygen transfer rate (OTR) and respiratory quotient (RQ) were determined by a respiration activity monitoring system (RAMOS) (Anderlei and Büchs 2001). During the measuring phase, this device measures the decrease of oxygen partial pressure in the gas phase of closed 250 mL flasks with a sensor mounted in the neck of each flask. From the slope of the oxygen partial pressure curve, the system calculates the OTR (Gomez-Pazarín et al. 2015). RQ was estimated from the quotient between the molar ratio of cumulative CO_2 production to cumulative O_2 utilization (Anderlei et al. 2004). The specific oxygen uptake rate (qO_2) was obtained from the quotient between the OTR_{max} value and the total protein content as previously described by Díaz-Barrera et al. (2011; 2021).

2.6 Analytical determinations

Biomass and alginate concentrations were determined gravimetrically (Peña et al. 1997). The number of colonies forming units mL^{-1} (CFU) was estimated by plate count (Strobel et al. 2018). Sucrose was assayed for reducing power with DNS reagent (Miller 1959). Samples were previously hydrolyzed using *β*-fructofuranosidase as described by Peña et al. (2011). The protein concentration was determined by the Lowry method using bovine serum albumin as standard (Lowry 1951).

All experiments were carried out by triplicate, and the results presented are the averages of independent samples. When needed, figures and tables show the mean values and standard deviations among replicates. Statistical analysis was carried out using an ANOVA with a multiple comparison Tuckey test (alpha<0.05).

2.7 Determination of chlorpyrifos (CP) and 3,5,6-trichloro pyridine-2-phenol (TCP)

The extracts resulting from the CP experiments described above (subsection 2.4. culture conditions) were evaluated to identify and quantify CP and its main metabolites (3,5,6-trichloro pyridine-2-phenol (TCP), Diethyl Phosphorothioate (DETP) and chlorpyrifos oxon). The samples were filtered through a 25 mm and a 0.22-µm PVDF membrane and then were diluted 100 and 1000 times with mobile phase prior to CP and TCP detection.

Each standard (Chlorpyrifos 99.5 % N-11459 ChemService, Inc; 3,5,6-trichloro-2-piridinol (TCP) 99.5 % (33972-BCBZ8746) Sigma Aldrich) and sample were automatically injected through a Sample-Manager system–FTN Acquity of Waters to equipment of Ultra Performance Liquid Chromatography (UPLC Acquity

Serie H) equipped with a column Brand Waters Acquity UPLC BEH C18 1.7µm, 2.1 x 50 mm, in a volume of 5.0 µL. The column temperature was kept at 40 °C. The chromatographic conditions were as follows: The mobile phase A was ammonium formate 5 mM, pH 3.0, and mobile phase B was methanol + ammonium formate 5 mM + 0.1 % of formic acid at a constant flow rate of 0.35 mL min^{-1}, with the following gradient: starting with 83 % of solvent A and 17 % of solvent B, reaching the 90% of solvent B at 5.5 min and remaining there for 2 min and returning to its first constitution at 7.51 min and remaining there for 2.5 min. With a total running time of 10 minutes. The autosampler injection needle was rinsed with a mobile phase after each injection. Nitrogen was used as the desolvation gas at a flow rate of 1000 Lh^{-1}. The desolvation temperature was 600 °C and the source temperature was 150 °C. Argon was used as the collision gas at a flow rate of 0.14 mL min^{-1}.

The identification and quantification were performed by means of ESI^+ (CP) and ESI^- (TCP) mode in a Mass Spectrometer Xevo TQ-S of Waters and workstation MassLynx. Ions were monitored using Multiple Reaction Monitoring (MRM) (Table 1).

Table 1. Tandem MS conditions.

Analyte	Parent m/z	Daughter m/z	Dwell (s)	Cone (V)	Collision (V)
Chlorpyrifos	349.90	97.00	0.115	27	32
		198.00	0.115	27	20
TCP	196.00	196.00	0.200	30	5
	198.00	198.00	0.200	30	5
	200.00	200.00	0.200	30	5

2.8 Mathematical analysis

The specific growth rate (μ) was calculated considering the growth from 0 h to 12 h of cultivation, the period at which the culture was growing exponentially. The equation used was: $dX/dt=\mu X$; where μ is the specific growth rate (h^{-1}) and X is the cell concentration (g L^{-1}) (Klimek and Ollis 1980). The percentage of degradation and the time in which the pesticide concentration was reduced by 50 % (DT_{50} values) was calculated (Abraham and Silambarasan 2016). The CP concentration profiles in each of the experiments were fitted to a pseudo-first-order degradation equation Ct=C0*e-kt where Ct is the concentration of the component at time t, C0 is the initial concentration, k is the degradation constant, and t is the time.

2.9 Microencapsulation by spray-drying

Azotobacter vinelandii alginate microcapsules were prepared by spray drying using Bowen Engineering BE-1448 equipment. A mixed suspension of 2.0 % (w/w) Na-alginate and *A. vinelandii* cells obtained by centrifugation from liquid cultures were prepared in 2 L Erlenmeyer flasks with 600 mL of BS medium. The mixture (6 L) was pumped into the dryer under identical operating conditions: the inlet air temperature was set at 130 °C and an inlet flow rate of 160 mL min^{-1}. Under these conditions, outlet temperatures ranged from 60 to 69 °C. Spray-dried powders were stored at room temperature in clear glass vials with exposure to ambient light for viability determinations.

3. RESULTS

3.1 *In vitro* growth

3.1.1 Growth and respirometric activity of A. vinelandii under different culture media

The respiratory activity parameters of *A. vinelandii* developed in NBRC, NBRCm, and BS media are shown in Fig. 1. Both the OTR and RQ profiles were different depending on the amount and type of carbon source present in the different media evaluated. In the three evaluated media, there were notable differences during cultivation time and presented characteristic profiles of oxygen limiting conditions distinguished by a higher sustained OTR value during the cell growth period (OTR_{max}). For the NBRC medium with mannitol as carbon source and a C:N ratio 21 (Fig.1, a), variations in OTR were observed at the begging of cultivation until reaching an average OTR_{max} of 2.65 mmol L^{-1} h^{-1} between 24 h and 55 h of culture. With the NBRCm medium (Fig.1, b), increasing the mannitol content (ratio C: N 29), the OTR_{max} increased at 5.87 mmol L^{-1} h^{-1} as expected because of the increase of the carbon source, indicating a high respiration activity and extending the culture time up to 80 h. Finally, in the BS medium (with sucrose as carbon source and a C: N ratio of 29) (Fig.1, c), an exponential increase in OTR was observed from 0 to 12 h, reaching an average OTR_{max} of 5.52 mmol L^{-1} h^{-1} until 36 h, when it decreased and then increased again until 48 h. Concerning the RQ values, in the NBRCm and BS media, similar values were obtained, both above 1 (1.2 and 1.1, respectively) indicating a less oxidative metabolic activity; whereas, the medium with a lower carbon concentration (NBRC) reached an average RQ of 0.7.

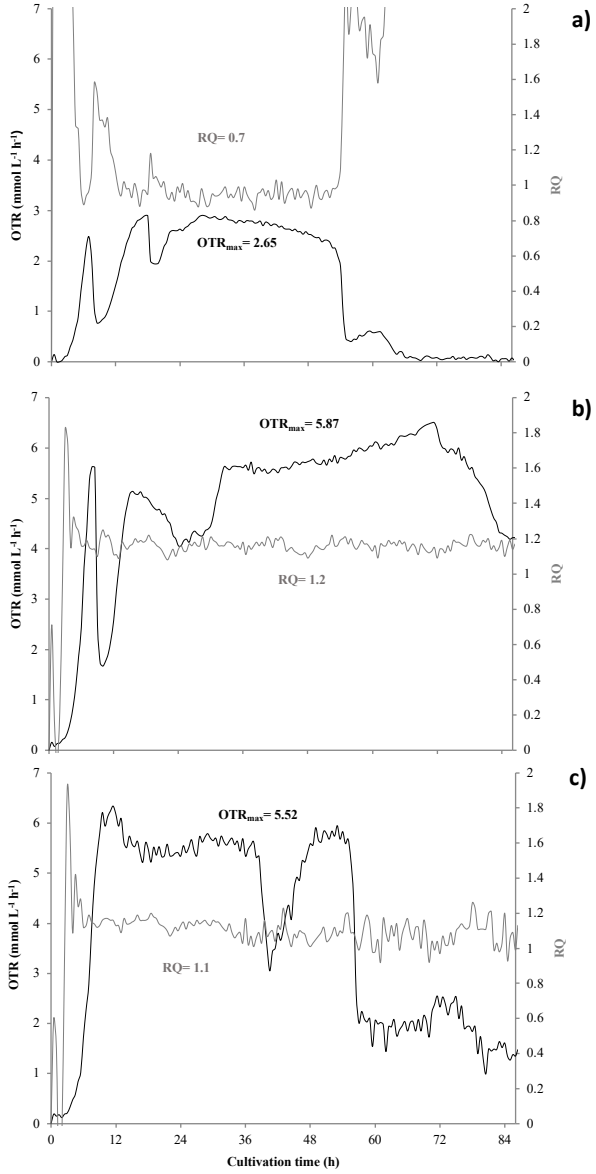


Fig. 1. Evolution of the oxygen transfer rate (OTR) and respiration quotient (RQ) in cultures of *A. vinelandii* grown in NBRC (a), NBRCm (b), and BS (c) media.

The growth of *A. vinelandii* determined by CFU and total protein in the different culture media is shown in Fig. 2. The maximum values of CFU mL^{-1} were 1.4 x10^{10} at 48 h in the BS medium, followed by NBRCm medium (9.16 x10^{9}) and NBRC (1.25 x10^{9}) at 72 h (Fig. 2 a). Similarly, total protein content (Fig. 2 b) increased exponentially up to 48 h and it was higher in BS medium compared to NBRCm medium using the same C: N ratio (29); whereas, the cultures where the NBRC medium (low carbon concentration) was employed showed notably lower growth.

Finally, the kinetic and respirometric parameters of *A. vinelandii* culture on the different media are summarized in Table 2. It is clear from the values of the table that a higher μ, number of CFU, and protein content were reached in the cultures with the BS medium when the OTR_{max} was 5.52 mmol L^{-1} h^{-1}. It is important to point out that the highest alginate content was obtained in the NBRCm medium, indicating that with mannitol as a carbon source, compared to sucrose medium (BS), the alginate synthesis was improved (5.17 g L^{-1}); whereas in BS medium, the alginate production was lower (0.97 g L^{-1}alginate).

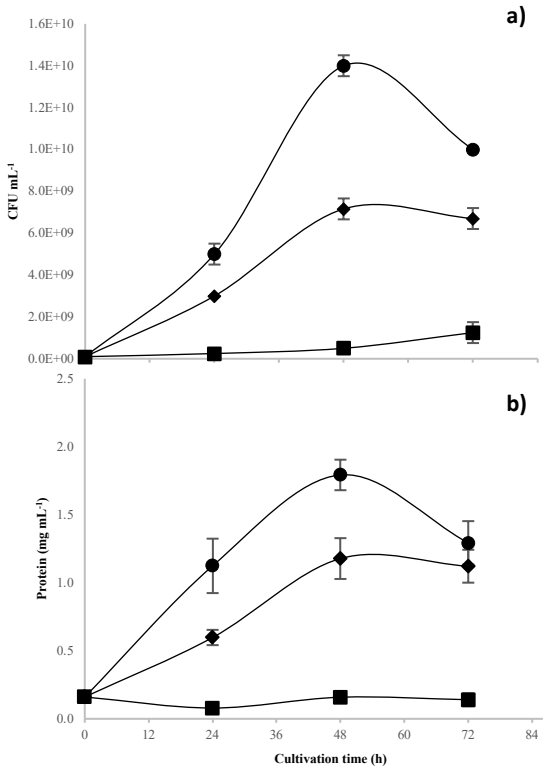


Figure 2. Growth of *A. vinelandii* in different culture media. CFU (a) and protein growth (b) of *A. vinelandii* cultures in shake flasks in BS (•), NBRCm (♦), and NBRC (■) medium. Data are presented as the mean and standard deviation from three experiments.

Table 2. Kinetic and respirometric parameters of *A. vinelandii* cultured in shake flasks in different culture media. Experiments were carried out in triplicate and the results presented are the means and standard deviation of independent runs.

