DESARROLLOS TECNOLÓGICOS PARA LA MEJORA Y CONTROL DEL PROCESO DE OBTENCIÓN DE BIOETANOL A PARTIR DE RESIDUOS AGROALIMENTARIOS

Tesis Doctoral

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Valencia, Junio 2017

Los Profesores, **Dr. Pedro Fito Maupoey**, Catedrático de Universidad, **Dra. Lucía Seguí Gil**, Profesora Contratada Doctora del Departamento de Tecnología de Alimentos y **Dr. Nicolás Laguarda Miró**, Profesor Contratado Doctor del Departamento de Ingeniería Química y Nuclear de la Universitat Politècnica de València,

CONSIDERAN: que la memoria titulada "Desarrollos tecnológicos para la mejora y control del proceso de obtención de bioetanol a partir de residuos agroalimentarios" que presenta Dª. Claudia Conesa Domínguez para aspirar al grado de Doctor por la Universitat Politècnica de València, reúne las condiciones adecuadas para constituir su Tesis Doctoral, por lo que **AUTORIZAN** a la interesada para su presentación.

Valencia, Junio 2017

Fdo. Pedro Fito Maupoey	**Fdo. Lucía Seguí Gil**	**Fdo. Nicolás Laguarda Miró**
Director de Tesis Doctoral	Directora de Tesis Doctoral	Director de Tesis Doctoral

A Nicolás,

A mis Padres,

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Por todos vosotros ha valido la pena este tiempo y esfuerzo.

Os quiero

RESUMEN

Durante la primera mitad de este siglo, se espera que la demanda mundial de energía aumente significativamente debido al previsible incremento de la población mundial y al desarrollo económico global, tal como afirman recientes estudios de la Departamento de Asuntos Económicos y Sociales de las Naciones Unidas. Por otro lado, los datos del Panel Intergubernamental del Cambio Climático vinculan el uso continuado de los combustibles fósiles con el aumento de la concentración de CO_2 y partículas contaminantes a la atmósfera causantes, entre otros, del cambio climático. En este contexto, es necesario seguir avanzando en la búsqueda de alternativas energéticas más limpias y medioambientalmente sostenibles, como es el caso del bioetanol de segunda generación obtenido a partir de residuos agroindustriales.

Así, la presente Tesis Doctoral plantea como objetivo profundizar en la revalorización de los residuos agroindustriales de frutas como la piña y el caqui. En concreto, se evaluaron diferentes estrategias para la mejora del proceso de obtención de bioetanol y el control en línea de la sacarificación y contenido en alcohol en los residuos.

Para ello, se estudiaron diferentes tecnologías para la mejora del rendimiento de la hidrólisis enzimática de los residuos de piña. En primer lugar se comparó la acción hidrolítica de las enzimas comerciales producidas por los hongos filamentosos *Aspergillus niger* y *Trichoderma reesei*. A continuación, se evaluó la aplicación de pretratamientos con microondas, solos o combinados con un medio alcalino, para la mejora del rendimiento de la sacarificación. Por otro lado, se analizó el potencial de revalorización del residuo industrial de caqui de la variedad “Rojo Brillante”. Finalmente se estudió la aplicación de técnicas basadas en la espectroscopía de impedancias para la monitorización de la sacarificación y la cuantificación de bioetanol en piña.

Los resultados obtenidos demostraron que la celulasa de *A. niger* es una alternativa eficaz a la obtenida a partir de *T. reesei* para la sacarificación de los residuos industriales de piña, especialmente cuando se combina con hemicelulasa. Por otra parte, la aplicación de pretratamientos con microondas a potencias y tiempos de exposición apropiados mejoraron significativamente el rendimiento de la hidrólisis enzimática. Esta mejora demostró ser particularmente destacable cuando se combinaron las microondas con un medio alcalino. Por otra parte, se demostró que los residuos industriales de caqui son una fuente de obtención de compuestos de alto valor añadido tales como: bioetanol y compuestos antioxidantes, principalmente carotenoides. Por último, se validó la espectroscopía de impedancias electroquímica como una metodología fácil, rápida, no destructiva, económica y alternativa a las técnicas de laboratorio tradicionales para el control de la sacarificación y fermentación. Esto se consiguió combinando la espectroscopía de impedancias con el desarrollo de modelos matemáticos basados en redes neuronales artificiales que se caracterizan por ser robustos, fiables, adaptativos y fácilmente implementables en sistemas electrónicos.

