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ECORFAN Journal Republic of Nicaragua

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Knowledge Area

The works must be unpublished and refer to topics of agriculture-forest, pathology-sustainable, forest, management, horticulture, engineering and integrated water useand other topics related to Biotechnology and Agricultural Sciences.

Presentation of Content

In Volume number eleven, as the first article we present, *Physicochemical characterisation of an anaerobic biodigestion process fed with agricultural wastes*, by AYALA, Alberto, HERRERA, Gabriel, GUTIERREZ, Elizabeth and RANGEL, Ulises, whith adscripction in the Instituto Tecnológico Superior de Irapuato, as a second article we present, *X-ray analysis of seed of Pinus devoniana*, by AVENDAÑO-LÓPEZ, A., QUINTANA-CAMARGO, M., PADILLA-GARCÍA, J. and ARRIAGA- RUÍZ, M., whith adscripction in the Universidad de Guadalajara and Centro Nacional de Recursos Genéticos (CNRG), as third article we present, *Cloning and characterisation of the xylose transporter coding gene of Debaryomyces hansenii in E. coli*, by DE LA RIVA-DE LA RIVA, Gustavo Alberto, COLLI-MULL, Juan Gualberto and JUÁREZ- SALDAÑA, Eric, whith adscripction in the Instituto Tecnológico Superior de Irapuato, as fourth article we present, *Molecular characterisation of the bacterial diversity potentially degrading triclosan present in the Xichú river basin, Guanajuato*, by GONZÁLEZ-LÓPEZ, Claudia Isela, RIVERA-MOSQUEDA, Ma. Cruz, COLLI-MULL, Juan Gualberto and NEGRETE-ALCALDE, Luis Jorge, whith adscripction in the Instituto Tecnológico Superior de Irapuato.

Content

Article **ECORFAN Journal-Republic of Nicaragua**
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Physicochemical characterisation of an anaerobic biodigestion process fed with agricultural wastes

Caracterización fisicoquímica de un proceso de biodigestión anaerobia alimentado con residuos agricolas

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Abstract

Contamination of water and soil for chemical fertilizers creates the need for efficient and economic alternatives of fertilization of soil, which decrease the negative impact of chemical fertilizers. A viable alternative is the use of vegetable waste through the anaerobic biodigestion, obtaining biogas and effluent solids and liquids such as by-products, with the latter being a potential of organic fertilizer residue. In order to use the organic waste and transform them waste to fertilizers, was carried out this work. It used 3-bio-digesters scale laboratory, which consist of a camera input of polyethylene of 3.8-litre capacity, one output of the same material and capacity of the first PVC tube, and a pair of valves of output, one for sampling and the remaining for the emission of biogas and thermometer. Determinations of physicochemical parameters were pH, cod, total phosphorus, total nitrogen, potassium, total volatile solids, total solids, fixed and volatile suspended solids. The first biodigester fed on remains of tomatoes and inoculum in a 2:1 ratio respectively, the second with cucumber waste and the proportions and the third was charged with a proportional mixture of both vegetables and inoculum.

Biodigester, Organic waste, Liquid effluents

Resumen

La contaminación de agua y suelo por los fertilizantes químicos genera la necesidad de encontrar alternativas económicas y eficientes de fertilización de suelo, que disminuyan el impacto negativo de los fertilizantes químicos. Una alternativa viable es la utilización de residuos vegetales, mediante la biodigestión anaerobia, obteniendo biogás y efluentes sólidos y líquidos como subproductos, siendo este último un residuo con potencial de fertilizante orgánico. Con el objetivo de utilizar los desechos orgánicos y transformarlos de residuos a biofertilizantes, se realizó el presente trabajo. Para ello, se utilizaron 3 biodigestores escala laboratorio, los cuales constan de una cámara de entrada de polietileno de 3.8 L de capacidad, una de salida del mismo material y capacidad del primero, PVC tubular, y un par de válvulas de salida, una de ellas para el muestreo y la restante para la emisión del biogás y termómetro. Se realizaron determinaciones de parámetros fisicoquímicos: pH, DQO, fósforo total, nitrógeno total, potasio, sólidos totales, solidos totales volátiles, sólidos suspendidos fijos y volátiles. El primer biodigestor se alimentó de restos de jitomates e inóculo en una relación 2:1 respectivamente, el segundo con desechos de pepino y las mismas proporciones y el tercero fue cargado con una mezcla proporcional de ambas hortalizas e inóculo.

Biodigestor, Residuos orgánicos, Efluentes líquidos

Citation: AYALA, Alberto, HERRERA, Gabriel, GUTIERREZ, Elizabeth and RANGEL, Ulises. Physicochemical characterisation of an anaerobic biodigestion process fed with agricultural wastes. ECORFAN Journal-Republic of Nicaragua. 2020. 6-11:1-4.

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Introduction

Global waste production has reached extremely high levels in recent years. Most of this waste is organic and represents up to 60% of total household waste, which is not recycled. These wastes are deposited in landfills or dumps where they occupy large spaces or are incorporated into the environment as a pollutant of water, soil, and atmosphere, due to their large volumes. (KRELING, 2006).

The lack of final management of agricultural waste in Mexico and the massive contamination due to the improper use of chemical fertilisers represent a serious environmental problem that is increasing day by day. This problem has led research into the search for a solution, and one of the various options found is anaerobic biodigestion, by means of which bioproducts such as methane gas and effluents are obtained. The latter have a positive impact on agriculture.

Biodigested organic matter added to the soil as a fertiliser or soil conditioner tends to produce significantly beneficial physical and chemical changes, the type and importance of which depends on many factors, including the quantity and quality of the organic matter applied.

Among the importance of organic fertiliser is its positive effect on the environment, since when applied in adequate proportions to soils that have lost their original characteristics (fertility, porosity), it helps the soil to recover them and contributes to preventing progressive deterioration of these soils.

This translates into the reincorporation of organic matter waste into the natural cycles, taking advantage of the available resources in almost their entirety, this at a low cost, contributing to sustainable environmental development.

Benefits in the use of biodigested effluents as biofertiliser:

Provides macro and micronutrients to the soil for direct consumption by plants. Increases ion exchange capacity. It achieves a buffer effect on soil pH. Reduces soil losses due to erosion. Prevents the loss of mineral nutrients (provided by chemical fertilisers) through leaching.

Development

The analyses (physical, biological, and chemical) were carried out in the laboratories of the Intituto Tenológico Superior de Irapuato with international methods applicable to our environment. The samples came from laboratory-scale batch-scale biodigesters located within the laboratory. The materials loaded into the biodigesters were agricultural vegetable wastes (tomato and cucumber).

Methodology

Sampling was performed only when determining physicochemical parameters, which were: Total Nitrogen, Total Phosphorus, Chemical Oxygen Demand (COD), Potassium, Total Solids (TS), Total Volatile Solids (TVS), Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS).

The equipment used for the measurement of parameters were:

Total phosphorus: Reactor HI839800 and photometer C 214 Multiparameter Bench Photometer hanna brand.

Total nitrogen: HI839800 reactor and hanna brand C 214 Multiparameter Bench Photometer.

Chemical Oxygen Demand (COD): HI839800 reactor and hanna brand C 214 Multiparameter Bench Photometer.

The standards used for the other parameters analysed were as follows:

Determination of dissolved solids and salts in natural, waste and treated wastewater: NMX-AA-034-SCFI-2001.