Culture medium	Specific growth rate (μ)	Duplication time (h)	CFU mL^{-1}	Protein ($mg\ mL^{-1}$)	OTR_{max} ($mmol\ L^{-1}\ h^{-1}$)	qO_2 ($mmol\ /mg\ mL^{-1}\ h^{-1}$)	Final alginate gL^{-1}
NBRC	0.03	9.45	$1.25x10^9$	0.14 ± 0.01	2.65	18.92	0.49 ± 0.03
NBRCm	0.08	6.30	$9.16x10^9$	1.12 ± 0.06	5.87	5.24	5.17 ± 0.56
BS	0.12	5.55	$1.4x10^{10}$	1.79 ± 0.02	5.52	3.08	0.97 ± 0.12

3.1.2 Growth and respirometric activity of A. vinelandii under oxygen and non-oxygen-limited conditions

Figure 3 shows the respiratory activity parameters of *A. vinelandii* developed in BS medium with different filling volumes 10 (a), 20 (b), and 50 mL (c). As it was expected, the OTR_{max} values increased considerably, decreasing the filling volume, obtaining values of 5.5, 11.45, and 22 mmol $L^{-1}\ h^{-1}$ with 50, 20, and 10 mL, respectively. With 50 and 20 mL, a typical oxygen limitation OTR profile was obtained for *A. vinelandii* cells.

On the other hand, in the cultures with 10 mL of filling volume, a typical non-oxygen-limited profile was observed. In that case, the OTR_{max} was reached at 20 h of culture, followed by a drop in the respiration rate, indicating the decrease in oxidative activity due to the rapid depletion of the sucrose. In the case of the cultures using 20 mL of filling volume, the same drop was presented at 20 h but increased again from 20 to 27 h. Finally, with 50 mL of filling volume, a previously described oxygen limitation profile was exhibited, showing an exponential increase in OTR during the first 6 h of culture and a stationary stage that remained until the carbon source in the medium was exhausted, prolonging the culture until 55 h. In contrast, the RQ was not modified by the filling volume, being in all cases RQ of 1.02-1.07.

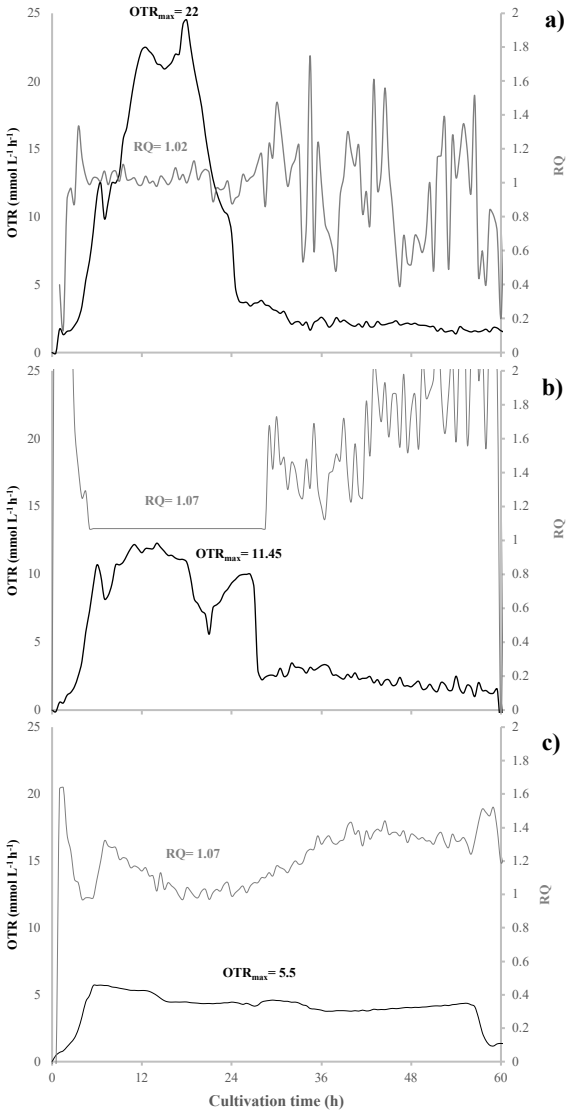


Fig. 3. Evolution of the oxygen transfer rate (OTR) and respiration quotient (RQ) in cultures of *A. vinelandii* grown in BS liquid medium with 10 (a), 20 (b), and 50 mL (c) of filling volumes.

As it is presented in Figure 4, the maximum growth values determined by the parameters of maximum biomass (9.7 gL^{-1}), CFU mL^{-1} ($1x10^{10}$), and total protein (2.78 mg mL^{-1}) were obtained in the lowest OTR condition, i.e., in the BS medium with 50 mL filling volume between 48 to 72h of culture. Similarly, the kinetic parameters (Table 3) with that condition showed a higher growth rate of 0.12 h^{-1} and thus a shorter doubling time (5.7 h) even under O_2-limiting conditions (5.5 mmol L^{-1} h^{-1}). It is clear that, the growth of strain ATCC 12837 in BS medium under limited oxygenation conditions did not significantly affect growth, and also presented better cell viability, protein content, and lower qO_2.

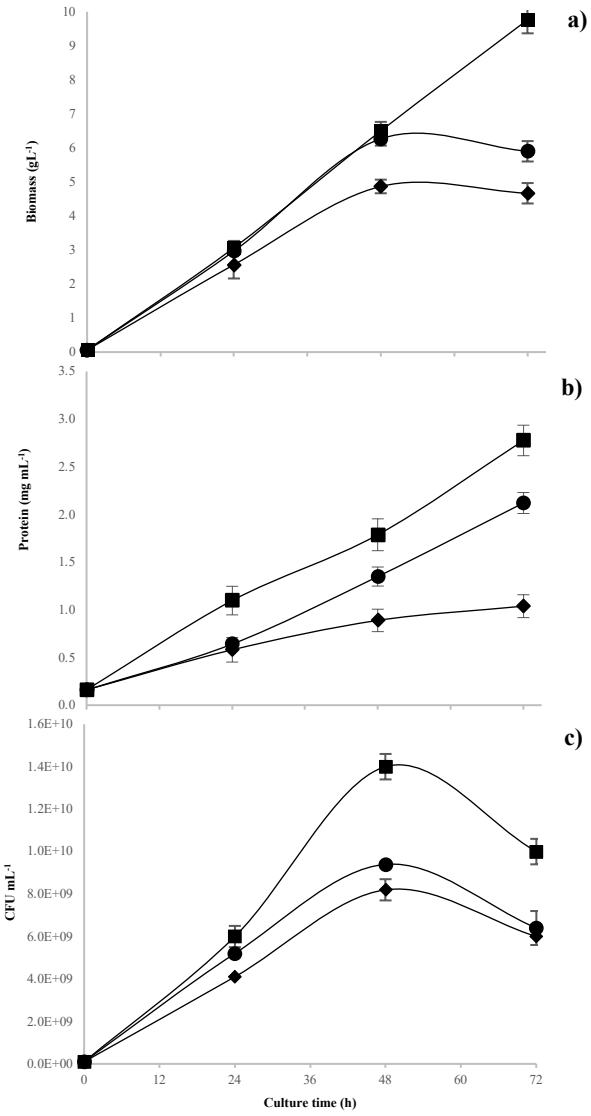


Figure 4. Biomass (a), Protein (b), and CFU growth kinetics (c) of *A. vinelandii* cultures in 250 mL shake flasks in BS medium at different filling volumes 10 mL (•), 20 mL (♦) and 50 mL (■). Data are presented as the mean and standard deviation from three experiments.

Table 3. Kinetic and respirometric parameters of *A. vinelandii* cultured in shake flasks with BS medium at different filling volumes. Experiments were carried out in triplicate and the results presented are the means and standard deviations of independent runs.

Filling volume (mL)	Specific growth rate (μ)	Duplication time (h)	Biomass ($g\ L^{-1}$)	Protein ($mg\ mL^{-1}$)	CFU mL^{-1}	OTR_{max} ($mmol\ L^{-1}\ h^{-1}$)	qO_{2max} ($mmol\ /mg\ mL^{-1}\ h^{-1}$)
50	0.12	5.77	9.77 ± 0.65	2.78 ± 0.26	$1x10^{10}$	5. 5	1.97
20	0.08	6.30	4.67 ± 0.18	1.04 ±0.28	$8.2x10^{8}$	11.45	11
10	0.11	8.66	5.90 ± 0.95	2.12 ±0.05	$9.4x10^{9}$	22	10.37

3.1.3 Growth and respirometric activity of A. vinelandii in media with chlorpyrifos

The respiratory activity parameters of *A. vinelandii* developed in BS and BS2 media with and without CP are shown in Fig. 5. In the first case, when the bacterium was cultured without a decrease in the carbon source (sucrose) in both BS medium without the contaminant (a) and BS with CP (b), clear differences at OTR in the first 12 h of culture were observed. Particularly in the BS medium with CP (Figure 5b), the increase in the OTR until reaching the maximal was slower compared to the medium without pesticide (BS) (Figure 5a) in which during the first 6h the maximum OTR was reached. However, the OTR_{max} values were higher in the medium with CP (7.9 mmol L^{-1} h^{-1}) in contrast to the BS medium (5.88 mmol L^{-1} h^{-1}), and a prolongation of the respiratory activity up to more than 60h of culture was observed, suggesting a higher metabolic activity. The average RQ values for the medium with CP were slightly lower (1.07) compared to the BS medium (1.12), in both cases greater than 1. In the second case, by decreasing the concentration of the carbon source (BS2) 10-fold and with the addition of the pesticide (B2+CP) (Fig. 5, c and d) a similar behavior to the previous one was obtained in terms of the increase in OTR_{max} for the medium with pesticide (6.9 mmol L^{-1} h^{-1}) compared to the BS2 medium (5.38 mmol L^{-1} h^{-1}) and a delayed activity in the increase of OTR in the first hours of culture, possibly linked to the adaptation of the bacteria to the presence of the contaminant. Finally, the RQ values for BS2 and BS2+CP media were 0.90 and 0.89, respectively.

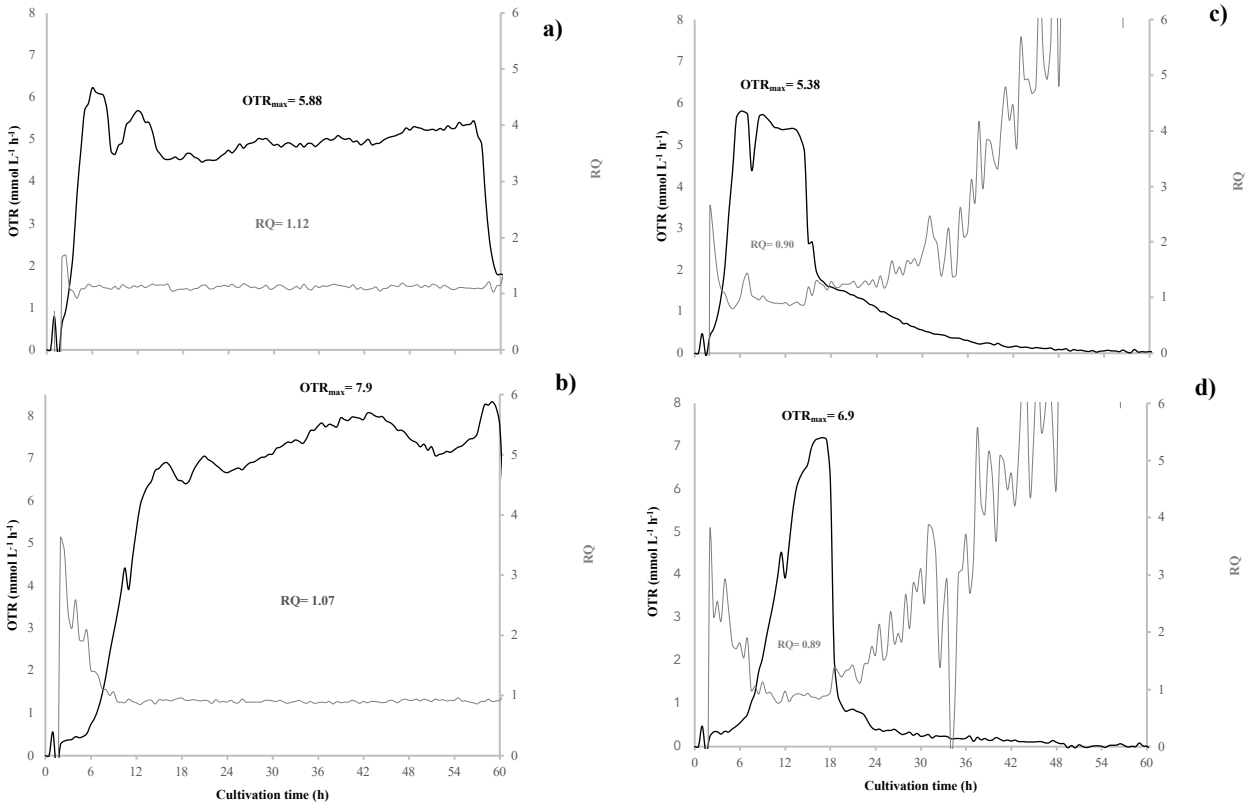


Fig. 5. Evolution of the oxygen transfer rate (OTR) and respiration quotient (RQ) in cultures of *A. vinelandii* grown in media: BS (a), BS+500 ppm CP (b), BS2 (c), and BS2+500 ppm CP (d).