A modo de conclusión, la presente Tesis Doctoral ha permitido avanzar en el conocimiento sobre el proceso de revalorización de los residuos industriales de frutas mediante la implementación de desarrollos tecnológicos para el aumento del rendimiento de la hidrólisis enzimática del residuo de piña y la obtención de compuestos de alto valor añadido a partir de caqui. Asimismo, se ha demostrado que es posible aplicar técnicas basadas en la espectroscopía de impedancias y modelos matemáticos específicos para mejorar la monitorización y control en línea de estos procesos, lo que supone un avance significativo en este campo.

RESUM

Durant la primera meitat d'aquest segle, s'espera que la demanda mundial d'energia augmente significativament a causa del previsible increment de la població mundial i al desenvolupament econòmic global, tal com afirmen recents estudis del Departament d'Assumptes Econòmics i Socials de les Nacions Unides. D'altra banda, les dades del Panell Intergovernamental del Canvi Climàtic vinculen l'ús continuat dels combustibles fòssils amb l'augment de la concentració de CO_2 i partícules contaminants a l'atmosfera causants, entre uns altres, del canvi climàtic. En aquest context, és necessari seguir avançant en la cerca d'alternatives energètiques més netes i mediambientalment sostenibles, com és el cas del bioetanol de segona generació obtingut a partir de residus agroindustrials.

Així, la present Tesi Doctoral planteja com a objectiu aprofundir en la revalorització dels residus agroindustrials de fruites com la pinya i el caqui. En concret, es van avaluar diferents estratègies per a la millora del procés d'obtenció de bioetanol i el control en línia de la sacarificació i contingut en alcohol en els residus.

Per a això, es van estudiar diferents tecnologies per a la millora del rendiment de la hidròlisi enzimàtica dels residus de pinya. En primer lloc es va comparar l'acció hidrolítica dels enzims comercials produïts pels fongs filamentosos *Aspergillus niger* i *Trichoderma reesei*. A continuació, es va avaluar l'aplicació de pretractaments amb microones, sols o combinats amb un medi alcalí, per a la millora del rendiment de la sacarificació. D'altra banda, es va analitzar el potencial de revaloració del residu industrial de caqui de la varietat “Rojo Brillante”. Finalment es va estudiar l'aplicació de tècniques basades en l’espectroscòpia d'impedàncies per al monitoratge de la sacarificació i la quantificació de bioetanol en pinya.

Els resultats obtinguts van demostrar que la cel·lulasa d’*A. niger* és una alternativa eficaç a l'obtinguda a partir de *T. reesei* per a la sacarificació

dels residus industrials de pinya, especialment quan es combina amb hemicel·lulasa. D'altra banda, l'aplicació de pretractaments amb microones a potències i temps d'exposició apropiats van millorar significativament el rendiment de la hidròlisi enzimàtica. Aquesta millora va demostrar ser particularment destacable quan es van combinar les microones amb un medi alcalí. D'altra banda, es va demostrar que els residus industrials de caqui són una font d'obtenció de compostos d'alt valor afegit tals com: bioetanol i compostos antioxidants, principalment carotenoides. Finalment, es va validar l'espectroscòpia d'impedàncies com una metodologia fàcil, ràpida, no destructiva, econòmica i alternativa a les tècniques de laboratori tradicionals per al control de la sacarificació i fermentació. Això es va conseguir combinant l'espectroscòpia d'impedàncies amb el desenvolupament de models matemàtics basats en xarxes neuronals artificials que es caracteritzen per ser robustos, fiables, adaptatius i fàcilment implementables en sistemes electrònics.

A manera de conclusió, la present Tesi Doctoral ha permès avançar en el coneixement del procés de revalorització dels residus industrials de fruites mitjançant la implementació de desenvolupaments tecnològics per a l'augment del rendiment de la hidròlisi enzimàtica del residu de pinya i l'obtenció de compostos d'alt valor afegit a partir de caqui. Així mateix, s'ha demostrat que és possible aplicar tècniques basades en l'espectroscòpia d'impedàncies i models matemàtics específics per a millorar el monitoratge i control d'aquests processos, fet que suposa un avanç significatiu en aquest camp.