Results

The physical and chemical characteristics of influents and effluents at the beginning, during and after the biodigesters are shown in the following tables 1 to 3:

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Parameter	Unit	Start	During
pH	$(H_3O)^+$	6.8	5
DQO	mg/L	24300	15460
Nitrogen	mg/L	250	7.1-7.2
Phosphorus	mg/L	1950	*
Temperature	℃	27	25
S.S.T.	mg/L	*	935
S.S.V.	mg/L	*	495
S.T.	mg/L	*	1570
S.V.T.	mg/L	*	1.17
NOTE: * = Could not determine			

Table 1 Biodigester No.1 Tomato

Parameter	Unit	Start	During
pH	$(H_3O)^+$	6.5	5
DQO	mg/L	24700	15715
Nitrogen	mg/L	745	460
Phosphorus	mg/L	600	*
Temperature	℃	27	25
S.S.T.	mg/L	*	215
S.S.V.	mg/L	*	225
S.T.	mg/L	*	710
S.V.T.	mg/L	*	0.485
NOTE: * = Could not determine			

Table 2 Biodigester No. 2 Cucumber

Parameter	Unit	Start	During
pH	$(H_3O)^+$	6.7	5
DQO	mg/L	25501	16225
Nitrogen	mg/L	360	1470
Phosphorus	mg/L	140	*
Temperature	℃	27	25
S.S.T.	mg/L	*	2140
S.S.V.	mg/L	*	1860
S.T.	mg/L	*	2480
S.V.T.	mg/L	*	3.92
NOTE: * = Could not determine			

Table 3 Biodigester No. 3 Tomato and Cucumber

Analysis

These results show a trend in the operation of the biodigester, as there is a clear decrease in COD and BOD5 as the process progresses. This means that the organic matter load that the system receives from the plant waste feed is being transformed, presumably into methane, carbon dioxide and biomass mainly. Although there is a slight increase in the organic load during the process, which could be due to the increase in bacterial biomass that makes the transformation possible, the decrease in the initial load is significant, slightly higher than 90% for COD and 70% for BOD5, which speaks of an efficient process as the removal is extremely high.

The quantitative determination of nitrogen and phosphorus shows a decrease in the amounts not so high since both elements can remain in the liquid phase, because the catabolism of the system favours the production of methane over ammonia, for example. Thus, both nutrients should remain in the effluent; if a high proportion is maintained in this liquid product, this effluent can be considered as an excellent organic fertiliser, as it would contain the proportions of both parameters like commercial inorganic fertilisers.

Elements	Percentages %	Elements	Percentages %
Total Nitrogen (N)	11.470	Timide hydrochloride	0.004
Phosphorus (P_2O_5)	8.000	Sulfur (S)	0.230
Potassium (K_2O)	6.000	Calcium (CaO)	0.025
Boron (B)	0.036	Cobalt (Co)	0.002
Copper (Cu)	0.040	Manganese (Mn)	0.036
Iron (Fe)	0.050	Magnesium (MgO)	0.025
Molybdenum (Mo)	0.005	Indoleacetic acid	0.003
Zinc (Zn)	0.080		

Table 4 Composition of Bayer's Bayfolan® Fertilizer

The composition of the fertiliser is shown in Table 4. It could even be used as a feed source for the cultivation of microalgae with the potential to produce biodiesel or other commercially important bioproducts such as carotenoids or other biofuels.

Undoubtedly, generating biogas and a biofertiliser from this process fed with organic matter is a remarkably interesting result to close process cycles and recycle nutrients such as carbon dioxide, nitrogen and phosphorus. It also generates new value chains and economics to agricultural systems, making them sustainable.

The results obtained show a clear decrease in COD (Chemical Oxygen Damage) as the process progresses. This means that the organic matter load that the system receives from the feeding of plant residues is transformed into methane and biomass. It should be noted that the decrease could be due to the increase in bacterial biomass that makes the transformation possible.

It was not possible to determine the results of solids and dissolved salts, for fear of air entering the biodigester process.

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The proportions of the waste could affect the functioning of the process, so it is necessary to investigate the design of biodigesters, as well as to determine other physicochemical parameters: metals such as iron, calcium and microbiological parameters such as total coliforms and faecal coliforms (Escherichia coli) to determine their safety and whether it is feasible to use them in soils.

Acknowledgements

We would like to thank the company 4e Power and Fuels S. de R.L. and Ing. Jesús Alberto Crespo Quintanilla for their valuable support. We also thank the CONACYT project for the grants awarded.

Conclusions

So far, the physicochemical analyses COD, BOD5, total nitrogen and phosphorus for the biodigestion process used by the company 4e Power and Fuels S. de R.L. have been carried out.

These results show an effective reduction of the organic load, measured as COD and BOD of up to 90%, so the biogas production should be very efficient as well.

The capacity of the biodigester to be fed with a higher flow rate will have to be analysed to determine the limit of maximum reduction of organic matter to transform it into biogas and generate useful biofertiliser.

Four laboratory-scale biodigesters were constructed to monitor and obtain data related to this process. The proportions of the waste could affect the functioning of the process, so it is necessary to investigate more experimental designs and carry out other physicochemical determinations, such as the content of total solids, soluble solids, volatiles, various metals such as potassium, iron, calcium, among others, and microbiological determinations in the biofertiliser to determine its safety or the need to carry out some other treatment.

Undoubtedly, generating biogas and a biofertiliser from this process fed with organic matter is a very interesting result to close the process cycles and recycle nutrients such as carbon dioxide, nitrogen and phosphorus. It also generates new value chains and economics to agricultural systems making them sustainable.

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X-ray analysis of seed of *Pinus devoniana*

Análisis de semilla de *Pinus devoniana* con rayos X

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Abstract

The aim of this study was to establish the relationship between a system based on X-ray and behavior seed viability test with tetrazolium and standard germination analysis. From radiographic classification was established relating seed physiological response; seeds that produce normal seedlings, abnormal seedlings, and dead seeds: 3 classes were established. Also, internal morphological characteristics were established: the embryonic cavity filling, developing embryo structures, defects, density and appearance, and even invasion and damage from pests. Correlation coefficients of .70 ** presented with seedling length; 796 ** for tetrazolium viability; ** 818 and between the filling cavity and classified as normal. Concluded that X-ray analysis, in addition to being useful in determining the physical quality of the seed, leads to the estimate of the physiological response in *Pinus devonian* seeds, as it is possible to estimate the relationship between the response variables and morphological functional, without being a destructive technique.

Analysis seeds X-Ray, *Pinus Devonian*

Resumen

El objetivo del presente trabajo fue establecer la relación entre un análisis basado en Rayos X y el comportamiento de la semilla con el ensayo de viabilidad con tetrazolio y de germinación estándar. A partir de imágenes radiográficas se estableció una clasificación de semillas relacionando la respuesta fisiológica; se establecieron 3 clases: semillas que generan plántulas normales, plántulas anormales y semillas muertas. Así mismo se establecieron características morfológicas internas: el llenado de la cavidad embrionaria, el desarrollo de estructuras de embrión, malformaciones, densidad y aspecto, e incluso invasión y daños causados por plagas. Se presentaron coeficientes de correlación de .70** con longitud de plántula; .796** para viabilidad con tetrazolio; y de .818** entre el llenado de cavidad y las clasificadas como normales. Concluyendo que un análisis de Rayos X, además de ser útil en la determinación de la calidad física de la semilla, conlleva a la estimación de la respuesta fisiológica en semillas de *Pinus devoniana*, ya que es posible estimar la relación entre variables morfológicas y la respuesta funcional, sin ser una técnica destructiva.

Analisis semillas, Rayos X, *Pinus devoniana*

Citation: AVENDAÑO-LÓPEZ, A., QUINTANA-CAMARGO, M., PADILLA-GARCÍA, J. and ARRIAGA- RUÍZ, M. X-ray analysis of seed of *Pinus devoniana*. ECORFAN Journal-Republic of Nicaragua. 2020. 6-11:5-9.

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Introduction

Seed quality is a concept made up of four main components: physical, physiological, genetic, and sanitary characteristics, the objective of which is to know the seed's potential during germination, development in the field and particular requirements during storage; the analyses are carried out in the laboratory and/or greenhouse, using standardised techniques, which ensure uniform and repeatable results.