Regarding the growth of *A. vinelandii* in BS medium with and without CP, Fig. 6 shows the biomass (a), protein (b), and CFU (c) content, as well as sucrose consumption of all treatments. The BS+CP and BS2+CP media obtained higher biomass production in relation to the controls without pesticide. The media with 20 gL^{-1} sucrose showed exponential growth until 60h of culture, while with 2 gL^{-1} sucrose, the exponential phase ended at 12h of culture. Similarly, the higher total protein content and CFU mL^{-1} were recorded in the media with CP at 60 and 24 h for BS+CP and BS2+CP media, respectively.

On the other hand, sucrose consumption (d) was slightly faster in the media without pesticide compared to the media with CP, indicating the use of alternative sources present in the medium with pesticide. Finally, the kinetic parameters showed a growth rate without statistical differences for the media with pesticide and their respective controls, as well as a lower qO_2 in the BS+CP medium (Table 4).

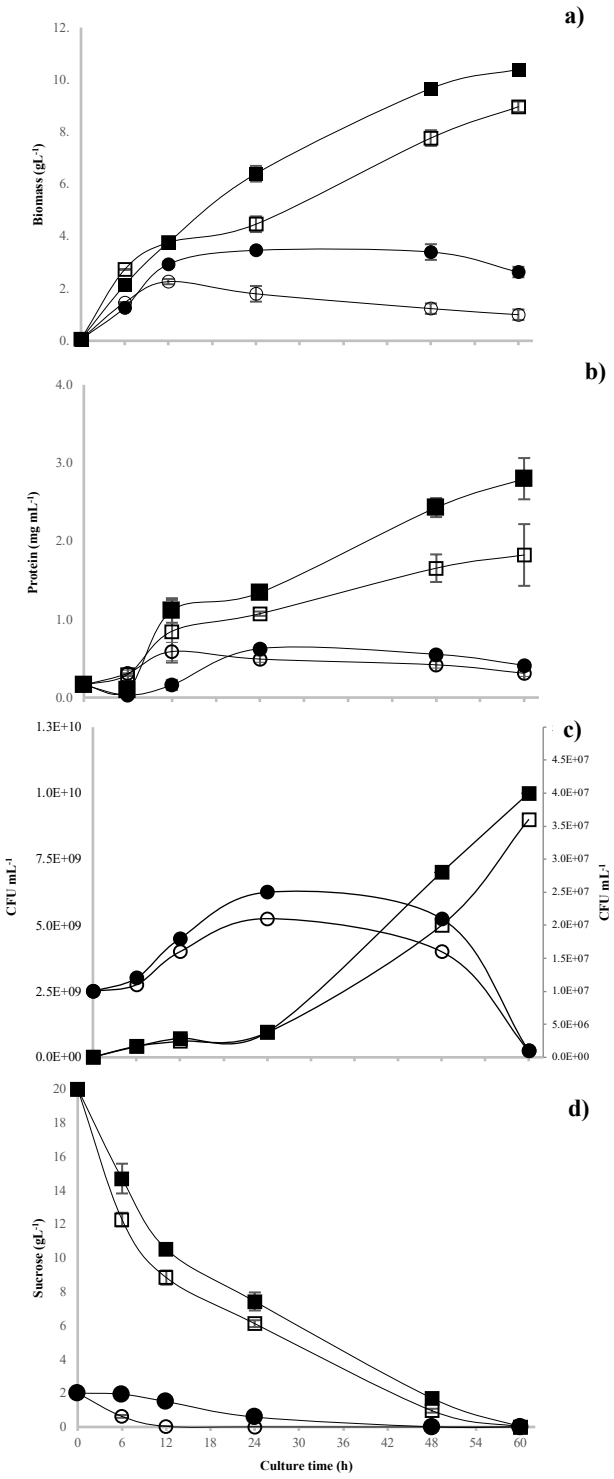


Figure 6. Biomass (a), Protein (b), CFU (c), and Sucrose consumption (d) of *A. vinelandii* growth in BS (☐), BS+CP (■), BS2 (○) and BS2+CP (•). Data are presented as the mean and standard deviation from three experiments.

Table 4. Kinetic and respirometric parameters of *A. vinelandii* cultured in shake flasks in BS and BS with 0 and 500 ppm of chlorpyrifos (CP). Experiments were carried out in triplicate and the results presented are the means and standard deviation of independent runs.

Medium	Specific growth rate (μ)	Biomass ($g\ L^{-1}$)	Protein ($mg\ mL^{-1}$)	CFU mL^{-1}	OTR_{max} ($mmol\ L^{-1}\ h^{-1}$)	qO_2 ($mmol\ /mg\ mL^{-1}\ h^{-1}$)
BS	0.26	8.9 ± 0.1	1.82 ± 0.21	9×10^9	5.88	2.66
BS+CP	0.26	10.4 ± 0.05	3.32 ± 0.18	1×10^{10}	7.9	2.37
BS2	0.14	2.27 ± 0.2	0.59 ± 0.06	2.1×10^7	5.38	9.11
BS2+CP	0.19	2.93 ± 0.02	0.63 ± 0.04	2.51×10^7	6.9	10.9

3.1.4 Tolerance and biodegradation of chlorpyrifos

The tolerance and biodegradation of CP and its major metabolite TCP were assessed using *A. vinelandii* ATCC 12837 in liquid culture. Although the ATCC 12837 strain was exposed to a concentration of 500 ppm of CP, this demonstrated not only tolerance to the compound but also increased growth and respirometric activity as described above in both BS and BS2 media (section 3.1.3).

According to the analysis of detection and quantification of CP and its intermediates in the supernatants of *A. vinelandii* cultures, both in BS and BS2 medium, a decrease in the concentration of the pesticide was determined in the medium (Fig. 7). Strain ATCC 12837 completely degraded the pesticide (500 mg L^{-1} of CP) with a DT_{50} of 6 h with BS medium. Whereas in the BS2 medium, when sucrose was reduced, the strain degraded 330 mg L^{-1} of CP and the time to reach DT_{50} was 30 h. The above, clearly was influenced by the decrease in growth in response to the reduction of sucrose concentration in the BS2 medium. Therefore, the degradation percentages of the strain after 60 h of culture were 99.5 % and 66.8 % in BS and BS2 media, respectively.

According to our results, no accumulation of TCP or formation of other metabolites (DETP or chlorpyrifos oxon) was detected in *A. vinelandii* growth supernatants with 500 ppm CP. This suggests that, under the

conditions tested, the bacteria apparently can metabolize CP and use it for growth and energy. In addition, a higher percentage of degradation was observed when grown on a nutrient-rich medium (BS medium).

Given that in our screening analysis of CP and TCP by UPLC/MS-MS no other intermediate compounds were detectable during *in vitro* culture development, we show a possible degradation pathway that *A. vinelandii* could follow (Bose et al. 2021) (Fig. 8). The hydrolysis of CP to TCP followed by reductive dechlorination of TCP and incorporation of the pyridine ring into the Krebs cycle which completes the degradation of CP; or the formation of DETP which is rapidly degraded to ethanol and phosphorothioic acid molecules and can be used as S, N, and P sources.

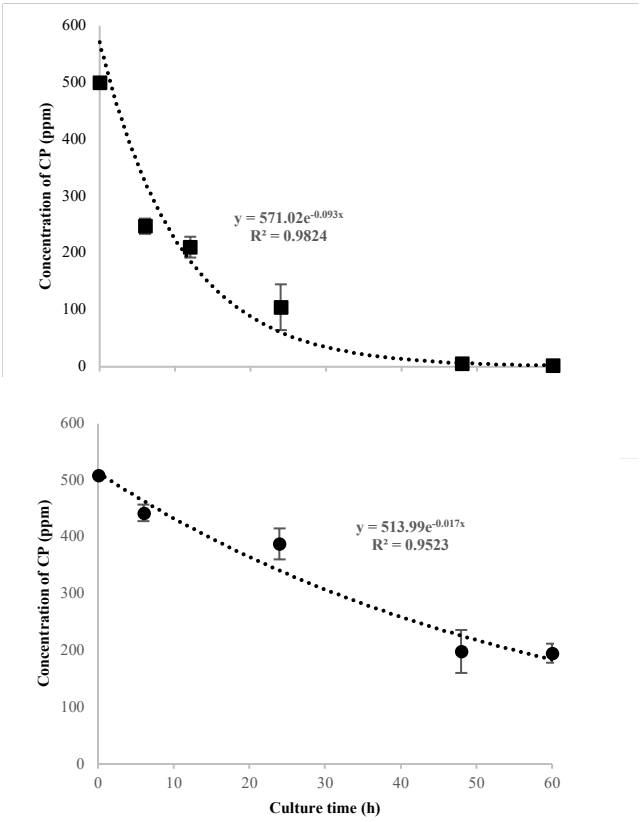


Figure 7. Chlorpyrifos degradation by *A. vinelandii* growth in BS+CP (■) and BS2+CP (•).

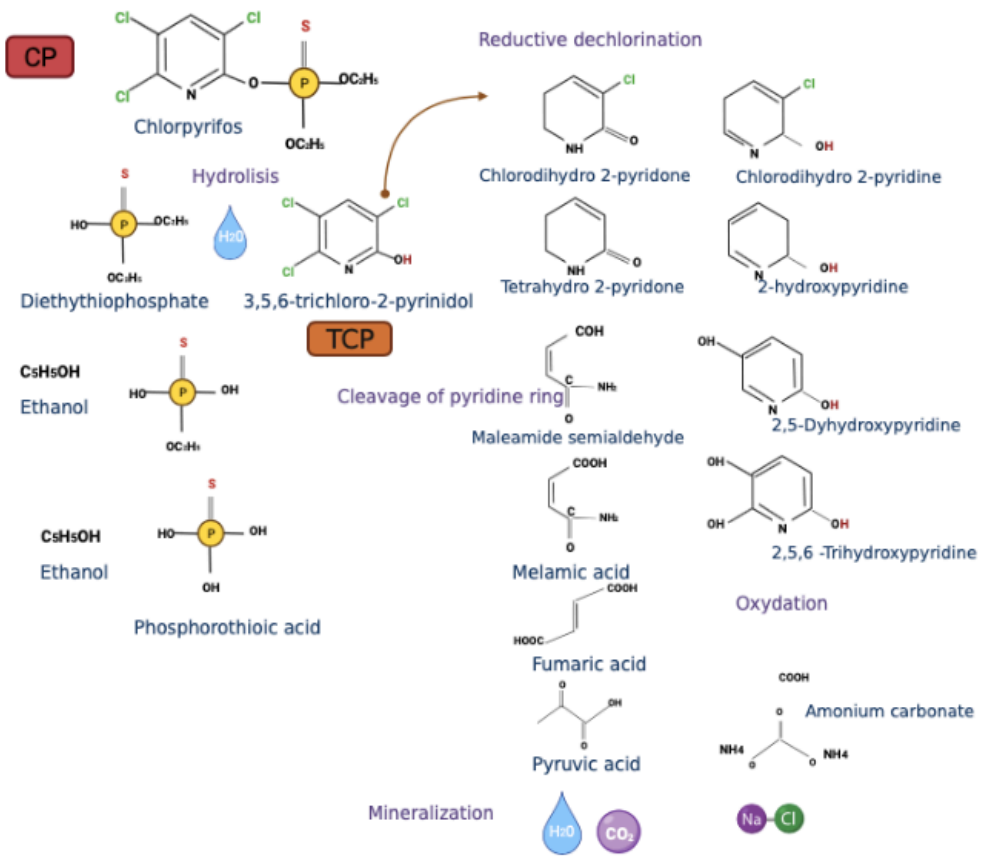


Fig. 8 Chlorpyrifos biodegradation pathway (modified of Vijayalakshmi and Usha 2012)

3.1.5. Microencapsulation by spray-drying, and powder A. vinelandii viability

In order to propose a powdered inoculant formulation with potential agro-environmental applications under an easily scalable method, microencapsulation of *A. vinelandii* with Na-alginate was carried out. The viability of *A. vinelandii* was monitored during the microencapsulation process. The microbial population was calculated in the shake flask cultures, in the input suspension containing the cell pellet dispersed in Na-alginate solution, and in the spray-dried powder. The shake flask cultures grew to approximately 5.2×10^9 CFU mL^{-1}, and the microbial population was not significantly reduced when the cell pellet was resuspended in the same volume of alginate solution (5×10^9 CFU mL^{-1}).