SUMMARY

As stated by the United Nations Department of Economic and Social Affairs, the first half of the present century will experience a significant increase in global energy demand due to the expected growth of world population and global economic development. On the other hand, recent reports from the Intergovernmental Panel on Climate Change definitely evidence the link between the continued use of fossil fuels and the increasing concentration of greenhouse gases into the atmosphere being responsible for climate change. In this context, a global commitment is needed in the search for cleaner, environmentally friendly and sustainable energy sources, such as second-generation bioethanol from agro-industrial waste.

Thus, this PhD Thesis aims to advance in the agro-industrial waste recovery of fruits such as pineapple and persimmon. Specifically, different strategies for enhancing the bioethanol production process were evaluated. Additionally on-line monitoring of the saccharification step and final alcohol content in the studied wastes were taken into consideration.

Thus, different technologies were studied to improve the enzymatic hydrolysis performance in pineapple waste. First, hydrolytic performances of commercial enzymes produced by the filamentous fungi *Aspergillus niger* and *Trichoderma reesei* were compared. Next, the use of microwave pretreatments, alone or combined with an alkali treatment, was evaluated to improve the saccharification performance. On the other hand, “Rojo Brillante” persimmon waste was studied as a potential source of high added value products. Finally, electrochemical impedance spectroscopy based techniques were evaluated for monitoring saccharification and quantifying ethanol in pineapple waste.

Results showed that *A. niger* cellulase is an effective alternative to that obtained from *T. reesei* for the saccharification of industrial pineapple

waste, especially when combined with hemicellulase. On the other hand, microwave pretreatments at appropriate power and exposure times significantly improved the enzymatic hydrolysis performance. This improvement was particularly remarkable when microwaves were combined with an alkali treatment. On the other hand, industrial persimmon waste was shown to be a low-cost source of bioethanol and antioxidant compounds, mainly carotenoids. Finally, electrochemical impedance spectroscopy was validated as an easy, fast, non-destructive, inexpensive and alternative methodology to the traditional laboratory ones for monitoring saccharification and fermentation processes. This validation was achieved by combining impedance spectroscopy with mathematical models based on artificial neural networks, being robust, reliable, adaptive and easily implementable in electronic systems.

To conclude, the present PhD Thesis has provided substantial progress towards agro-industrial waste recovery processes. In fact, several technological developments have been implemented in order to increase the saccharification yield in pineapple waste. Moreover, high added value products have been obtained from persimmon residue. Likewise, these processes can be accurately controlled on-line by electrochemical impedance spectroscopy based techniques combined with specific mathematical models, representing a significant advance in this field.

PRÓLOGO

PRESENTACIÓN

El documento que se presenta es la descripción de los trabajos de investigación realizados por la autora para la obtención del Título de Doctora, que han sido dirigidos por los Doctores Dª Lucía Seguí Gil, D. Pedro Fito Maupoey y D. Nicolás Laguarda Miró y que llevan por título "Desarrollos tecnológicos para la mejora y control del proceso de obtención de bioetanol a partir de residuos agroalimentarios" desarrollados dentro del "Programa de Doctorado en Ciencia, Tecnología y Gestión Alimentaria" que imparte la Universitat Politècnica de València (UPV).

La presente Tesis Doctoral se ha desarrollado a caballo entre dos líneas de investigación. La primera, titulada "Valorización de residuos de la industria agroalimentaria", se lleva a cabo en el Instituto Universitario de Ingeniería de Alimentos para el Desarrollo (IuIAD) de la UPV. La segunda, desarrollada por el Group of Electronic Development and Printed Sensors (GED+PS) del Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) de la UPV, lleva por título "Desarrollo de equipos y dispositivos electrónicos como sistema de detección y actuación basados en nuevas tecnologías electrónicas" y se enmarca dentro del proyecto del Ministerio de Industria, Economía y Competitividad del Gobierno de España: MAT2015-64139-C4-3-R.

Así, esta Tesis Doctoral pretende por un lado mejorar el proceso de obtención de bioetanol a partir de residuos industriales de frutas como la piña y el caqui. Por otro lado, pretende también monitorizar en línea las etapas de sacarificación enzimática y fermentación mediante el uso de la espectroscopía de impedancias electroquímica (Electrochemical Impedance Spectroscopy, EIS).