The X-ray technique in seed analysis is a non-destructive method, useful in internal studies such as anatomy, imperfections, physiological changes that occur during maturation and insect attack, among others, based on digitalised radiographic images that, through software, allow measurements and observations of the embryonic cavity of the seed (Altzugaray, et al., 2006). This technique has been used in the analysis of grass seeds, establishing a density profile of specific regions in embryonic structures that caused problems during germination (Pérez-Talavera, 1999). Iglesias, et al (2006) evaluated the morphometry, viability, and variability of *Pinus hartwegii* seed from Perote, Veracruz based on X-ray irradiated seed plates, which allowed the characterisation of seed lots from different populations.

However, its use in seed analysis laboratories in Mexico is not common, largely due to the lack of established protocols, as international protocols have been designed for seed of species. *Pinus devoniana* Lildl is native to Mexico.

General objective of the work

To establish a protocol for seed quality analysis of *Pinus devoniana* based on seed classification through digitised X-ray images.

Methodology

The work was carried out in the seed analysis laboratory of the Germplasm Bank "El Centinela" of the National Forestry Commission (CONAFOR). It was also carried out at the Seed Analysis Laboratory of the Institute of Science and Technology (INCITES) of the Agricultural Production Department of the CUCBA.

A sample of a batch of seeds of *Pinus devoniana* Lindl. was used.

A FAXITRON MX-20 seed X-ray machine was used. Using 400 seeds distributed in 4 replicates of 100. Each seed was identified to follow its physiological condition. Once the images were digitised, the external characteristics were obtained, such as length, width and thickness, and internal characteristics: embryo cavity, embryo length and width of each seed. It was necessary to carry out tests with different numbers of seeds to establish the appropriate calibration of the equipment and the optimum sample size; in subsequent tests of radiographic plates, exposure time and distance between the X-ray emitting tube and the seed were determined with an automatic calibration that varied between 1.3 and 2.7, which allowed a clearer visualisation of the internal structures of the seed, as can be seen in figure 1. It was thus established that, according to the characteristics of the equipment used, the sample size per plate or image is 10 seeds.

Figure 1 X-ray plate image of *Pinus devoniana* seed using a sample of 10 seeds at and speed of 20 seconds

According to ISTA (1996), the regulation of the X-ray equipment depends on several factors such as thickness, density, and composition of the seed.

Viability test with Tetrazolium

The viability test gives a relatively quick indication of the germination potential of a batch of seeds by reacting the tetrazolium salt and the hydrogen released during seed respiration, generating a red staining of living cells. Seeds stained red in all their structures are considered live seeds (figure 2).

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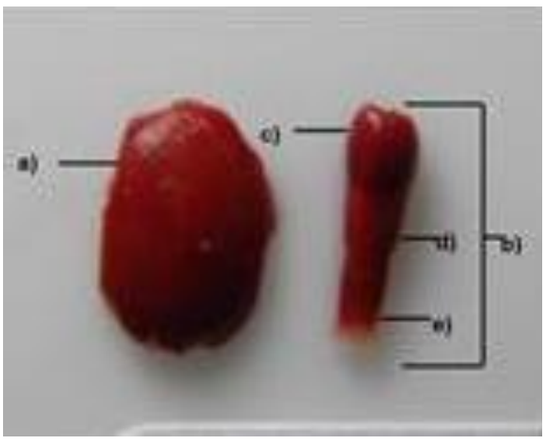


Figure 2 Seed completely stained red in all its structures. a) Endosperm, b) Embryo, consisting of c) Cotyledonary leaves, d) Hypocotyl and e) Radicle.

Figure 3 Dead seeds

Germination analysis

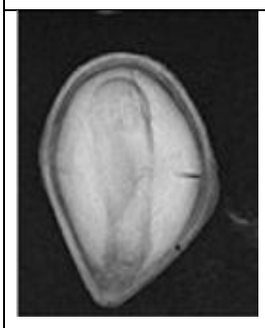
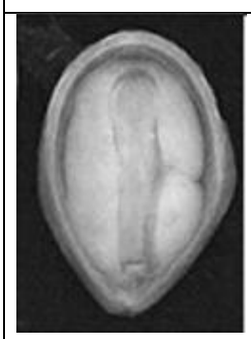
The standard germination analysis was carried out by incubating the seed at 25°C for 14 days using germination paper as substrate. 14 days using germination paper as substrate, the seedlings obtained were classified as normal, abnormal, in addition to counting the dead seeds, figure 4 shows the irradiated seedlings obtained in this test.

Figure 4 Seedlings of *P. devoniana* from the St. George germination test

From the X-ray images and the results obtained in the initial standard germination test, a seed classification was established by relating the physiological response of the seed and internal morphological characteristics such as: the percentage of filling of the embryo cavity, the number of seeds in the embryo cavity, the percentage of seeds in the embryo cavity and the percentage of seeds in the embryo cavity.

Visualisation of development of embryo structures, malformations, density and glassy or hyaline appearance, embryo detachment and even invasion and pest damage. This classification of irradiated seed was the basis for seed classification used in the vigour analyses carried out.

Seed grading pattern

	Visually healthy seed, with defined internal structures. Cover without damage or cracks. Entire endosperm does not present dark, vitreous areas or fissures. Embryonic cavity with more than 90% filled by the embryonic axis. Embryo attached to the basal area of the endosperm, without fissures or alterations. It develops a normal seedling. Viable seed in tetrazolium analysis.
	Seed without external damage. Endosperm with dark, opaque vitreous areas or fissures. Vitreous embryo, with dark areas, without defined embryonic axis structures. Cotiledoeas illegible leaves, have dark or vitreous areas. Visible deterioration due to physical damage. It does not germinate. Seed NOT viable in tetrazolium analysis.
	Damaged endosperm. Embryo attached the basal area of the endosperm shows alterations, the cotyledonary axis is vitreous. Cotyledonary leaves may or may not be legible with dark or vitreous areas. It develops abnormal or low vigor seedlings. Seed NOT viable in tetrazolium analysis.

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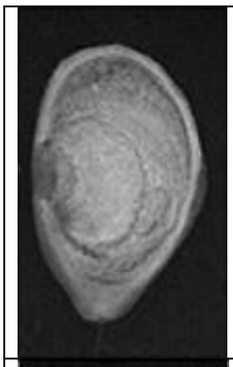
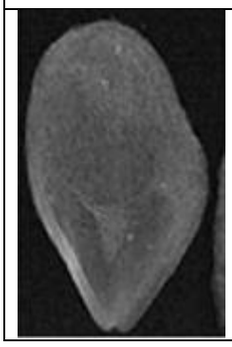
	Seed with hardly visible external damage. Sweeps for plague. Vain seed.
	Seeds without external damage. Dark and vitreous colorations in embryo and endosperm. Not filling of embryonic cavity and embryo thinning. Abnormal seedling.
	Seed without external damage. Intact endosperm with no fissures or vitreous, opaque or dark areas. It does not present defined embryonic axis structures, amorphous, vitreous tissue. It does not germinate, it is classified as dead seed. NON-viable seed in tetrazolium assay.
	Seed without external dali. Vana.

Table 1

Results

Seeds without visual damage showed up to 26.4% and 31.6% physiological damage, respectively, when analysed radiographically, which indicates the usefulness of the X-ray method in the evaluation of internal damage in relation to visual analysis. Similar results were observed by Carvalho et al (1999) in the analysis of pre-harvest internal damage in maize seeds.

Correlation coefficients of .70** with the seedling length test; .796** for viability with tetrazolium; .37NS with accelerated ageing and .818** between cavity filling and those classified as more vigorous.

Seed viability was also found to be affected by storage time, reflected in a lower percentage of stained seeds in the Tz viability test between the first and second samples.

According to McDonald (1994), although the X-ray test is a physical test, it provides information that can assist in viability assessments and can reveal morphological deficiencies that indicate the structural potential for viability.