The resulting viability of the spray-dried cells was 4×10^9 CFU g^{-1}, which represents a 20 % reduction within the same order of magnitude relative to the population in the spray-dryer input suspensions (5×10^9 CFU mL^{-1}). Spray drying under the conditions evaluated did not compromise the survival of the microencapsulated

bacteria because *A. vinelandii* cells in solution with sodium alginate survived the drying process with a well viable count and the advantage of avoiding alginate cross-linking at some stage.

4. DISCUSSIONS

4.1 Growth and respirometric activity of A. vinelandii in different culture media

Regarding to the respiratory activity parameters of *A. vinelandii* grown under different culture media, the OTR and RQ were dependent on the amount and type of carbon source available (Fig. 1), a similar behavior that has been observed previously (Noguez et al. 2008). This is explained because both parameters are substrate-dependent (Kahraman and Altin et al. 2020). Factors such as oxygen availability and the amount or type of carbon source modify the metabolic response of aerobic organisms to oxidize compounds and produce CO_2 which can be monitored by OTR and RQ values (Gomez-Pazarín et al 2015).

In this context, characteristic profiles of oxygen limiting conditions were observed in the three evaluated media, similar to those previously reported for *A. vinelandii* (Peña et al. 2011). Those profiles are characterized by maintaining a sustained OTR_{max} value during the culture time and RQ values of 1 or higher, as those obtained in the NBRCm and BS media. RQ values higher than 1 are generally attributed to anaerobic or microaerophilic conditions, where the oxygen availability is not sufficient to oxidize the carbon source present in the media, whereas, values below 1 are related to aerobic processes (Dilly 2001, 2003; Lamy et al. 2013).

In our results, the OTR_{max} values and the growth of *A. vinelandii* determined by CFU and total protein in the different culture media were higher when C:N ratio of 29 was used (Fig. 2). It is known that high concentrations of organic carbon in the form of sugars, alcohols, and organic acids (25%) are used to improve the growth of *Azotobacter* (Tejera et al. 2005). In contrast, Castillo et al. (2017) found that using ratios between16 and 32 gC gN^{-1}, there were no significant differences in the growth of *A. vinelandii* when using sucrose and yeast extract as carbon and nitrogen sources, respectively. In our results with *A. vinelandii* ATCC 12837, the type of C source and the increase of the C:N ratio, positively impacted the cell growth, viability, and respirometric parameters as expected, even in oxygen limiting conditions.

In this line, recently Díaz-Barrera et al. (2021) reported that, under oxygen limitation conditions (5.0 ± 0.9 mmol L^{-1} h^{-1}) and no-nitrogen fixation, similar to those carried out in our work, *A. vinelandii* ATCC 9046 channeled the carbon source mainly to the production of biomass and intracellular polymers like PHB.

The above is consistent with that reported by Peña et al. (2007; 2011), who obtained higher viability, biomass production, and specific growth rate with the increase in the OTR in cultures in shaken flasks (OTR_{max} 6 mmol L^{-1} h^{-1} compared to an OTR_{max} of 2.5 mmol L^{-1} h^{-1}). Also, when *A. vinelandii* was grown at OTR_{max} of 5.5 mmol L^{-1} h^{-1}, (similar to that obtained in our study for BS and NBRCm medium) the carbon source was

mainly directed to growth with an increase in the biomass concentration, polymer and CO_2 production, which may be affected by the strain qO_2 (Diaz-Barrera et al. 2011). That is coherent with our results because a lower qO_2 was obtained in the BS medium, the same with the higher growth and respirometric activity.

4.2 Growth and respirometric activity of A. vinelandii under oxygen and non-oxygen-limited conditions

As it was expected, the OTR_{max} values in the cultures of *A. vinelandii* in BS medium increased considerably by decreasing the filling volume (Fig. 3), obtaining a typical oxygen limitation and non-oxygen limitation profiles as previously reported in cultures of *A. vinelandii* in stirrer tank and shaken flasks (Peña et al. 2007; Díaz-Barrera et al. 2007; Moral et al. 2016). In contrast, the RQ values were not modified by the filling volume. These data contrast with those previously reported for *A. vinelandii* ATCC 9046 (Peña et al. 2011), where at higher OTR, RQ values are less than 1 and conversely at low OTR, higher than 1. This could be related to a lower respiration rate observed in the strain ATCC 12837 used in the present study. For the case of *A. vinelandii* ATCC 9046 strain, it has been documented that it possesses mechanisms that regulate its respiration efficiency depending on the modifications of its respiratory chain by the activation or deactivation of terminal oxidases. These oxidases respond to environmental and nutritional changes such as oxygen availability to maximize energy conservation or produce intracellular or extracellular polymers and it may vary slightly among strains (Castillo et al. 2020).

On the other hand, previous studies with *A. vinelandii* in shaken flasks, under high and low aeration conditions, showed that changes in oxygen availability had a considerable impact on the growth profiles (Castillo et al. 2013), especially on growth, measured as biomass, and protein production (Peña et al. 2011). In the study of García et al. (2018), the highest protein yield of *A. vinelandii* (0.15 g protein g glucose^{-1}) was obtained in the cultures developed under the lowest OTR (2.4 mmol L^{-1} h^{-1}). In our case, the maximum growth values were also obtained at the lowest OTR condition (Fig. 4). Other authors have reported that when OTR_{max} was reduced, the μ value also decreased, although without changes in the final biomass concentration (Peña et al. 2007; Peña et al. 2011; Díaz-Barrera et al. 2021). In our study, the growth of strain ATCC 12837 in BS medium under limited oxygenation conditions did not significantly affect growth, and also presented better cell viability, protein content, and lower qO_2. The last is a relevant characteristic to consider as a scale-up criterion, owed to the high requirements that characterize other strains like *A. vinelandii* ATCC 9046, and are usually a limiting condition for process scale-up.

4.3 Growth and respirometric activity of A. vinelandii in media with chlorpyrifos

Due to the typically high respiration rates of *A. vinelandii*, oxygen limitations and preferences for carbon sources usually occur in the early stages of fermentation (Peña et al. 2007). Besides, in the presence of toxic substances, a period of adaptation or reduced respiration rates could occur early in the culture (Chennappa

et al. 2013). In our case, we observed differences in OTR values in the cultures of *A. vinelandii* developed in BS and BS2 media with CP at the first 12 h of cultivation, in OTR_{max}, and the prolongation of respiratory activity, relative to their respective control conditions (Fig. 5).

It is important to point out that, this is the first time when OTR and RQ online values have been estimated for *A. vinelandii* in response to the presence of CP in a liquid medium with sucrose. The OTR_{max} values recorded were significantly higher in media with the pesticide, and prolongation of respiratory activity was observed in both conditions (BS and BS2). The above suggested an increase in metabolic activity related to the addition of the pesticide as a carbon source.

Regarding the RQ values, the average values were slightly lower by decreasing 10-fold the carbon concentration (BS2) with and without the addition of pesticide. The latter was related with the decrease of the carbon availability and presence of pesticide, which resulted in an oxygen non-limiting condition, where the lower metabolic activity and CO_2 production was reflected in the RQ value (Lamy et al. 2013). All the above was also then supported by growth parameters.

In the cultures of *A. vinelandii* in the presence of CP, the growth was better than when the pesticide was not used (Fig. 6). This suggests the use of CP as a carbon source by *A. vinelandii* ATCC 12837, since it not only tolerated the high concentration of the pesticide (500 ppm) but also had a significantly higher growth compared to the reference treatments, as well as a lower qO_2 in the BS+CP medium. This is consistent with other *Azotobacter* strains that did not show any *in vitro* growth impairment in the presence of CP (Chennappa et al. 2014a). On the other hand, slightly faster sucrose consumption in the media without pesticide, indicating the use of alternative sources present in the medium with CP.

Although other *Azotobacter* strains have shown tolerance to CP (Gurikar et al. 2016; Chennappa et al. 2019), this is the first time that respirometric parameters are measured and related to the growth of *A. vinelandii* in presence of a pesticide. In the present study, we highlight that strain ATCC 12837 growth in a high CP concentration in contrast with previously reported (100 ppm and 1-5 %) (Mac-Rae and Celo 1974; Chennappa et al. 2019); without adversely affecting its growth or respiratory activity. In contrast, according to Mac-Rae and Celo (1974), despite showing tolerance, the oxygen consumption rate of *A. vinelandii* was considerably reduced when using 100 ppm of OP (Naled, Terracur-P, coumaphos, malathion, CP). This could be related to the strain ATCC 12837 high tolerance to the pollutant, since decrease in its respiration activity is not observed as a result of exposure to CP.

4.4 Tolerance and biodegradation of chlorpyrifos

The tolerance and biodegradation of toxic compounds by *Azotobacter* spp. have not been fully addressed, especially concerning pesticides. Recently, it has been suggested that strains of this genus can show

tolerance to compounds such as CP, and even degrade it (Chennappa et al. 2014a; Chennappa et al. 2019). In the present study, even though the strain was exposed to a concentration of 500 ppm of CP, higher than those used in other reports for *Azotobacter* spp. and other genera (from 10 to 300 ppm) (Maya et al. 2011; Rayu et al. 2017; Akbar and Sultan et al. 2016; Liu et al. 2011: Yang et al. 2005; Abraham and Silambarasan 2016; Shi et al. 2019), the strain ATCC 12837 demonstrated not only tolerance to the compound, but also increased growth and respiration activity.

Commonly, some microorganisms can be tolerant to low concentrations of pesticides such as CP, thanks to primary protective mechanisms mediated by oxidative enzymes as cytochrome p450, peroxidases, and polyphenol oxidases (Abraham and Lambarasan 2018), but high CP concentrations could strongly affect the bacterial growth (Singh et al. 2011) and drastically decrease the number of tolerant organisms at concentrations above 100 ppm (Hernández-Ruíz et al. 2017).

In addition, one of the limiting factors in the complete degradation of CP is usually the generation of secondary metabolites such as TCP. TCP is the main degradation product of CP and tends to be resistant to biodegradation or bactericidal due to its composition, as it contains a pyridinol ring with 3 chlorine atoms (Jabeen et al. 2015). The above limits the number of organisms capable of fully mineralizing the compound (Abraham and Silambarasan 2016). Some strains, such as *Pseudomonas* sp. and *Bacillus megaterium*, have been able to degrade CP (100 mgL^{-1}) but not completely TCP (Barman et al. 2014: Zhu et al. 2019). This situation may be reflected with the accumulation of TCP and other intermediates, which prevents the complete elimination of the parent compound (Barman et al. 2014) and may allow further dissipation of contaminants, as e.g. TCP is more soluble than the parent molecule (John and Shaike 2015) and acts as an endocrine disruptor (Fishel 2013).

In our study, *A. vinelandii* tolerated, grew, and efficiently degrade a high CP concentration *in vitro*, both in BS and BS2 media, without the accumulation of TCP or formation of other metabolites (DETP or chlorpyrifos oxon). This suggests that, under the conditions tested, the bacteria can completely metabolize CP and use it for growth and energy. However, the mechanisms of CP degradation or the involvement of enzymes associated with its degradation (such as organophosphate hydrolases (Li et al. 2007; Barman et al.2014)) have not yet been fully elucidated or reported for *Azotobacter* spp.

One of the closest genera to *Azotobacter* that has shown efficiency in CP degradation is *Pseudomonas*, as it can use it as a carbon source and energy (Gilani et al. 2016); and it has been particularly documented in strains such as ATCC 700113 (Feng et al 1988). *Pseudomonas syringae* was able to degrade 99.1 % of 100 mg L^{-1} of CP in 5 d also presenting degradation-associated phosphoesterase enzymatic activity (Zhu et al. 2019). However, the initial concentration is important and another limiting factor in CP degradation. e.g., although *Pseudomonas* spp. can degrade CP, it decreases its growth or stops degrading TCP at

concentrations higher than 200 ppm (Li et al. 2007). In contrast, *A. vinelandii* ATTCC 12837 strain showed higher tolerance (500 ppm) and degradation efficiency compared to *Pseudomonas* spp.