Para tal fin, se han desarrollado cinco apartados: Introducción, Objetivos, Resultados, Discusión General y Conclusiones. En la **Introducción** se plantea el panorama energético actual, se describe el proceso de obtención de bioetanol a partir de residuos

agroalimentarios y la necesidad de su control a partir de técnicas electroquímicas como la EIS. El apartado de **Objetivos** presenta el objetivo principal y los objetivos específicos de la Tesis Doctoral. La sección **Resultados** se organiza en siete capítulos, correspondientes cada uno de ellos a una publicación científica:

- En primer lugar, se compara en el Artículo 1 *"Hydrolytic performance of Aspergillus niger and Trichoderma reesei cellulases on lignocellulosic industrial pineapple waste intended for bioethanol production"* la acción hidrolítica de las celulasas comerciales producida por los hongos filamentosos *A. niger* y *T. reesei*.
  - En el Artículo 2, titulado *"Microwaves as a pretreatment for enhancing enzymatic hydrolysis of pineapple industrial waste for bioethanol production"*, se estudia la aplicación de microondas como pretratamiento alternativo de los residuos industriales de piña. Para ello, se evalúa su efecto sobre el rendimiento de la sacarificación enzimática, la liberación de compuestos inhibidores de la fermentación y los cambios estructurales observados.
  - Las microondas pueden combinarse con pretratamientos químicos convencionales para aumentar el efecto del pretratamiento. Es por ello que en el Artículo 3 *"Microwave-Assisted Alkali Pretreatment for Enhancing Pineapple Waste Saccharification"* se investiga el efecto del uso de microondas aplicadas en un medio alcalino para mejorar el rendimiento de la hidrólisis enzimática de dichos residuos.
- En el Artículo 4 *"Evaluation of "Rojo Brillante" persimmon industrial residues as a source for antioxidant compounds and substrate for bioethanol production"*, se evalúa el potencial del residuo industrial de caqui de la variedad "Rojo Brillante" como fuente de obtención de bioetanol y de compuestos antioxidantes.
  - Por otro lado, en el Artículo 5 *"An Electrochemical Impedance Spectroscopy-Based Technique to Identify and Quantify Fermentable Sugars in Pineapple Waste Valorization for Bioethanol Production"* se estudia la validez de las técnicas

basadas en la EIS para identificar y cuantificar azúcares fermentables en residuos industriales de piña.

- De esta manera, en el Artículo 6 *"An Electrochemical Impedance Spectroscopy System for Monitoring Pineapple Waste Saccharification"* se pretende validar esta metodología para la monitorización de la hidrólisis enzimática de los residuos de piña.
- Por último, en el Artículo 7 *"Ethanol quantification in pineapple waste by an electrochemical impedance spectroscopy-based system and artificial neural networks"* se comprueba que dicha metodología es capaz de determinar el contenido de etanol disuelto en el residuo y de generar modelos capaces de ser programados en sistemas portátiles de medida.

En el apartado **Discusión General**, se presenta una discusión global de los resultados más relevantes obtenidos durante la realización de la investigación. Finalmente, se presentan las **Conclusiones** más importantes de la Tesis Doctoral y las líneas de trabajo futuras.

DIFUSIÓN DE RESULTADOS

Hasta la fecha de la publicación de esta Tesis Doctoral, la producción científica derivada de la misma ha sido la siguiente:

1) Publicaciones en revistas indexadas en Journal Citation Reports (JCR):

- Conesa, C.; Seguí, L.; Fito, P. (2017). Hydrolytic Performance of *Aspergillus niger* and *Trichoderma reesei* Cellulases on Lignocellulosic Industrial Pineapple Waste Intended for Bioethanol Production. *Waste and Biomass Valorization*, 140: 1-10. Índice de impacto (JCR 2016): 1,337. Q3 Environmental Science (162/229).
- Conesa, C.; Gil Sánchez, L.; Seguí, L.; Fito, P.; Laguarda-Miró, N. (2017). Ethanol quantification in pineapple waste by an electrochemical impedance spectroscopy-based system and artificial neural networks. *Chemometrics and Intelligent Laboratory Systems,* 161 (15): 1-7. Índice de impacto (JCR 2016): 2,303. Q1 Statistic & Probability (12/124); Q1 Mathematics,

Interdisciplinary Applications (20/100); Q2 Automation & Control Systems (23/60); Q2 Instruments & Instrumentation (18/58); Q2 Chemistry, Analytical (35/76); Q2 Computer Science & Artificial Intelligence (48/133).