Acknowledgements

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Conclusions

The X-ray analysis test gives a quick and useful insight into the quality of the seed lot of *Pinus devoniana*, as it shows clear and accurate images of the physical condition of the seeds without the need to destroy the sample.

In general, X-ray analysis of pine seed was effective in detecting damage and abnormalities and provides comprehensive information about the association of seed morphology and seedling development and should be used as a supplementary procedure to assess the physiological potential of the seed.

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Cloning and characterisation of the xylose transporter coding gene of *Debaryomyces hansenii* in *E. coli*

Clonación y caracterización del gen codificador del transportador de xylose de *Debranomyces hansenii* en *E. coli*

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Abstract

Xilitol is 5 C alcohols, and noncariogenic noncaloric sweetener usually obtained by a chemically reaction of D-xylose hydrogenation. An alternative to this reduction reaction is the use of highly polyol producing yeast from genus *Debaryomyces* and *Candida*. In our project we have amplified by PCR and cloned the gen encoding for a transmembranal transpoter of xylose. The cloned gene was sequenced and characterized for further transfer to Saccharomyces cerevisiae to study the capacity of transformed yeast to use xylose as a carbon source and its possible role in xylitol production.

***D. hansenii*, Xylose, Conveyor**

Resumen

El xilitol es un alcohol de 5 C, es un edulcorante no calórico, es obtenido por la reducción química del azúcar D-xilosa por hidrogenación. Una alternativa de la producción química es la utilización de levaduras altamente productoras de polioles del género *Debaryomyces* y *Candida*, aplicando ingeniera genética. En nuestro proyecto hemos amplificado por PCR y clonado en E. coli el gen codificador del transportador de xilosa, para posteriormente transferirlos expresarlos en cepas de Saccharomyces cerevisiae para estudiar la capacidad de utilizar xilosa como fuente de carbono y su posible rol en la producción de de xilitol.

***D. hansenii*, Xilosa, Transportador**

Citation: DE LA RIVA-DE LA RIVA, Gustavo Alberto, COLLI-MULL, Juan Gualberto and JUÁREZ- SALDAÑA, Eric. Cloning and characterisation of the xylose transporter coding gene of *Debaryomyces hansenii* in *E. coli*. ECORFAN Journal-Republic of Nicaragua. 2020. 6-11:10-14.

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Introduction

Fermentation processes have always been a field of permanent interest in biotechnological research and industrial biotechnology development. The production of ethanol and xylitol by fermentative processes is one of the areas where fermentative processes are constantly being improved for applications in both the food industry and biofuel production. Currently, fermentative processes are used to convert residues from primary forest products into industrial by-products such as ethanol and xylitol. To improve this type of process we sought to create genetically modified Saccharomyces cerevisiae strains capable of using xylose as a carbon source. These attempts have not resulted in more efficient processes because S. cerevisiae is a very efficient yeast in hexose fermentation processes, but incapable when it comes to using pentoses as a carbon source. For this reason, we tried to improve this situation by transferring the xylose transporter coding gene from *Debaryomyces hansenii* into S. cereviciae strains. This is a hylotrophic yeast that efficiently uses pentoses and xylose as a carbon source.

Xylitol is a major by-product in the production of ethanol from lignocellulosic by-products that is widely used in the food and pharmaceutical industries, as it has useful characteristics, including its use for the prevention of dental caries, as a sugar substitute for insulin-independent diabetics, and as a natural food sweetener (Makinen, 1992).

Xylitol is currently produced on an industrial scale by catalytic reduction (hydrogenation) of xylose obtained from wood sources such as white birch trees. *Candida intermedia* has been reported (Leandro et al; 2006) as a yeast with the ability to grow in xylose-rich media and to transport this pentose by two different transport systems: a high-affinity system, in which it is carried out by H^+ symport, and a low-affinity system by facilitated diffusion; both systems use glucose as substrate. *D. hansenii* has been described as a halophilic-halotolerant yeast (González-Hernández, J. C. et al., 2004, 2005), which can metabolise D-xylose to xylitol (Gírio et al., 2000). This characteristic is an interesting and potential biotechnological aspect due to the dietary and clinical characteristics of xylitol.

At the same time, a molecular approach will be followed to identify the genes involved in this process (GXF1, GXS1, XR). Our aim is to have basic knowledge of the xylose transport and metabolism systems in *D. hansenii*; and in the immediate future to establish and propose this technology for xylitol production. The enzyme xylose reductase (XR) is responsible for the first step in xylose metabolism in yeast (Chiang and Knight, 1960). In a reaction catalysed by this enzyme, xylose is reduced to xylitol which can be oxidised to xylulose by the enzyme xylitol dehydrogenase (XDH) or can be released into the environment, depending on the conditions of the culture medium (Kern et al., 1997; Ho et al., 1998). Studies on the extraction and purification of XR from yeast have been conducted with the aim of characterising the enzyme to implement better fermentation processes or to obtain a purified solution of XR to be used directly for the conversion of xylose to xylitol (Mayerhoff et al., 2001; Cortez et al., 2001).

Candida magnoliae is a yeast capable of growing and utilising xylose as a carbon source and, on the other hand, accumulating xylitol, preventing it from being transformed into D-xylulose. *D. hansenii* is a highly polyol-producing yeast that can have a potential use for the utilisation of lignocellulosic hydrolysates, which has been extensively studied for this bioconversion to take place.

We have designed a strategy for the study and characterisation of the xylose transporter gene of *D. hansenii*. PCR amplification of the complete gene and separate PCR amplification of the promoter and coding region fragments was performed. The amplified fragments were cloned into an E. coli vector and sequenced. We also included a strategy to manipulate these fragments for transfer to an ethanol-overproducing *Saccharomyces cerevisiae* strain isolated from industrial processes to evaluate their behaviour and fermentation efficiency in both ethanol and xylitol production.

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Materials and methods

Strains and culture media

Four different yeast strains were used, two of them from *D. hansenii* and *C. magnoliae*, which grow efficiently using xylose as carbon source and two *Saccharomyces cerevisiae*, one of them, *Saccharomyces cerevisiae* W303-1 Ura-, is an auxotrophic mutant to test the constructs to be tested and the other is the *Saccharomyces cerevisiae* strain ITM 2014 and is characterised by its high efficiency in fermentative ethanol production (Table 1). The layers were grown on various culture media, YPD being the most suitable.

It included yeast extract, usually 1% mass/volume ratio with water, 2% peptone, 2% glucose. When glucose was required, it was replaced by 2% xylose and called YPX. *E. coli* strain DH5 alpha was used for cloning. And grown on LB medium. When required, the medium was supplemented with 75 mg/l ampicillin, and/or 40 µg/l 5- bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 40 µg/l isopropyl-β-D-thiogalacpyranoside (IPTG).

Yeast kinetics in YPD and YPX media

A growth kinetics of the yeasts of interest for this project was performed to determine their behaviour both on glucose and xylose in the culture medium. These yeasts were grown in 50 mL batches of YPD (Yeast Extract, Peptone and Glucose 2%) or YPX (Yeast Extract, Peptone and Xylose 2%) media at 110 rpm and a temperature of 28°C. Samples were taken for 24 hours every 2 hours and optical density parameters were measured at 595 by spectrophotometry in visible light. The pH was also measured, the number of cells per mL of culture was determined (seeding dilutions every 4 hours).

Design and synthesis of primers for PCR amplification

Based on the comparative analysis of nucleotide sequence information of *C. intermedia and D. hansenii* from the NCBI GenBank and EMBL databases (www.ncbi.nlm.nih.gov; www.embl.de), different pairs of primers or oligonucleotides are designed to facilitate the cloning of the regulatory and coding regions of the genes from total DNA.

The characteristics of the primers are summarised and their purpose is summarised in the table below.