On the other hand, *Pseudomonas putida* is among the most efficient strains in CP degradation (Gilani et al. 2016). Especially when it was developed under optimal growth conditions in glucose supplemented medium (Vijayalakshmi and Usha 2012). In the case of *A. vinelandii*, a higher percentage of degradation was observed when grown on a nutrient-rich medium (BS medium) (Fig. 7). This is consistent with what was also reported by Gilani et al. (2010), who point out that the degradation of CP in the presence of nutrients increases due to better cell growth by greater availability of easily metabolizable compounds, which allows the pesticide degradation in a co-substrate condition.

Furthermore, it has been described that, under neutral pH conditions, as our experiment was conducted, CP can be hydrolyzed and follow different biodegradation pathways; and under aerobic conditions, the breaking of the aromatic rings is favored (Jayasri et al. 2014).

Given that in our screening analysis and the fact that no other intermediate compounds were detectable during *in vitro* culture development, *A. vinelandii* could follow two possible degradation pathways (Fig. 8) (Bose et al. 2021). The hydrolysis of CP to TCP, followed by reductive dechlorination of TCP and incorporation of the pyridine ring into the Krebs cycle which completes the degradation of CP and this has also been identified in *Pseudomonas* (Vijayalakshmi and Usha 2012); or the formation of DETP which is rapidly degraded to ethanol and phosphorothioic acid molecules and can be used as a S, N, and P source for microorganisms (Rokade and Mali 2013).

Regarding efficiency, in our study, *A vinelandii* degraded CP 10 times faster (200 mg L^{-1} in 4.8 h in BS medium), compared to the bacterium *Cupriavidus nantogensis* (200 mg L^{-1} in 48 h) and similarly could tolerate up to 500 mg L^{-1} (Shi et al. 2019). On the other hand, the fungus *Cladosporium cladosporioides* degraded only 50 ppm of CP in 5 d and tolerated 500 mgL^{-1} as well, and although it generated TCP as an intermediate, it degraded rapidly without leading to accumulation; and similar to our results, they did not detect traces of compounds in chromatographic analysis (Chen et al. 2012).

The elimination of CP in the medium suggests that the strain utilizes the pesticide as a carbon source and energy efficiently compared to other strains in addition to being highly tolerant so it could maintain its activities as a PGPR. This has been previously described in *Azotobacter salinestris* which maintained the highest production of indoleacetic acid (auxin) on medium supplemented with 1 mg tryptophan and CP (1%), indicating that CP did not negatively affect its growth or phytohormone synthesis (Chennappa 2016).

It is worth noting that, decades of research on the effect of pesticides such as CP on the development of *Azotobacter* spp. generally reported growth impairment, respiratory inhibition, changes in oxygen

consumption rate, and no degradation (Mac-Rae and Celo 1974; Omar and Abd-Alla 1992). Recent studies have identified that certain strains have shown greater tolerance to different compounds, particularly to CP (Chennappa et al. 2014a; Farhan et al. 2021). This is attributable, according to some authors, to the fact that rhizospheric microorganisms that have been chronically exposed to pesticides have created resistance and accumulated adaptations to use them as a carbon source and energy (Roy et al. 2020); while, maintaining and even favoring their PGPR activities (Shahgoli and Ahangar 2014; Pant et al. 2016).

This allows that *A. vinelandii* ATTCC 12837 to be an excellent candidate to be used in CP remediation, both *in vitro* and *in situ*, since microorganisms that can degrade pesticides *in vitro* usually maintain this capacity in soil (Vidya Lakshmi et al. 2009); although considering a decrease in the speed and efficiency of degradation (Deng et al. 2015) due to multiple edaphoclimatic factors that may vary their behavior (Aasfar et al. 2021).

4.5. Microencapsulation by spray-drying, and powder A. vinelandii viability

The viability of *A. vinelandii* after the microencapsulation process by spray-drying without alginate cross-linking was $4x10^9$ CFU g^{-1}. It was achieved in the experimental conditions proposed, with a relatively low reduction (20 %) of the population in the spray-dryer input suspension. This resulted in a higher viability in contrast to other studies e.g., *Pseudomonas fluorescens-putida* which suffered a decrease of 2 logarithmic units in its viability after spray drying for microencapsulation with a methacrylic copolymer, modified starch, and ethyl cellulose (Amiet-Charpentier et al. 1998). In addition, the microencapsulated viable cells obtained in this report present advantages over liquid formulations (Albareda et al. 2008) and inoculants encapsulated in alginate by the conventional gelation method (Bashan et al. 2014). This study employed an easily scalable method, an easily handled dry formulation, and maintained an acceptable viable count compared to other reports (Amiet-Charpentier et al. 1998; Digat 1991; Strobel et al. 2016) and in contrast with the usual viability of A. vinelandii in soil (<10 4 UFC g-1 soil) (Kennedy et al. 2004).

Few reports in the literature have suggested the use of microencapsulation with alginate by spray drying for agricultural and environmental purposes (John et al. 2011). Similar to our results, *Enterobacter* cells microencapsulated with maltodextrin and alginate maintained their survival after spray drying, even when the polymer was also not cross-linked (Campos et al. 2014). In the present study, spray drying under the conditions evaluated did not compromise the survival of the microencapsulated bacteria, contrary to claims that it is an aggressive process for preparing viable formulations of agri-environmental inoculants (John et al. 2011; Schoebitz et al. 2013). This could be explained by the conditions tested in this report, the cell density achieved in the culture, the alginate protection, and the drying conditions, especially the outlet temperature (60 to 69°C) and the feed rate (160 mL min^{-1}). Regarding the latter, it has been reported that increases in the feeding rate improve viability due to the increased entry of cells subjected to rapid desiccation of the matrix at time zero of the process (John et al. 2011).

Finally, cross-linking of alginate to form gels is often an additional step during microencapsulation or taken inside the equipment to favor the formation of microcapsules to provide further protection (Strobel et al. 2016); but excessive crosslinking also reduces cell viability by limiting intermembrane interactions and diffusibility phenomena (Park and Chang 2000). In our case, *A. vinelandii* cells in solution with sodium alginate survived the drying process with a well viable count and the advantage of avoiding alginate cross-linking at some stage.

5. CONCLUSIONS

The excessive use of pesticides as CP is related to multiple environmental alterations. Degradation strategies using rhizospheric microorganisms that also favor the development of crops have become more interesting in the search for alternatives that contribute to the reduction of fertilizers and toxic agents. In this context, we proposed a cultivation strategy to evaluate the growth of *A. vinelandii* ATTC 12837 and degradation of CP; as well as the development of a microencapsulated formulation as an inoculant for agricultural and environmental purposes. Our strategy to optimize bacterial growth allowed us to confirm that sucrose as a carbon source favored the *in vitro* development of *A. vinelandii* ATCC 12837, as well as the degradation of CP. Furthermore, despite the high oxygen consumption rates that are often a limiting step for large-scale production of *Azotobacter* spp., oxygen-limiting conditions did not affect the growth of strain ATTC 12837. Also, this is the first time when online respirometric parameters have been estimated in response to the presence of CP for this bacterium. On the other hand, the results demonstrate that the model organism *A. vinelandii* ATTC 12837 (deeply studied as a PGPR), is also highly tolerant and efficiently degraded chlorpyrifos, without accumulation of toxic secondary metabolites, and with the potential to develop into a promising candidate for improving the productivity of crops in pesticide-contaminated soils.

Finally, microencapsulation of the bacteria with Na-alginate by spray drying did not compromise their viability and this was achieved without the need to implement an alginate cross-linking step. This report proposes the development of practical applications for *A. vinelandii* ATTC 12837 as a decontaminating agent. From a technological point of view, the above is very relevant since it would allow the generation of a formulation for large-scale inoculation.

6. ACKNOWLEDGMENTS

We thank the Instituto de Biotecnología-UNAM and the Laboratorio Nacional para la investigación en Inocuidad Alimentaria CIAD for the facilities provided for the development of the experimental work. The authors are grateful for the financial support obtained from CONACyT (277600) and DGAPA-UNAM (AG2002019). We thank Dr. Celia Flores for technical assistance.

7. DECLARATIONS

Competing Interests

There is no statement on conflict of interes

8. REFERENCES

Aasfar A, Bargaz A, Yaakoubi K, Hilali A, Bennis I, Zeroual Y, Meftah Kadmiri I (2021) Nitrogen Fixing *Azotobacter* Species as Potential Soil Biological Enhancers for Crop Nutrition and Yield Stability. Front Microbiol doi: 10.3389/fmicb.2021.628379

Abo-amer A, Abu-gharbia M, Soltan E, Abd El-Raheem W (2014) Isolation and Molecular Characterization of Heavy Metal-Resistant *Azotobacter chroococcum* from Agricultural Soil and Their Potential Application in Bioremediation. Geomicrobiol J 31:551-561. doi: 10.1080/01490451.2013.850561

Abraham J, Silambarasan S (2016) Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol using a novel bacterium *Ochrobactrum* sp. JAS2: A proposal of its metabolic pathway. Pestic Biochem Phys 126:13-21. doi: 10.1016/j.pestbp.2015.07.001

Abraham J, Silambarasan, S (2018) Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by fungal consortium isolated from paddy field soil. Environ Eng Manag J 17(3): 523–528. doi:10.30638/eemj.2018.052

Akbar S, Sultan S (2016) Soil bacteria showing a potential of chlorpyrifos degradation and plant growth enhancement. Braz Journal Microbiol 47(3): 563-570. doi: 10.1016/j.bjm.2016.04.009

Albareda M, Rodríguez-Navarro DN, Camacho M, Temprano FJ (2008) Alternatives to peat as a carrier for rhizobia inoculants: Solid and liquid formulations. Soil Biol Biochem 40: 2771–2779. doi: 10.1016/j.soilbio.2008.07.021

Amiet-Charpentier C, Gadille P, Digat B, Benoit JP (1988). Microencapsulation of rhizobacteria by spray-drying: Formulation and survival studies. J Microencapsul. 15(5):639–659. 10.3109/02652049809008247

Anderlei T, Büchs J (2001) Device for sterile online measurement of the oxygen transfer rate in shaking flasks. Biochem Eng J 7:157-162. doi: 10.1016/s1369-703x(00)00116-9

Anderlei T, Zang W, Papaspyrou M and Büchs J (2004) Online respiration activity measurement (OTR, CTR, RQ) in shake flasks. Biochem Eng J 17:187–194. doi: 10.1016/S1369-703X(03)00181-5

Anupama K, Paul S (2009) Ex situ and in situ biodegradation of lindane by *Azotobacter chroococcum*. J Environ Sci Health - B 45:58-66. doi: 10.1080/03601230903404465

Askar A, Khudhur M (2013) Effect of some pesticides on growth, nitrogen fixation and nif genes in *Azotobacter chroococcum* and *Azotobacter vinelandii* isolated from soil. J Toxicol Environ Health Sci 5:166-171. doi: 10.5897/jtehs12.029

Barman D, Haque M, Islam S, Yun H, Kim M (2014) Cloning and expression of ophB gene encoding organophosphorus hydrolase from endophytic *Pseudomonas* sp. BF1-3 degrades organophosphorus pesticide chlorpyrifos. Ecotoxicol Environ Saf 108:135-141. doi: 10.1016/j.ecoenv.2014.06.023

Barman S, Das S, Bhattacharya SS (2019) Chapter 17-The Prospects of Bio-Fertilizer Technology for Productive and Sustainable Agricultural Growth. In: Singh JS, Singh DP (eds) New and Future Developments in Microbial Biotechnology and Bioengineering Elsevier 233–253. doi: 10.1016/b978-0-444-64191-5.00017-1

Bashan Y, de-Bashan L, Prabhu S, Hernandez J (2014) Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant and Soil 378:1-33. doi: 10.1007/s11104-013-1956-x

Bhosale H, Kadam T, Bobade A (2013) Identification and production of *Azotobacter vinelandii* and its antifungal activity against Fusarium oxysporum. J Environ Biol 34:177-182.