- Conesa, C.; Seguí, L.; Laguarda-Miró, N.; Fito, P. (2016). Microwaves as a pretreatment for enhancing enzymatic hydrolysis of pineapple industrial waste for bioethanol production. *Food and Bioproducts Processing*, 100 (Part A): 203–213. Índice de impacto (JCR 2016): 1,970. Q2 Food Science & Technology (45/129); Q2 Engineering, Chemical (56/135); Q3 Biotechnology & Applied Microbiology (86/158).
- Conesa, C.; Seguí, L.; Laguarda-Miró, N.; Fito, P. (2016). Microwave-Assisted Alkali Pretreatment for Enhancing Pineapple Waste Saccharification. *Bioresources,* 11 (3): 6518-6531. Índice de impacto (JCR 2016): 1,321. Q2 Materials Science, Paper & Wood (6/21).
- Conesa, C.; Ibáñez Civera, J.; Seguí, L.; Fito, P.; Laguarda-Miró, N. (2016). An Electrochemical Impedance Spectroscopy System for Monitoring Pineapple Waste Saccharification. *Sensors*, 16 (2): 188. Índice de impacto (JCR 2016): 2,677. Q1 Instruments & Instrumentation (10/58); Q2 Chemistry, Analytical (25/76); Q2 Electrochemistry (12/29).
- Conesa, C.; García Breijo, E.; Loeff, E.; Seguí, L.; Fito, P.; Laguarda Miró, N. (2015). An Electrochemical Impedance Spectroscopy-Based Technique to Identify and Quantify Fermentable Sugars in Pineapple Waste Valorization for Bioethanol Production. *Sensors*, 15 (9): 22941-22955. Índice de impacto (JCR 2015): 2,033. Q1 Instruments & Instrumentation (12/61); Q2 Chemistry, Analytical (36/76); Q3 Electrochemistry (16/27).

2) Actas de congresos internacionales publicadas en editorial:

- Conesa, C.; Laguarda-Miró, N.; Olguín Pinatti, C.; Loeff, E.; Seguí, L.; Fito, P. (2016). An approach to the determination and quantification of sugars in fruits by Impedance Spectroscopy. International Conference on Food Innovation (FoodInnova 2014): 409 – 409.
- Conesa, C.; Gómez-Cocera, J.; Laguarda Miró, N.; Olguín Pinatti, C. (2015). Monitorización mediante espectroscopía de Impedancias de la hidrólisis enzimática de los residuos de piña para la obtención de bioetanol de segunda generación. IX International Workshop on Sensors and Molecular Recognition: 348 – 351.
- Conesa, C.; Ferrando, B.; Laguarda-Miró, N.; Seguí, L.; Ibáñez Civera, J. (2015). Aplicación de la espectroscopía de impedancias para la identificación y cuantificación de bioetanol de segunda generación a partir de residuos de piña. IX International Workshop on Sensors and Molecular Recognition: 348 – 351.
- Conesa, C.; Fombuena, V.; Loeff, E.; Olguín Pinatti, C.; Seguí, L.; Laguarda-Miró, N. (2014). Discriminación de azúcares mono y di-sacáridos presentes en alimentos mediante espectroscopía de impedancias. VIII International Workshop on Sensors and Molecular Recognition: 198 – 202.
- Conesa, C.; Bernat-Senent, I.; Seguí, L.; Fito, P. (2014). Pretratamiento con microondas para la obtención de bioetanol a partir de residuos industriales de piña: una propuesta de mejora de la hidrólisis enzimática. IX Congreso Iberoamericano de Ingeniería de Alimentos: 435 – 443.

3) Actas de congresos nacionales:

- Conesa, C.; Tirone, A.; Seguí, L.; Fito, P. (2013). Efecto del pretratamiento con microondas en el contenido en aceites esenciales y en la hidrólisis enzimática de los residuos cítricos para la obtención de bioetanol. VII Congreso Nacional de Ciencia y Tecnología de los Alimentos (CyTA 2013): 153 – 154.