Extraction of total DNA from *Debaryomyces hansenii*

We established the optimal growth conditions for *D. hansenii* by using two methods: one based on a commercial extraction kit (Yeastar Genomic DNA kitTM, Zymo Research) and the other based on breaking with glass beads and detergent solution (Winston Solution: 2% Triton, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1mM EDTA).

PCR amplification of DNA fragments corresponding to the complete xylose transporter gene, its promoter region and coding region.

Conditions were optimised for separate amplification of the promoter region (1'-95°C, 1'-53°C, 1'-72°C), the coding region and its terminator (1'-95°C, 1'-53°C, 1'-72°C). Coding region and its terminator (1'-95°C, 1'-53°C, 2'-72°C) and the whole gene (1'-95°C, 1'-53°C, 3'-72°C). The reactions were carried out by standard Polymerase Chain Reaction methodology using DreamTaq™ (ThermoscietificTM). The amplified fragments were analysed by electrophoresis in TA buffer and purified by a commercial kit (ZymocleanTM Gel, Biosys TM).

Cloning in *E. coli* and sequencing

The purified fragments were ligated into a commercial TOPO vector (TOPOTM PCR Cloning, Life Science) and the ligation was transformed into E. coli DH5 alpha competent cells and selected in LB medium supplemented with 100 mg/l ampicillin, 40 µg/l 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 40 µg/l isopropyl-β-D-thiogalacpyranoside (IPTG).

And incubated at 37°C for 18 hours. Possible clones were analysed by restriction with the enzymes Xho1, Spe1 and Sac1 and by analytical PCR using the primers corresponding to each type of amplified fragment (Table 1). The selected E. coli clones were purified and sent for sequencing (T4 OligoTM).

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Results and Discussion

Optimisation of culture conditions. Yeast kinetics in YPD and YPX media

Optimal growth conditions for *Debaryomyces hansenii* and *Candida magnoliae* and the two *Saccharomyces cerevisiae* strains were established. Different rich media were tested including YNB (Yeast Nitrogen Base) and YPD (Yeast Extract, Peptone and Glucose or Xylose) medium. The most suitable medium proved to be the but in the case of *Saccharomyces cereviciae* strain W303-1 Ura-, growth, even on YPD, was always slower compared to the other strains.

Kinetics of *D. hansenii* and *S. cereviciae* yeasts in YPD and YPX media

It was verified that *Debaryomyces hansenii* and *Candida magnoliae* grow satisfactorily both in the presence of glucose (YPD medium) and in the presence of xylose (YPX) while *S. cereviciae* grows efficiently only in the presence of glucose as carbon source. With this experiment we verified that our *Debaryomyces hansenii* and *Candida magnoliae* strains do indeed possess the ability to internalise xylose.

First, the growth conditions were optimised for each of the four yeast strains that will be used throughout this project. The results show that the strains (Fig. 1).

The growth kinetics (Fig. 1) allowed us to determine the behaviour of both the strains that will be sources of the gene of interest and those that will be hosts of the heterologous gene, determining that the former have the capacity to internalise xylose and metabolise it and the latter cannot, although a certain basal level of xylose internalisation is observed. This is since the YPX medium has a certain number of hexoses that allow basal growth. From this result, we have developed a minimal medium, like M9, composed of salts and where we can completely manipulate the carbon source for each type of yeast we transform. In this way, the transformants that grow on xylose medium will only be those that have received and correctly expressed the gfx gene, which codes for a fully functional protein in the host.

Comparative study of xylose transporter protein sequences

We performed a comparative analysis of the known sequences of the xylose transporters (GenBank www.ncbi.nlm.nhi.gov) of *Candida intermedia* with the sequences corresponding to the two reported complete genomes of *D. hansenii*. The analysis allowed the determination of the region and predicted nucleotide sequence of these homologous genes. We used the *C. intermedia* PYCC 4715 genes gxf1 (glucose/xylose facilitator) and gxs1 (glucsa/xylose symporter, accession number AJ875406) as well as the reported *D. hansenii* genome sequences (*D. hansenii* MTCC 234 and *D. hansenii* CBS767). From this analysis we designed the primers used for PCR amplification (Table 2).

Amplification of the *D. hansenii* gfx gene by PCR and cloning into *E. coli* vector

We first standardised conditions for the extraction of total yeast DNA. As this is a novel yeast, we used the method of total DNA extraction with glass beads and Winston solution but introduced the use of ximolase and the columns of the commercial kit.

With the primers designed, PCR amplification was carried out to amplify the fragments corresponding to the promoter region, coding region, terminator, and complete gene (Figure 2), which were subsequently ligated into a commercial TOPO vector (TOPOTM PCR Cloning, Life Science) and transformed by electroporation into electrocompetent cells.

E. coli DH5 alpha cells. These were seeded onto plates of LBA xgal/IPTG medium and white colonies were selected, which indicated that these cells contained the DNA insert.

The selected clones were analysed by restriction with the enzymes Spe1, Sac1 and Xho1 and by analytical PCR, which gave certainty to the clones of the pPTX series, containing the promoter fragment, pRCTX containing the coding region fragment and PGTX containing the complete gene. The result was corroborated by sequencing of the cloned fragments and their subsequent comparison with the known sequences of the homologous genes.

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Further manipulation of this gene is aimed at cloning it into the plasmid vector of pYES and introducing it into S. cerevisiae W303-1A Ura-la allowing selection by auxotrophy and evaluating in this system the expression of the gene and the functioning of the recombinant protein in medium using xylose as a carbon source.

Subsequently, an integrative vector will be used to transform the S. cerevisiae ITM 2014 strain that is able to use both hexoses and xylose as a carbon source. This, together with the fact that it is a very efficient strain in ethanol production, offers the possibility of optimising industrial alcoholic fermentation processes in the mezcal and wine industries using various fruit substrates, biofuel production and xylitol production.

Conclusions

D. hansenii and *C. magnoliae* can use either hexoses (glucose) or pentoses (xyloses) as carbon source whereas *S. cerevisiae* only grows efficiently on substrates containing hexoses. If S. cerevisiae acquires the ability to internalise xylose more efficiently, this may result in optimisation of various industrial fermentation processes, including better utilisation of lignocellulosic hydrolysers, production of wines and mezcal, and the possibility of designing an alternative process for xylitol production.

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Article **ECORFAN Journal-Republic of Nicaragua**
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Molecular characterisation of the bacterial diversity potentially degrading triclosan present in the Xichú river basin, Guanajuato

Caracterización molecular de la diversidad bacteriana potencialmente degradadora de triclosán presente en cuenca del rio Xichú, Guanajuato

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Abstract

Triclosán (TCS), is a trichlorinated phenoxyphenol having antibacterial properties. The aim of this study was to molecularly characterize bacterial diversity with potential to degrade Triclosan in isolated samples Xichú River in the Biosphere Reserve Sierra Gorda of Guanajuato. For molecular characterization of bacteria-based amplification techniques 16SrRNA gene sequences were used. The amplification products were purified and analyzed and compared sequences in databases. These affiliations allowed to infer phylogenetic relationships among prokaryotes. The analysis shows that the microbial diversity with potential to degrade triclosan is dominated by members of the genus *Bacillus* belonging to fermicute taxa, and to a lesser extent *Aeromonas* belonging to taxa Gama-proteobacteria, both groups considered as potential organisms for bioremediation sites contaminated. This work is the first report documenting the molecular characterization of bacteria with the capacity to resist and degrade Triclosan in Guanajuato. These data are of great value when implementing future technologies for the remediation of contaminated in the field of bioremediation environments.