Bose S, Kumar PS, N. Vo DV (2021) A review on the microbial degradation of chlorpyrifos and its metabolite TCP. Chemosphere 283: 131447. doi: 10.1016/j.chemosphere.2021.131447

Campos DC, Acevedo F, Morales E, Aravena J, Amiard V, Jorquera M, Inostroza NG, Rubilar M (2014) Microencapsulation by spray drying of nitrogen-fixing bacteria associated with lupin nodules. World J Microbiol Biotechnol 30(9):2371–2378. doi: 10.1007/s11274-014-1662-8

Castillo J, Casas J, Romero E (2011) Isolation of an endosulfan-degrading bacterium from a coffee farm soil: Persistence and inhibitory effect on its biological functions. Sci Total Environ 412-413:20-27. doi: 10.1016/j.scitotenv.2011.09.062

Castillo T, Flores C, Segura D, Espín G, Sanguino J, Cabrera E, Barreto J, Díaz-Barrera A, Peña C (2017) Production of polyhydroxybutyrate (PHB) of high and ultra-high molecular weight by *Azotobacter vinelandii* in batch and fed-batch cultures. J Chem Technol Biotechnol 92(7): 1809–1816. doi: 10.1002/jctb.5182

Castillo T, García A, Padilla-Córdova C, Díaz-Barrera A, Peña C (2020) Respiration in *Azotobacter vinelandii* and its relationship with the synthesis of biopolymers. Electron J Biotechnol 48:36-45. doi: 10.1016/j.ejbt.2020.08.001

Castillo T, Heinzle E, Peifer S, Schneider K, Peña M C (2013) Oxygen supply strongly influences metabolic fluxes, the production of poly(3-hydroxybutyrate) and alginate, and the degree of acetylation of alginate in *Azotobacter vinelandii*. Process Biochem 48:995-1003. doi: 10.1016/j.procbio.2013.04.014

Chen S, Liu S, Peng C, Liu H, Hu M, Zhong G (2012) Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-Trichloro-2-Pyridinol by a new fungal strain *Cladosporium cladosporioides* Hu-01. PloS One 7: 1-12. doi: 10.1371/journal.pone.0047205

Chennappa G, Adkar-Purushothama C, Naik M, Suraj U, Sreenivasa M (2014a) Impact of Pesticides on PGPR Activity of *Azotobacter* sp. Isolated from Pesticide Flooded Paddy Soils. Greener J Agric Sci 4:117-129. doi: 10.15580/gjas.2014.4.010314003

Chennappa G, Adkar-Purushothama C, Suraj U, Tamilvendan K, Sreenivasa M (2013) Pesticide tolerant *Azotobacter* isolates from paddy growing areas of northern Karnataka, India. World J Microbiol Biotechnol 30:1-7. doi: 10.1007/s11274-013-1412-3

Chennappa G, Adkar-Purushothama CR, Suraj U, Tamilvendan K, Sreenivasa MY (2014b) Pesticide tolerant *Azotobacter* isolates from paddy growing areas of northern Karnataka, India. World J Microbiol Biotechnol 30(1):1–7. doi: 10.1007/s11274-013-1412-3

Chennappa G, Naik MK, Adkar-Purushothama CR, Amaresh YS, Sreenivasa MY (2016). PGP potential, abiotic stress tolerance, and antifungal activity of *Azotobacter* strains isolated from paddy soils. Indian J Exp Biol 54 5, 322-31. http://nopr.niscair.res.in/handle/123456789/34155

Chennappa G, Naik MK, Amaresh YS, Nagaraj H, Sreenivasa MY (2018a) *Azotobacter* - A potential bio-fertilizer and bio inoculants for sustainable agriculture. In: Panpatte D (ed) Microorganisms for green revolution Springer Nature, Singapore, pp 78–87.

Chennappa G, Sreenivasa M, Nagaraja H (2018b) *Azotobacter salinestris*: A Novel Pesticide-Degrading and Prominent Biocontrol PGPR Bacteria. In: Naveen Kumar A (ed) Microorganisms for Sustainability 23-43. doi: 10.1007/978-981-10-7146-1_2

Chennappa G, Udaykumar N, Vidya M, Nagaraja H, Amaresh Y, Sreenivasa M (2019) *Azotobacter*—A Natural Resource for Bioremediation of Toxic Pesticides in Soil Ecosystems. New and Future Developments

in Microbial Biotechnology and Bioengineering 267-279. doi: 10.1016/b978-0-444-64191-5.00019-5

Chitara M, Chauhan S, Singh R (2021) Bioremediation of Polluted Soil by Using Plant Growth–Promoting Rhizobacteria. Microbial Rejuvenation of Polluted Environment 203-226. doi: 10.1007/978-981-15-7447-4_8

Chobotarov A, Volkogon M, Voytenko L, Kurdish I (2017) Accumulation of Phytohormones By Soil Bacteria *Azotobacter Vinelandii* And *Bacillus Subtilis* Under The Influence Of Nanomaterials. J Microbiol Biotechnol Food Sci 7:271-274. doi: 10.15414/jmbfs.2017/18.7.3.271-274

Conde-Avila V, Ortega-Martínez L, Loera O, El Kassis E, Dávila J, Valenzuela C, Armendáriz B (2020) Pesticides degradation by immobilised microorganisms. Int J Environ Anal Chem 1-31. doi: 10.1080/03067319.2020.1715375

Cycoń M, Żmijowska A, Wójcik M, Piotrowska-Seget Z (2013) Biodegradation and bioremediation potential of diazinon-degrading *Serratia marcescens* to remove other organophosphorus pesticides from soils. J Environ Manage 117:7-16. doi: 10.1016/j.jenvman.2012.12.031

Deng S, Chen Y, Wang D, Shi T, Wu X, Ma X, Li X, Hua R, Tang X, Li QX (2015) Rapid biodegradation of organophosphorus pesticides by *Stenotrophomonas* sp. G1. J. Hazard Mater., 297: 17-24. doi: 10.1016/j.jhazmat.2015.04.052

Díaz-Barrera A, Aguirre A, Berrios J and Acevedo F (2011) Continuous cultures for alginate production by *Azotobacter vinelandii* growing at different oxygen uptake rates. Process Biochem 46:1879–1883. doi: 10.1016/j.procbio.2011.06.022

Díaz-Barrera A, Peña C, Galindo E (2007) The oxygen transfer rate influences the molecular mass of the alginate produced by *Azotobacter vinelandii*. Appl Microbiol Biotechnol 76:903–910. doi: 10.1007/s0025 3-007-1060-3

Díaz-Barrera A, Sanchez-Rosales F, Padilla-Córdova C, Andler R, Peña C (2021) Molecular weight and guluronic/mannuronic ratio of alginate produced by *Azotobacter vinelandii* at two bioreactor scales under diazotrophic conditions. Bioprocess Biosyst Eng 44:1275-1287. doi: 10.1007/s00449-021-02532-8

Digat B (1991) A new encapsulation technology for bacterial inoculants and seed bacterization. In: Kell C, Koller B, Defago G (Eds) Plant Growth Promoting Rhizobacteria-Progress and Prospects Zurich: I.O.B.C./W.P.R.S. bulletin. https://hal.archives-ouvertes.fr/hal-01607138/document

Dilly O (2001) Microbial respiratory quotient during basal metabolism and after glucose amendment in soils and litter. Soil Biol Biochem 33:117-127. doi: 10.1016/s0038-0717(00)00123-1

Dilly O. (2003). Regulation of the respiratory quotient of soil_microbiota by availability of nutrients. FEMS Microbiol Ecol 43(3): 375–381. doi: 10.1111/j.1574-6941.2003.tb01078.x

Farhan M, Ahmad M, Kanwal A. et al. (2021) Biodegradation of chlorpyrifos using isolates from contaminated agricultural soil, its kinetic studies. Sci Rep 11: 10320. doi: 10.1038/s41598-021-88264-x

Feng Y, Minard R, Bollag J (1998) Photolytic and microbial degradation of 3, 5, 6-trichloro-2-pyridinol. Environ Toxicol Chem 17: 814–819. doi: 10.1002/etc.5620170508

Fishel FM (2013) EPA's Endocrine Disruptor Screening Program (EDSP). doi: 10.32473/edis-pi227-2013

García A, Ferrer P, Albiol J, Castillo T, Segura D, Peña C (2018) Metabolic flux analysis and the NAD(P)H/NAD(P)+ ratios in chemostat cultures of *Azotobacter vinelandii*. Microb Cell Fact. doi: 10.1186/s12934-018-0860-8

Gilani RA, Rafique M, Rehman A, Munis MFH, Rehman SU, Chaudhary HJ (2016) Biodegradation of chlorpyrifos by bacterial genus *Pseudomonas*. J Basic Microbiol 56(2):105–119. doi: 10.1002/jobm.201500336

Gilani S, Ageen M, Shah H, Raza S (2010) Chlorpyrifos degradation in soil and its effect on soil microorganisms. J Anim Plant Sci 20: 99–102. http://www.thejaps.org.pk/docs/20-2-2010/Gilani-et-al.pdf

Giri K, Rai J (2012) Biodegradation of endosulfan isomers in broth culture and soil microcosm by *Pseudomonas fluorescens* isolated from soil. Int J Environ Stud 69:729-742. doi: 10.1080/00207233.2012.702480

Gómez-Pazarín K, Flores C, Castillo T, Büchs J, Galindo E, Peña C (2015) Molecular weight and viscosifying power of alginates produced in *Azotobacter vinelandii* cultures in shake flasks under low power input. J Chem Technol Biotechnol 91:1485-1492. doi: 10.1002/jctb.4747

Gurikar C, Naik MK, Sreenivasa MY (2016) *Azotobacter*: PGPR Activities with Special Reference to Effect of Pesticides and Biodegradation. In: Singh D, Singh H, Prabha R (eds) Microbial Inoculants in Sustainable Agricultural Productivity. Springer, New Delhi. doi: 10.1007/978-81-322-2647-5_13

Hernández-Ruiz GM, Álvarez-Orozco NA, Ríos-Osorio LA (2017) Biorremediación de organofosforados por hongos y bacterias en suelos agrícolas: revisión sistemática. Ciencia y Tecnología Agropecuaria 18(1): 139-159. https://www.redalyc.org/articulo.oa?id=449949161008

Jabeen H, Iqbal S, Anwar S (2015) Biodegradation of Chlorpyrifos and 3, 5, 6-Trichloro-2-Pyridinol by a Novel Rhizobial Strain *Mesorhizobium* sp. HN3. Water Environ J. doi: 10.1111/wej.12081

Jayasri Y, Naidu MD, Mallikarjuna M (2014) Review article microbial degradation of chlorpyrifos. Int J Recent Sci Res 5: 1444-1450. http://recentscientific.com/sites/default/files/1678.pdf

John EM, Shaike JM (2015) Chlorpyrifos: pollution and remediation. Environ Chem Lett 13:269–291. 2015). doi: 10.1007/s10311-015-0513-7

John RP, Tyagi RD, Brar SK, Surampalli RY, Prévost D (2011). Bio-encapsulation of microbial cells for targeted agricultural delivery. Crit Rev Biotechnol 31(3): 211–226. doi: 10.3109/07388551.2010.513327

Kahraman B, Altın A (2020) Evaluation of Different Approaches for Respiratory Quotient Calculation and Effects of Nitrogen Sources on Respiratory Quotient Values of Hydrocarbon Bioremediation. Water Air Soil Pollut 231, 38.1doi: 10.1007/s11270-020-04763-z

Kennedy IR, Choudhury ATM, Kecskés ML (2004) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biol. Biochem 36:1229–1244. doi: 10.1016/j.soilbio.2004.04.006

Klimek J, Ollis D (1980) Extracellular microbial polysaccharides: Kinetics of *Pseudomonas* sp., *Azotobacter vinelandii*, and *Aureobasidium pullulans* batch fermentations. Biotechnol Bioeng 22:2321-2342. doi: 10.1002/bit.260221109

Krell V, Jakobs-Schoenwandt D, Vidal S, Patel A (2018) Encapsulation of *Metarhizium brunneum* enhances endophytism in tomato plants. Biol Control 116:62-73. doi: 10.1016/j.biocontrol.2017.05.004

Kumar A, Singh VK, Tripathi V, Singh PP, Singh AK (2018) Chapter 16 - Plant Growth-Promoting Rhizobacteria (PGPR): Perspective in Agriculture Under Biotic and Abiotic Stress. In: Prasad R, Gill SS, Tuteja N (Eds) Crop Improvement Through Microbial Biotechnology, Elsevier, 333-342 pp. doi: 10.1016/B978-0-444-63987-5.00016-5.

Kumar V, Singh S, Upadhyay N (2019) Effects of organophosphate pesticides on siderophore producing soils microorganisms. Biocatalysis and Agricultural Biotechnology 21:101359. doi: 10.1016/j.bcab.2019.101359.