Triclosan, Emerging contaminants, Bacterial diversity, Biodegradation

Resumen

El triclosán (TCS), es un fenoxifenol triclorado con propiedades antibacterianas. El objetivo de este estudio fue caracterizar molecularmente la diversidad bacteriana con potencial para degradar Triclosán en muestras del Río Xichú en la Reserva de la Biosfera "Sierra Gorda" de Guanajuato. Para la caracterización molecular se utilizaron técnicas basadas en la amplificación de secuencias del gen 16SrRNA. Los productos de la amplificación fueron purificados y las secuencias analizadas y comparadas en bases de datos. Estas afiliaciones permitieron inferir relaciones filogenéticas entre los organismos. El análisis muestra que la diversidad microbiana con potencial para degradar triclosán es dominada por el género *Bacillus* que pertenecen al taxa fermicute, y en menor proporción el género *Aeromonas* perteneciente al taxa gamma- proteobacteria, ambos grupos considerados como organismos potenciales la biorremediación de sitios contaminados. Este trabajo es el primer reporte que documenta la caracterización molecular de bacterias con capacidad para resistir y degradar Triclosán en Guanajuato. Los datos son de gran valor a la hora de implementar futuras tecnologías para la recuperación de ambientes contaminados en el ámbito de la biorremediación.

Triclosán, Contaminante emergente, Diversidad bacteriana, Biodegradación

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Introduction

In the last decades, among the organic pollutants in water, so-called emerging pollutants have been increasingly detected, which correspond in most cases to unregulated pollutants, which may be candidates for future regulation, depending on research on their potential health effects and monitoring data regarding their occurrence. Pharmaceuticals used in humans and animals have been identified as emerging environmental contaminants (Daughton, 2004).

The list of new pollutants includes analgesics, anti-inflammatory drugs, anti-epileptics, blockers and antibiotics, and even personal care products such as soaps, toothpastes, and deodorants among others, which are discharged into sewage systems. The presence of these emerging pollutants in municipal effluents has been dispersed into the environment causing a negative impact on both health and ecosystems (Ternes et al., 2004). Some of these compounds can alter the endocrine system by blocking or disrupting functions in humans and animals even when found at exceptionally low concentrations (García-Gómez et al. 2011). Recent studies show that it is increasingly common to find concentrations of nanogram to micrograms per litre for many of these products in wastewater, making treatment techniques for these compounds insufficient (Vienoa et al. 2007). A clear example of emerging contaminants is Triclosan, which is used as a bactericide. This emerging contaminant has no industrial regulation and is dispersed in the environment, affecting the lives of different organisms.

The suggested mechanisms by which TCS is removed from surface waters are biodegradation, photolysis, chlorination and association with surface solids. Since conventional methods have not shown convincing results for the removal of these pollutants, biodegradation emerges as an attractive alternative. In that sense, the search for triclosan degrading microorganisms is a tool that can be used in biological digestion to complement and/or increase the effectiveness in the removal of triclosan and other persistent pollutants.

Literature review Triclosan

Triclosan [5-chloro-2-2,4-dichlorophenoxyphenol], TCS, is a trichlorinated phenoxyphenol, with the chemical formula $C_{12}H_7Cl_3O$, has a molecular weight of 289,546, and a function point of 55-57 ℃, is a potent antibacterial and fungicidal agent. It is a colourless solid with a slight phenol odour. It is a chlorinated aromatic compound which has functional groups representative of ethers and phenols. Its solubility is, 0.01 g/L for water; 0.1 N NaOH, 23.5 g/L; ethanol, and in acetone it is highly soluble (FDA, 2008).

Triclosan is present in many disinfection-related products such as soaps, deodorants, cleansers, shampoos, and cosmetics. In addition, it is suitable for introduction into polymers and fibres, mattress pads, cutting boards, shoes, and sportswear (Glaser A; 2004).

Triclosan transformation products and related compounds

Due to their reactivity, their main transformation products as hydrolysis products of triclosan in aqueous media are the chlorophenols: methyltriclosan, 2,4-dichlorophenol, 2,3,4-trichlorophenol and 2,4,6-trichlorophenol.

Dioxins are one of the most dangerous products of triclosan photodegradation, can be highly carcinogenic and can cause such serious health problems as suppression of the immune system, decreased fertility, disruption of sex hormones, birth defects, miscarriages, and cancer. TCS is listed as a "possible" precursor contaminant of dioxins (Latch, et al, 2005).

Antibacterial properties

Triclosan is a phenolic derivative that at low concentrations inhibits essential enzymes of metabolism or binds to essential cell wall metabolites, causing wall degradation in some groups of bacteria. It exhibits bactericidal properties on Gram+ and Gram- bacteria as well as fungi and yeasts. (Canosa-Rodríguez, 2009)

At high concentrations it causes cell lysis and inhibits the enzyme enoyl-ACP (acyl carrier protein) reductase which is involved in fatty acid synthesis (Trilla, 2005).

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Environmental problem of Triclosan: Sources of contamination

Its production began in the 1970s for personal care, as a control of bacteria that could affect human health.

Between 1976 and 2010, the US Patent and Trademark Office issued more than 2,900 patents containing the word "Triclosan".

As it is present in multiple products related to disinfection such as veterinary products, medicines, cosmetics, fragrances etc. The main sources of environmental contamination by triclosan considered according to Dann and Hontela (2011) are domestic wastewater discharges as well as incomplete removal of triclosan in wastewater treatment plants, allowing its distribution on soil and water surfaces.

In different countries around the world, including Mexico, triclosan has been detected in treated and untreated water, as well as in effluents and effluents from lakes, rivers, and seawater among others (Canosa-Rodriguez, 2009).

Effects of Triclosan

One of the biggest problems arising from the introduction of triclosan into the environment is the adverse effects it can cause. The main affected by this problem are aquatic flora and fauna, such as unicellular algae and cyanobacteria. According to a study by Orvos in 2002, these unicellular organisms suffered growth inhibition using Triclosan concentrations of between 1.3 and 13 ng/mL. About amphibians, in the species *Rana pipiens* (leopard frog) it was observed that the organism lost weight, as well as a high mortality rate at Triclosan concentrations of 230 ng/L (Fraker, 2004).

For the species *Rana catesbiana* (bullfrog), thyroid hormone-mediated changes in their premature metamorphosis have been observed, affecting their phenotype as an increase in tadpole tail size and decrease in weight using a concentration of 150 ng/mL (Veldhoen, 2006).

Reports for mammals state that in rats Triclosan decreases thyroid hormone levels but the concentrations needed to observe these changes are higher for aquatic organisms as 30 mg/kg per day is necessary (Crofton, 2007).

Triclosan degradation processes

Because Triclosan is dispersed in the environment and difficult to remove by conventional methods, several mechanisms of Triclosan degradation have been studied. One alternative is advanced oxidation processes (AOP), where the use of different photocatalysts such as hydrogen peroxide, ozone, and metal oxides of zinc and titanium have been investigated, the results have been relatively good at low concentrations, some methods use Pd/Fe nanoparticles, photodegradation, even ultraviolet radiation and free chlorine (Molina, 2014). In the case of free chlorine, degradation varies according to the pH of the sample; the more neutral the pH, the more effective the degradation will be (Canosa et al; 2005).

Other little studied methods are the biodegradation of Triclosan with bacteria. Recently it has been found that some bacteria are able to resist this compound and even degrade it, some of the genera of these bacteria are Pseudomonas (Molina, 2014) and Achromobacter xylosoxidases (Canosa, 2008).

These resistance capabilities have been documented for two types: Intrinsic and Acquired which arise by mutation or by the acquisition of genetic material in the form of plasmids or transposons; these configurations allow large arrays of resistance genes (Cabrea et al; 2007).

Other wastewater microorganisms that have been documented to have the ability to degrade triclosan include *Sphingomonas* spp. Rd1, *Nitrosomonas europaea*, *Sphingomonas* spp. PH-07 and *Sphingophyxis Strain* KCY1 (Hay et al. 2001, Roh et al. 2009, Lee et al. 2012 in Lee, et al., 2013).

In most cases, treatment to remove a compound does not necessarily involve mineralisation, so the likelihood is that the parent compound has been transformed, changing its functionality and toxicity.

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In the case of treatments with microorganisms, the problem lies in the pathogenicity of most of the microorganisms studied. On the other hand, the studies carried out to determine the presence of triclosan-degrading microorganisms in Mexico are quite scarce, without specifying the specific basins investigated.

It is necessary to use new technologies aimed at solving this problem and guaranteeing the complete elimination of these pollutants, which can have serious consequences on the health of human beings, flora, and aquatic fauna in the effluents where they are discharged.

Analysis of bacterial diversity

Using molecular techniques using phylogenetic markers such as the 16S ribosomal gene, genomic libraries have been constituted whose members of different groups and sub-groups include in their order: Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes and Firmicutes (as the most abundant), (Forney, 2004).

Our working group has recently reported physicochemical and microbiological parameters of water quality, as well as the isolation of triclosan resistant and degrading microorganisms in the micro-watershed of the Xichú river and its intersection with the Laja river in the hydrological sub-watershed of the Santa María river in the Sierra Gorda Biosphere Reserve of Guanajuato (González C. et al, 2014).

In this context and given the importance of the use of microorganisms in biodegradation processes, this work proposes to contribute to the identification and molecular characterisation of the bacterial diversity involved in the degradation of this type of pollutants.

Methodology

Microbial resources

The bacterial strains were previously collected from water bodies in the state of Guanajuato, obtaining five strains from the municipality of Xichú, Gto. with the key M1SA, M310-3, M4AA, M1Sp and M110-3ª (González C., et al., 2014) and one strain from the municipality of Yuriria, Gto. with the key E4. (Rodríguez-Rodríguez, et al., 2013) for a total of 6 strains selected for their ability to use Triclosan as a carbon source.

DNA extraction

Bacterial isolates were inoculated in liquid LB medium (5 ml) for 48 hr. A pellet is obtained after centrifugation at 110 rpm. Lysis was carried out in 300 µl of buffer (TRIS- EDTA) and vortexed for 1 min followed by mechanical lysis with glass beads (Atashpaz et al; 2010). Subsequently 10% SDS was added and subjected to shaking. Then 50 µl NaCI (5 M) and 50 µl LiCL (5 M) were added and allowed to stand for 5 min at room temperature. 5ul RNA was added to each tube. They were then incubated at 37°C for 10 min. (Eguiarte et al; 2007). 500 µl of the supernatant was taken and a volume of phenol-chloroform was added to the samples. 400 µl of the aqueous phase was taken and 2 volumes of absolute ethanol were added with vigorous shaking. Finally, the genetic material pellet could dry. Finally, it was suspended in 50 µl of sterile water, shaken gently to dissolve and left to freeze at -20°C (Zavala, 2005).

Horizontal agarose gel electrophoresis

DNA visualisation was performed in a horizontal electrophoresis chamber on 1% agarose gels in TAE 1X buffer. 2µl of DNA extraction sample and 1µl of GelGreen loading buffer were loaded, mixed and placed in the wells. Finally, the chamber was set to 100 volts for 30 min. The molecular marker was placed in the last well. The displacement of the samples was observed through a transilluminator. The image was transferred to a PC.

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Amplification of the bacterial 16S rRNA gene by polymerase chain reaction (PCR)

For the amplification of the 16S rRNA genes by PCR technique, the universal primers F27 and R1492 (Wang, 1996) specified in Table 1 were used. 25 µl of 2x Dream Taq Mix, 1µl of the universal primers F27, 1µl of DNA sample and 22µl of sterile water were placed in an ependorff tube, thus obtaining a volume of 50 µl.

Indicator code	Sequence (5´- 3´)	Size
F27	AGAGTTTGATCMTGGCTCAG	20
R1492	TACGGYTACCTTGTTACGACTT	22

Table 1 Initiators Used for Amplification and Sequencing

They were then placed in the Multigene thermal cycler with the following amplification conditions: First stage at 95 °C, 5 min, which at 35 cycles as shown in Table 2.

	(1 cycle)		(35 cycles)		(1 cycle)	
Temp.	105°C	94°C	94°C	53°C	72°C	4°C
Time	3:00 min	0:50 min	1:00 min	1:30 min	5:00 min	∞

Table 2 Temperatures and times used in the Polymerase Chain Reaction process at a volume of 50µl

This amplification process was carried out twice to ensure a significant sample in the purification process, thus obtaining a total volume of 100 µl per sample. The PCR products were then subjected to electrophoresis in a 1% Agarose gel for 30 minutes at 80 volts.

Purification of Amplification Products

The purification of the genetic material was carried out with the Zymo DNA Clean & Concentrator™-25 Purification Kit (Zymo Research) obtaining a total volume of 40 µl per sample. Finally, electrophoresis was performed by placing 2 µl of the purified sample and 1µl of GelGreen to observe the purified sample.

16S Ribosomal Gene Sequencing

After purification, approximately 38 µl of each sample was sequenced at the Cinvestav-Langebio Genomic Services Department using a Sanger sequencing process.

Molecular analysis of the 16S ribosomal gene of the bacterial diversity with potential for Triclosan degradation.

The last step was the comparison of the sequences obtained with those deposited in the databases. The first step was to use the Blast software of the NCBI (National Center for Biotechnology Information) website to compare the sequences with those of this database. In the next step, the GreenGen database was used, which contains only bacterial sequences of the 16s gene, with the aim of downloading type sequences to observe the similarities between groups, at this point the type sequences of the species with affinity were downloaded. Finally, the 6 sequenced samples and the type sequences were subjected to a maximum likelihood analysis in the MEGA software for the elaboration of a phylogenetic tree, thus observing the phylogenetic relationships between each group.

Results and discussions DNA extraction and visualisation

The extraction process was essential for DNA purification and well defined bands were achieved as shown in Figure 1. The amount of DNA was sufficient and reliable for the amplification process shown below. From the DNA integrity analysis it is concluded that the samples present an acceptable concentration and purity for subsequent amplification.

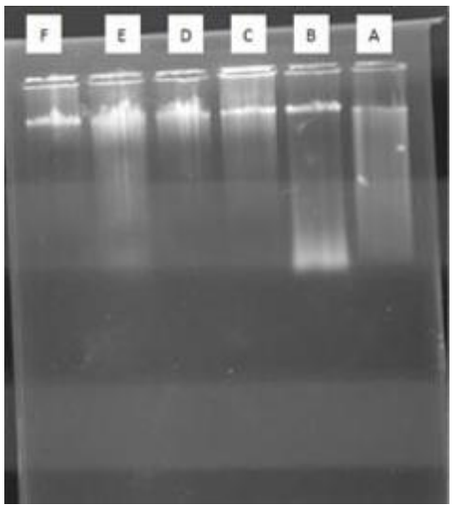

Figure 1 DNA integrity of Chromosomal DNA samples from bacterial strains with the ability to use Triclosan as a sole carbon source, A.- E4, B.- M1SA, C.- M310-3, D.- M4AA, E.- M1Sp, F.- M110-3

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PCR amplification products

Once the 16s rRNA gene was amplified to a volume of 50µl, an electrophoresis was carried out to determine if the observed bands were reliable for purification, the band corresponding to the amplification product of approximately 1500 bp was observed in the samples.

This process had to be repeated several times because not all amplifications were successful, even when the genetic material looked reliable, this is due to substances that interfere with the polymerase by partially or totally blocking its catalytic activity such as some salts (Newlester-Microbial 2009), a factor in reproducing the amplification was the required volume of 100µl of each sample. Figure 2 shows the PCR products for the amplification of the 16S rRNA gene of the bacterial samples M310-3, M4AA and M1Sp. In the other three samples, the 16s gene is not observed. Finally, after making the corresponding adjustments, the amplified products were obtained for samples M1SA and M110-3 and E4 (not shown).