Lamy E, Tran TC, Mottelet S, Pauss A, Schoefs O (2013) Relationships of respiratory quotient to microbial biomass and hydrocarbon contaminant degradation during soil bioremediation. Int Biodeter Biodegradation 83: 85–91. doi: 10.1016/j.ibiod.2013.04.015

Lenart A (2012) In Vitro effects of various xenobiotics on *Azotobacter chroococcum* strains isolated from soils of southern Poland. J Environ Sci Health B 47:7-12. doi: 10.1080/03601234.2012.601942

Li X, He J, Li S (2007) Isolation of chlorpyrifos degrading bacterium, *Sphingomonas* sp. strain Dsp-2, and cloning of the mpd gene. Res Microbiol.158:143–149. doi:10.1016/j.resmic.2006.11.007

Liu ZY, Chen X, Shi Y, Su ZC. 2011. Bacterial degradation of chlorpyrifos by *Bacillus cereus*. Adv Mater Res 356–360: 676-680. doi: 10.4028/www.scientific.net/AMR.356-360.676

Lowry O, Rosebrough N, Farr A, Randall R (1951) Protein Measurement with The Folin Phenol Reagent. J Biol Chem 193:265-275. doi: 10.1016/s0021-9258(19)52451-6

Lozano E, Galindo E. Peña CF (2011) Oxygen transfer rate during the production of alginate by *Azotobacter vinelandii* under oxygen-limited and non-oxygen-limited conditions. Microb Cell Fact 10 (13). doi: 10.1186/1475-2859-10-13

Mac-Rae IC, Celo JS (1974) The effects of organo-phosphorus pesticides on the respiration of *Azotobacter vinelandii*. Soil Biol Biochem 6(2):109–111. doi: 10.1016/0038-0717(74)90068-6

Maya K, Singh RS, Upadhyay SN, Dubey SK (2011) Kinetic analysis reveals bacterial efficacy for biodegradation of chlorpyrifos and its hydrolyzing metabolite TCP. Process Biochem 46(11): 2130–2136. doi: 10.1016/j.procbio.2011.08

Menon P, Gopal M, Prasad R (2004) Influence of Two Insecticides, Chlorpyrifos and Quinalphos, on Arginine Ammonification and Mineralizable Nitrogen in Two Tropical Soil Types. J Agric Food Chem 52:7370-7376. doi: 10.1021/jf049502c

Miller G (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem 31:426-428. doi: 10.1021/ac60147a030

Moneke A, Okpala G, Anyanwu C (2010) Biodegradation of glyphosate herbicide in vitro using bacterial isolates from four rice fields. Afr J Biotechnol 9:4067–4074.

Moral ÇK, Ertesvåg H, Sanin FD (2016) Guluronic acid content as a factor affecting turbidity removal potential of alginate. Environ Sci Pollut Res 23:22568–22576. doi: 10.1007/s11356-016-7475-6

Mousa N, Adham A, Merzah N, Jasim S (2021) *Azotobacter* spp. Bioremediation Chemosate. Asian J Water Environ Pollut 18:103-107. doi: 10.3233/ajw210034

Mustapha M, Halimoon N, Wan Johari W, Abd Shakur M (2018) Soil Microorganisms and Their Potential in Pesticide Biodegradation; A Review Article. J Sustain Agric Sci 0:0-0. doi: 10.21608/jsas.2018.3465.1061

Muttawar AS and Wadhai VS (2014) Isolation of Pesticide Tolerant *Azotobacter* Species from Rhizospheric Region of the Crop. Int J Res Biosci Agric Techn. doi: 10.29369/ijrbat.2014.03.iii.0006

Myresiotis C, Vryzas Z, Papadopoulou-Mourkidou E (2011) Biodegradation of soil-applied pesticides by selected strains of plant growth-promoting rhizobacteria (PGPR) and their effects on bacterial growth. Biodegradation 23:297-310. doi: 10.1007/s10532-011-9509-6

Nagaraja H, Chennappa G, Rakesh S, Naik M, Amaresh Y, Sreenivasa M (2016) Antifungal activity of *Azotobacter nigricans* against trichothecene-producing *Fusarium* species associated with cereals. Food Sci Biotechnol 25:1197-1204. doi: 10.1007/s10068-016-0190-8

Nakkeeran S, Fernando W, Siddiqui Z (2005) Plant Growth Promoting Rhizobacteria Formulations and its Scope in Commercialization for the Management of Pests and Diseases. Biocontrol and Biofertilization 257-296. doi: 10.1007/1-4020-4152-7_10

Noar J, Bruno-Bárcena J (2018) *Azotobacter vinelandii*: the source of 100 years of discoveries and many more to come. Microbiology 164:421-436. doi: 10.1099/mic.0.000643

Noguez R, Segura D, Moreno S, Hernandez A, Juarez K, Espín G (2007) Enzyme INtr, NPr, and IIANtr are involved in regulation of the poly-*β*-hydroxybutyrate biosynthetic genes in *Azotobacter vinelandii*. J Mol Microbiol Biotechnol 15:244-254. doi: 10.1159/000108658

Omar SA, Abd-Alla MH (1992) Effect of pesticides on growth, respiration, and nitrogenase activity of *Azotobacter* and *Azospirillum*. World J Microbiol Biotechnol 8:326–328 doi: 10.1007/BF01201891

Pant R, Pandey P, Kotoky R (2016) Rhizosphere mediated biodegradation of 1, 4-dichlorobenzene by plant growth-promoting rhizobacteria of *Jatropha curcas*. Ecol Eng 94:50–56. doi: 10.1016/j.ecoleng.2016.05.079

Park JK, Chang HN (2000) Microencapsulation of microbial cells. Biotechnol Adv 18(4):303–31. Doi: 10.1016/S0734-9750(00)00040-9

Peña C, Campos N, Galindo E (1997) Changes in alginate molecular mass distributions, broth viscosity and morphology of *Azotobacter vinelandii* cultured in shake flasks. Appl Microbiol Biotechnol 48:510-515. doi: 10.1007/s002530051088

Peña C, Galindo E, Büchs J (2011) The viscosifying power, degree of acetylation and molecular mass of the alginate produced by *Azotobacter vinelandii* in shake flasks are determined by the oxygen transfer rate. Process Biochem 46:290-297. doi: 10.1016/j.procbio.2010.08.025

Peña C, Peter CP, Büchs J, Galindo E (2007) Evolution of the specific power consumption and oxygen transfer rate in alginate-producing cultures of *Azotobacter vinelandii* conducted in shake flasks. Biochem Eng J 36(2) 73-80. doi: 10.1016/j.bej.2007.02.019.

Plunkett M, Knutson C, Barney B (2020) Key factors affecting ammonium production by an *Azotobacter vinelandii* strain deregulated for biological nitrogen fixation. Microb Cell Fact. doi: 10.1186/s12934-020-01362-9

Praveen Kumar G, Mir Hassan Ahmed S, Desai S, Leo Daniel Amalraj E, Rasul A (2014) In Vitro Screening for Abiotic Stress Tolerance in Potent Biocontrol and Plant Growth Promoting Strains of *Pseudomonas* and *Bacillus* spp. Int J Bacteriol 2014:1-6. doi: 10.1155/2014/195946

Rani R, Kumar V (2017) Endosulfan Degradation by Selected Strains of Plant Growth Promoting Rhizobacteria. Bull Environ Contam Toxicol 99:138-145. doi: 10.1007/s00128-017-2102-x

Rani R, Kumar V, Gupta P, Chandra A (2019a) Effect of endosulfan tolerant bacterial isolates (*Delftia lacustris* IITISM30 and *Klebsiella aerogenes* IITISM42) with *Helianthus annuus* on remediation of endosulfan from contaminated soil. Ecotoxicol Environ Saf 168:315-323. doi: 10.1016/j.ecoenv.2018.10.059

Rani R, Kumar V, Gupta P, Chandra A (2019b) Application of plant growth-promoting rhizobacteria in remediation of pesticides contaminated stressed soil. New and Future Developments in Microbial Biotechnology and Bioengineering 341-353. doi: 10.1016/b978-0-12-818258-1.00029-7

Rayu S, Nielsen UN, Nazaries L, Singh BK (2017) Isolation and molecular characterization of novel chlorpyrifos and 3,5,6-trichloro-2-pyridinol-degrading bacteria from sugarcane farm soils. Front. Microbiol 8:1-16. doi: 10.3389/fmicb.2017.00518

Revillas J, Rodelas B, Pozo C, Martínez-Toledo M, González-López J (2000) Production of B-group vitamins by two *Azotobacter* strains with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions. J Appl Microbiol 89:486-493. doi: 10.1046/j.1365-2672.2000.01139.x

Rokade KB, Mali GV (2013) Biodegradation of chlorpyrifos by Pseudomonas desmolyticum NCIM 2112. Int J Pharma Bio Sci 4(2):(B)609–616. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.302.2334&rep=rep1&type=pdf

Roy T, Das N, Majumdar S (2020) Pesticide Tolerant Rhizobacteria: Paradigm of Disease Management and Plant Growth Promotion. In: Varma A, Tripathi S, Prasad R (eds) Plant Microbe Symbiosis. Springer, Cham 221-239. doi: 10.1007/978-3-030-36248-5_12

Santos MS, Rondina ABL, Nogueira MA, Hungria M (2020) Compatibility of *Azospirillum brasilense* with Pesticides Used for Treatment of Maize Seeds. Int J Microbiol ID 8833879. doi:10.1155/2020/8833879

Sarker A, Nandi R, Kim J, Islam T (2021) Remediation of chemical pesticides from contaminated sites through potential microorganisms and their functional enzymes: Prospects and challenges. Environ Technol Innov 23:101777. doi: 10.1016/j.eti.2021.101777

Schoebitz M, Lopez MD, Roldan A (2013) Bioencapsulation of microbial inoculants for better soil-plant fertilization. A review. Agron Sustain Dev 33(4):751–765. doi: 10.1007/s13593-013-0142-0

Sethi S, Gupta S (2013) Impact of Pesticides and Biopesticides on Soil Microbial Biomass Carbon. Univers J Environ 3(2):326-330. http://web.a.ebscohost.com/ehost/pdfviewer/pdfviewer?vid=1&sid=bd5623b9-c38a-4afd-b9ea-d316a3f69789%40sdc-v-sessmgr01

Shahgholi H, Ahangar A (2014) Factors controlling degradation of pesticides in the soil environment: A Review. Agric Sci Dev 3(8): 273-8. https://www.semanticscholar.org

Shahid M, Zaidi A, Ehtram A, Khan M (2019) In vitro investigation to explore the toxicity of different groups of pesticides for an agronomically important rhizosphere isolate *Azotobacter vinelandii*. Pestic Biochem Phys 157:33-44. doi: 10.1016/j.pestbp.2019.03.006

Shi T, Fang L, Qin H, Chen Y, Wu X, Hua R (2019) Rapid biodegradation of the organophosphorus insecticide chlorpyrifos by *Cupriavidus nantongensis* x1T. Int J Environ Res Publ Health 16(23): 4593. doi: 10.3390/ijerph16234593

Singh DP, Khattar JIS, Nadda, J, et al. (2011) Chlorpyrifos degradation by the cyanobacterium *Synechocystis* sp. strain PUPCCC 64. Environ Sci Pollut Res 18:1351–1359. doi: 10.1007/s11356-011-0472-x

Strobel S, Allen K, Roberts C, Jimenez D, Scher H, Jeoh T (2018) Industrially-Scalable Microencapsulation of Plant Beneficial Bacteria in Dry Cross-Linked Alginate Matrix. Ind Biotechnol 14:138-147. doi: 10.1089/ind.2017.0032

Sumbul A, Ansari R, Rizvi R, Mahmood I (2020) Azotobacter: A potential bio-fertilizer for soil and plant health management. Saudi J Biol Sci 27:3634-3640. doi: 10.1016/j.sjbs.2020.08.004

Talwar M, Ninnekar H (2015) Biodegradation of pesticide profenofos by the free and immobilized cells of *Pseudoxanthomonas suwonensis* strain HNM. J Basic Microbiol 55:1094-1103. doi: 10.1002/jobm.201400978

Tejera N, Lluch C, Martìnez-Toledo M, Gonzàlez-López J (2005) Isolation and characterization of *Azotobacter* and *Azospirillum* strains from the sugarcane rhizosphere. Plant and Soil 270:223-232. doi: 10.1007/s11104-004-1522-7

Then C, Wai O, Elsayed E, Mustapha W, Othman N, Aziz R, Wadaan M, Enshsay H (2016) Comparison between Classical and Statistical Medium Optimization Approaches for High Cell Mass Production of *Azotobacter Vinelandii*. J Sci Ind Res 75:231-238.

Vidya Lakshmi C, Kumar M, Khanna S (2009) Biodegradation of chlorpyrifos in soil enriched cultures. Curr Microbiol 58:35-38. doi: 10.1007/s00284-008-9262-1

Vijayalakshmi P, Usha MS (2012) Degradation of chlorpyrifos by free cells and calcium-alginate immobilized cells of *Pseudomonas putida*. Adv Appl Sci Res 3:2796–2800. https://www.cabdirect.org/cabdirect/abstract/20123377370

Walvekar V, Bajaj S, Singh D, Sharma S (2017) Ecotoxicological assessment of pesticides and their combination on rhizospheric microbial community structure and function of *Vigna radiata*. Environ Sci and Pollut R 24:17175-17186. doi: 10.1007/s11356-017-9284-y

Wu X, Xu J, Dong F, Liu X, Zheng Y (2014) Responses of soil microbial community to different concentration of fomesafen. J Hazard Mater 273:155-164. doi: 10.1016/j.jhazmat.2014.03.041

Yang L, Zhao YH, Zhang BX, Yang CH, Zhang X (2005) Isolation and characterization of a chlorpyrifos and 3,5,6-trichloro-2- pyridinol degrading bacterium. FEMS Microbiol Lett 251(1): 67-73. doi: 10.1016/j.femsle.2005.07.031

Young C, Rekha P, Lai W, Arun A (2006) Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. Biotechnol Bioeng 95:76-83. doi: 10.1002/bit.20957

Zhu J, Zhao Y, Ruan H (2019) Comparative study on the biodegradation of chlorpyrifos-methyl by *Bacillus megaterium* CM-Z19 and *Pseudomonas syringae* CM-Z6. An Acad Bras Cienc 91(3). doi: 10.1590/0001-3765201920180694

BIBLIOGRAFÍA

Abraham J, Silambarasan S (2016) Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol using a novel bacterium *Ochrobactrum* sp. JAS2: A proposal of its metabolic pathway. Pestic Biochem Phys 126:13-21. doi: 10.1016/j.pestbp.2015.07.001

Badii MH, Varela S (2008) Insectidas organofosforados: efectos sobre la salud y el ambiente. Culcyt/Toxicología de Insecticidas. 28: 5-17.

Barman S, Das S, Bhattacharya SS (2019) Chapter 17-The Prospects of Bio-Fertilizer Technology for Productive and Sustainable Agricultural Growth. In: Singh JS, Singh DP (eds) New and Future Developments in Microbial Biotechnology and Bioengineering Elsevier 233–253. doi: 10.1016/b978-0-444-64191-5.00017-1

Bose S, Kumar PS, N. Vo DV (2021) A review on the microbial degradation of chlorpyrifos and its metabolite TCP. Chemosphere 283: 131447. doi: 10.1016/j.chemosphere.2021.131447

Castillo J, Casas J, Romero E (2011) Isolation of an endosulfan-degrading bacterium from a coffee farm soil: Persistence and inhibitory effect on its biological functions. Sci Total Environ 412-413:20-27. doi: 10.1016/j.scitotenv.2011.09.062

Castillo T, Flores C, Segura D, Espín G, Sanguino J, Cabrera E, Barreto J, Díaz-Barrera A, Peña C (2017) Production of polyhydroxybutyrate (PHB) of high and ultra-high molecular weight by *Azotobacter vinelandii* in batch and fed-batch cultures. J Chem Technol Biotechnol 92(7): 1809–1816. doi: 10.1002/jctb.5182

Chennappa G, Adkar-Purushothama C, Naik M, Suraj U, Sreenivasa M (2014a) Impact of Pesticides on PGPR Activity of *Azotobacter* sp. Isolated from Pesticide Flooded Paddy Soils. Greener J Agric Sci 4:117-129. doi: 10.15580/gjas.2014.4.010314003

Chennappa G, Naik MK, Adkar-Purushothama CR, Amaresh YS, Sreenivasa MY (2016). PGP potential, abiotic stress tolerance, and antifungal activity of *Azotobacter* strains isolated from paddy soils. Indian J Exp Biol 54 5, 322-31. http://nopr.niscair.res.in/handle/123456789/34155

Chennappa G, Naik MK, Amaresh YS, Nagaraj H, Sreenivasa MY (2018a) *Azotobacter* - A potential bio-fertilizer and bio inoculants for sustainable agriculture. In: Panpatte D (ed) Microorganisms for green revolution Springer Nature, Singapore, pp 78–87.

Chennappa G, Udaykumar N, Vidya M, Nagaraja H, Amaresh Y, Sreenivasa M (2019) *Azotobacter*—A Natural Resource for Bioremediation of Toxic Pesticides in Soil Ecosystems. New and Future Developments in Microbial Biotechnology and Bioengineering 267-279. doi: 10.1016/b978-0-444-64191-5.00019-5

Chobotarov A, Volkogon M, Voytenko L, Kurdish I (2017) Accumulation of Phytohormones By Soil Bacteria *Azotobacter Vinelandii* And *Bacillus Subtilis* Under The Influence Of Nanomaterials. J Microbiol Biotechnol Food Sci 7:271-274. doi: 10.15414/jmbfs.2017/18.7.3.271-274

Colla TS, Andreazza R, Bucker F, de Souza MM, Tramontini L, Prado GR, Frazzon, APG, Camargo FADO, Bento FM (2014) Bioremediation assessment of diesel-biodiesel-contaminated soil using an alternative bioaugmentation strategy. Environ Sci Poll Res. 21:2592–2602.

Costa LG. (2006) Current issues in organophosphate toxycology. Clinica Chimica Acta. 366(1-2): 1-13.

Cycoń M, Mrozik A, Piotrowska-Seget S (2017) Bioaugmentation as a strategy for the remediation of pesticide-polluted soil: A review. Chemosphere. 172:57-71.

Cycoń M, Żmijowska A, Wójcik M, Piotrowska-Seget Z (2013) Biodegradation and bioremediation potential of diazinon-degrading *Serratia marcescens* to remove other organophosphorus pesticides from soils. J Environ Manage 117:7-16. doi: 10.1016/j.jenvman.2012.12.031

Das R, Das SJ, Das AC (2016) Effect of synthetic pyrethroid insecticides on N2-fixation and its mineralization in tea soil. Euro J Soil Biol. 74: 9–15.

Firozjaei SAA, Latifi AM, Khodi S, Abolmaali S, Choopani A (2015) A review on biodegradation of toxic organophosphate compounds. J Appl Biotechnol Rep. 2(2):215-224.

Giri K, Rai J (2012) Biodegradation of endosulfan isomers in broth culture and soil microcosm by *Pseudomonas fluorescens* isolated from soil. Int J Environ Stud 69:729-742. doi: 10.1080/00207233.2012.702480

Gurikar C, Naik MK, Sreenivasa MY (2016) *Azotobacter*: PGPR Activities with Special Reference to Effect of Pesticides and Biodegradation. In: Singh D, Singh H, Prabha R (eds) Microbial Inoculants in Sustainable Agricultural Productivity. Springer, New Delhi. doi: 10.1007/978-81-322-2647-5_13

Hernández-León R, Velázquez-Sepúlveda, Orozco-Mosqueda MC, Santoyo G (2010) ΦYTON Rev Int Bot Exp 79: 133-139.

Hussain S, Siddique T, Saleem M, Arshad M, Khalid A (2009) Impact of pesticides on soil microbial diversity, enzymes and biochemical reactions. Adv Agron 102:159-200.

Ipsilantis I, Samourelis C, Karpouzas DG (2012) The impact of biological pesticides on arbuscular mycorrhizal fungi. Soil Biol Biochem 45: 147-155.

Kumar V, Singh S, Upadhyay N (2019) Effects of organophosphate pesticides on siderophore producing soils microorganisms. Biocatal Agric Biotechnol 21:101359. doi: 10.1016/j.bcab.2019.101359.

Lenart A (2012) In vitro effects of various xenobiotics on *Azotobacter chroococcum* strains isolated from soils of southern Poland. J Environ Sci Health B 47:7-12. doi: 10.1080/03601234.2012.601942

Mrozik A y Piotrowska-Seget Z (2010). Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiol Res 165: 363–375.

Muñoz P, Antón A, Nuñez M, Paranipe A, Ariño J, Castells X, Montero JI, Rieradevall J (2008) Comparin the envirionmental impacts of greenhouse versus open-field tomato production in the Mediterranean region. ISHS Acta Hortuculturae 801. DOI 10.17600/ActaHortic.2008.801.197.

Nagaraja H, Chennappa G, Rakesh S, Naik M, Amaresh Y, Sreenivasa M (2016) Antifungal activity of *Azotobacter nigricans* against trichothecene-producing *Fusarium* species associated with cereals. Food Sci Biotechnol 25:1197-1204. doi: 10.1007/s10068-016-0190-8

Ortega Martínez LD, Martínez Valenzuela C, Huerta de la Peña A, Ocampo Mendoza J, Sandoval Castro E, Jaramillo Villanueva JL (2014) Uso y manejo de plaguicidas en invernaderos de la región norte del estado de Puebla, México. Acta Universitaria, 24(3), 3-12. doi: 10.15174.au.2014.570

Ortiz-Hernández ML, Sánchez-Salinas E, Dantan-González E, Castrejón-Godínez ML (2013) Pesticide Biodegradation: Mechanisms, Genetics and Strategies to Enhance the Process. In *Biodegradation-Life of Science* Chamy R, Rosenkranz F (Eds). pp. 251. doi:10.5772/52777.

Pasiani JO, Torres P, Silva JR, Diniz BZ, Caldas ED (2012) Knowledge, actitudes, practices and Biomonitoring of farmers and residents exposed to pesticides in Brazil. Int J Environ Res Pub Healt 9(9): 3051-3068.

Praveen Kumar G, Mir Hassan Ahmed S, Desai S, Leo Daniel Amalraj E, Rasul A (2014) In vitro Screening for Abiotic Stress Tolerance in Potent Biocontrol and Plant Growth Promoting Strains of *Pseudomonas* and *Bacillus* spp. Int J Bacteriol 2014:1-6. doi: 10.1155/2014/195946

Rani R, Kumar V (2017) Endosulfan Degradation by Selected Strains of Plant Growth Promoting Rhizobacteria. Bull Environ Contam Toxicol 99:138-145. doi: 10.1007/s00128-017-2102-x

Rani R, Kumar V, Gupta P, Chandra A (2019a) Effect of endosulfan tolerant bacterial isolates (*Delftia lacustris* IITISM30 and *Klebsiella aerogenes* IITISM42) with *Helianthus annuus* on remediation of endosulfan from contaminated soil. Ecotoxicol Environ Saf 168:315-323. doi: 10.1016/j.ecoenv.2018.10.059

Revillas J, Rodelas B, Pozo C, Martínez-Toledo M, González-López J (2000) Production of B-group vitamins by two *Azotobacter* strains with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions. J Appl Microbiol 89:486-493. doi: 10.1046/j.1365-2672.2000.01139.x

Santos MS, Rondina ABL, Nogueira MA, Hungria M (2020) Compatibility of *Azospirillum brasilense* with Pesticides Used for Treatment of Maize Seeds. Int J Microbiol ID 8833879. doi:10.1155/2020/8833879

Sarker A, Nandi R, Kim J, Islam T (2021) Remediation of chemical pesticides from contaminated sites through potential microorganisms and their functional enzymes: Prospects and challenges. Environ Technol Innov 23:101777. doi: 10.1016/j.eti.2021.101777

Sethi S, Gupta S (2013) Impact of Pesticides and Biopesticides on Soil Microbial Biomass Carbon. Univers J Environ 3(2):326-330. http://web.a.ebscohost.com/ehost/pdfviewer/pdfviewer?vid=1&sid=bd5623b9-c38a-4afd-b9ea-d316a3f69789%40sdc-v-sessmgr01

Shahid M, Zaidi A, Ehtram A, Khan M (2019) In vitro investigation to explore the toxicity of different groups of pesticides for an agronomically important rhizosphere isolate *Azotobacter vinelandii*. Pestic Biochem Phys 157:33-44. doi: 10.1016/j.pestbp.2019.03.006

Suaza-García ER y Coy-Barrera E (2014) Alquilresorcinoles: Compuestos Naturales con Importancia Biológica. Rev Fac Cienc Basic 10(2):210-222. doi:10.18359/rfcb.332

Walvekar V, Bajaj S, Singh D, Sharma S (2017) Ecotoxicological assessment of pesticides and their combination on rhizospheric microbial community structure and function of *Vigna radiata*. Environ Sci and Pollut R 24:17175-17186. doi: 10.1007/s11356-017-9284-y

Yañez-Ocampo G, Sánchez-Salina E, Jimenez-Tobon GA, Penninckx M, Ortiz-Hernández ML (2009) Removal of two organophosphate pesticides by bacterial consortium immobilized in alginate or tezontle. J Hazard Mater 168: 1554-1561.

Zhang Z, Qu Y, Li S, Feng K, Wang S, Cai W, Liang Y, Li H, Xu M, Yin H, Deng Y (2017) Soil bacterial quantification approaches coupling with relative abundances reflecting the changes of taxa. Sci Rep 7:4837. DOI:10.1038/s41598-017-05260-w