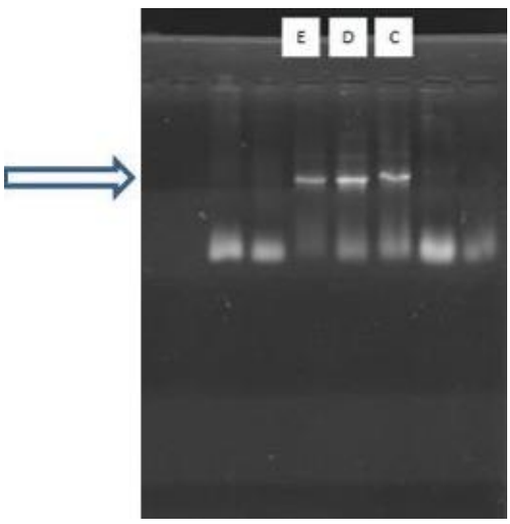

Figure 2 Amplification product of approximately 1500 bp C.- M310-3, D.- M4AA, E.- M1Sp

Purification of amplification products

Samples were purified by the Zymo DNA Clean & Concentrator™-25 Purification Kit. As a product of the purification two fragments are obtained for most of the samples as can be seen in figure 3, both fragments differ from the expected size of 1500 bp.

The results are reproduced by repeating the experiment, however, the purification method proved to be effective for sequencing the amplified fragment. A possible explanation could be interference in the gel run due to an overload of genetic material.

Sequence analysis and identification of Triclosan-degrading bacterial diversity

The edited sequences were run in the BLAST algorithm of NCBI, to determine the degree of homology of the sequences of the isolated strains with respect to the type sequences deposited in the GreenGen database, which contains only bacterial sequences of the 16s gene, twelve type sequences of several species of the genus *Bacillus* and the genus *Aeromonas* were downloaded. The 6 sequenced samples and the twelve type sequences were subjected to a maximum likelihood analysis in MEGA software for the elaboration of a phylogenetic tree (Figure 4) observing the phylogenetic relationships between each group. Where sample E4, M1AA and M110-3 have an affinity with B. *safensis* and pumilus, while sample M1Sp has a phylogenetic relationship with *B. subtilis*, sample M310-3 has a strong resemblance with B. cereus and thuringiensis, and finally sample M1SA has a strong phylogenetic relationship with *Aeromonas hydrophila*.

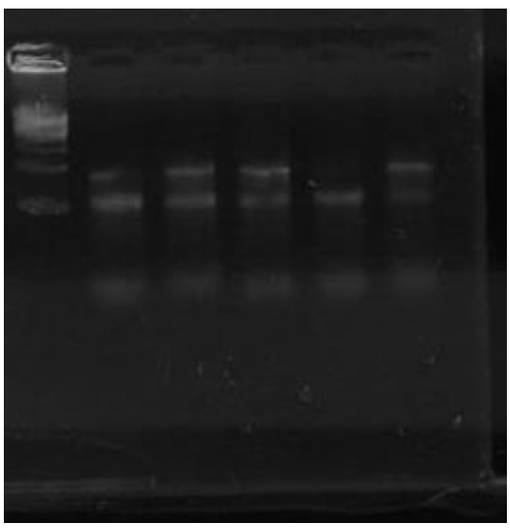

Figure 3 1% Agarose Gel purified amplification fragments, lane 1 molecular marker 1kb (left) lanes 2-6 Samples E4, M1SA, M310-3, M4AA, M1Sp

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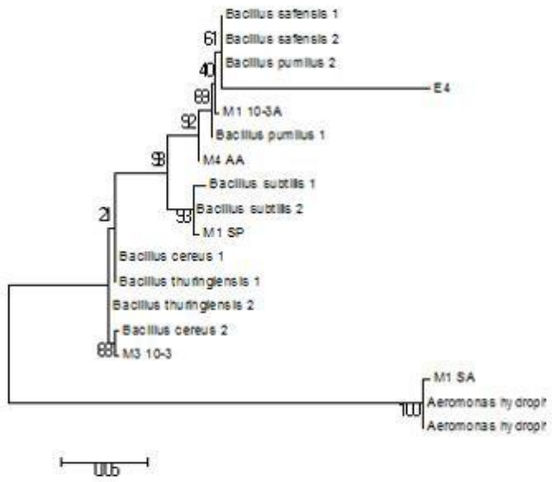

Figure 4 Phylogenetic tree made in MEGA, showing the phylogenetic relationships between the sequenced samples and the type sequences.

The microorganisms of the genus *Bacillus* are large (4-10 µm), Gram-positive, strict aerobic or encapsulated facultative anaerobic bacilli.

An important characteristic is that they form spores that are extraordinarily resistant to unfavourable conditions (Bartram et al; 2003), which may be a key feature for their ability to grow in contaminated water bodies and/or in the presence of Triclosan. B. safensis and pumilus bacteria are closely related based on phenotypic characteristics and 16s sequences (Branquinho et al; 2007), both species are reported to be found in industrial wastewater environments (Satomi, 2006), which could be another indicator for Triclosan resistance. However, *B. pumilus* species have been found in food-borne infections and even skin infections in humans (Bentur, 2007), which represents a more sensitive handling for this species.

B. subtilis species are generally found in sediments and in the rhizosphere and produce heat resistant endospores and have a resistance to chemical disinfectants (Cuervo, 2010) and possibly gaining resistance and degradation to Triclosan. B. cereus is a food pathogen and is closely related phylogenetically to *B. thuringiensis* and this species can be differentiated from *B. cereus* because it produces crystals inside its cell during sporulation, however there is no report that *B. thuringiensis* has a parasitic life cycle, both have been isolated from water bodies as they are found in almost any environment (Perez, 2005) and it can be deduced that they would be found in contaminated sites.

For *Aeromonas hydrophila*, the characteristics of the genus refer to short Gram-negative bacilli between 0.3-1.0 µm (Altweeg, 1999), which are pathogenic organisms of reptiles, but there are reports of skin infections and diarrhoea in humans.

The life cycle is aquatic and previous isolates have been reported from sewage and chlorinated water (Kühn et al; 1997), thus possibly providing the ability to degrade to Triclosan.

All the bacterial strains found could have developed resistance to Triclosan thanks to intrinsic or acquired capacities and which could be indicators of contaminated water as they are resistant to several contaminants thanks to their previous isolations in contaminated water which has given them this resistance to the compound Triclosan, however, more studies are needed on the degradation pathways that these strains possess.

Conclusions

Six potentially Triclosan-degrading bacterial strains were found, of which five belong to the genus Bacillus and one to the genus Aeromonas. The characterised bacteria could be used to degrade Triclosan in further studies due to their biodegradation potential. However, it is necessary to consider that some species are classified as pathogenic and care should be taken in their handling, such as B. cereus and Aeromonas.

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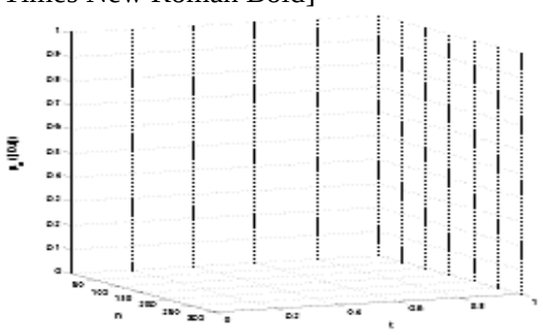

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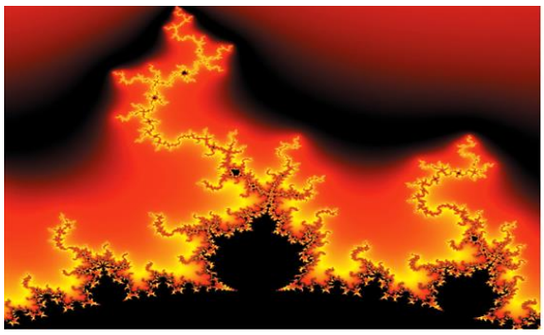

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