- Conesa, C.; Bernat-Senent, I.; Seguí, L.; Fito, P. (2014). Efecto del pretratamiento con microondas en la hidrólisis enzimática de los residuos industriales de la piña para la obtención de bioetanol. VII Congreso Nacional de Ciencia y Tecnología de los Alimentos (CyTA 2013): 152 – 152.

4) Actas de congresos publicadas sin ISBN:

- Conesa, C.; Bernat-Senent, P.; Seguí, L.; Fito, P. (2013). Microwave-assisted pretreatments for enhancing enzymatic hydrolysis of industrial pineapple waste for bioethanol production. EFFoST Annual Meeting (EFFoST 2013): 1 – 2.
- Conesa, C.; Hurtado Abad, A.L.; Seguí, L.; Fito, P. (2012). Enzymatic hydrolysis of industrial pineapple waste with commercial enzyme mixtures for bioethanol production. International Conference of Food Science and Technology Innovation (FoodInnova 2012): 51 – 51.
- Conesa, C.; Bernat-Senent, I.; Seguí, L.; Fito, P. (2012). Saccharification of Industrial Pineapple Waste with *Aspergillus niger* Enzymes and the effect of pH and Temperature for obtaining Bioethanol. International Conference of Food Science and Technology Innovation (FoodInnova 2012): 197 – 198.
- Conesa, C.; Fito, P.J.; Fito, P. (2012). Extension of the project for obtaining bioethanol from citrus waste. 12th International Citrus Congress: 362 – 362.
- Conesa, C.; Seguí, L.; Fito, P. (2012). Saccharification of industrial pineapple waste with commercial enzyme mixtures. EFFoST Annual Meeting (EFFoST 2012): 19 – 19.

Durante el periodo doctoral, la autora ha realizado una estancia de investigación de tres meses en el Centro Interdipartimentale di Ricerca Industriale Agroalimentare (CIRI-Agroalimentare) de la Università di Bologna (Italia) liderado por el Profesor Marco Dalla Rosa y que la habilita para la Mención Internacional en el título de Doctor.

Como se detalla en el apartado Anexos, la autora ha participado en un proyecto de investigación subvencionado en convocatorias públicas y

como miembro del comité organizador de un congreso. En el ámbito de la docencia, ha ejercido como directora experimental en la elaboración de siete Trabajos Final de Carrera o Grado (T.F.C. / T.F.G.) y en un Trabajo Final de Máster (TFM), ha colaborado en la docencia de asignaturas y posee dos actas de congresos nacionales y tres internacionales publicadas en editorial.


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New York
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Hawai
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Sydney
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San Francisco
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ÍNDICES

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1. INTRODUCCIÓN

CONTENIDOS

1.1. JUSTIFICACIÓN DEL ESTUDIO

El último informe presentado por la División de Población del Departamento de Asuntos Económicos y Sociales de Naciones Unidas (DESA, 2015) prevé que la población mundial aumente hasta alcanzar los 9.700 millones de habitantes en 2050, principalmente en países en vías de desarrollo. Esto, unido al crecimiento económico global generará un fuerte incremento de la demanda y consumo energético y un consiguiente encarecimiento de la energía.

Además, el Panel Intergubernamental del Cambio Climático (IPCC) afirma que las concentraciones de gases de efecto invernadero (GEI) llevan aumentando debido a la actividad humana desde la revolución industrial y son los principales causantes del "calentamiento global". El IPCC estima que la temperatura promedio de la superficie terrestre aumentará entre 1,1 °C y 6,4 °C para finales de siglo. Esto provocará el aumento del nivel del mar, cambios irreversibles en los ecosistemas y un aumento de los fenómenos meteorológicos extremos (IPCC, 2007).

Ante esta perspectiva, es necesario apostar por políticas que favorezcan el desarrollo económico, social y medioambiental sostenible (WCED, 1987). En el ámbito de la energía, existen diferentes iniciativas que pretenden garantizar la sostenibilidad. Este es el caso de los biocarburantes, combustibles líquidos o gaseosos obtenidos a partir de biomasa para su uso en vehículos de transporte, regulados por la Directiva Europea de Energías Renovables (ORDEN ITC/2877/2008) del Ministerio de Industria, Turismo y Comercio (2008). Su producción y uso de forma sostenible puede contribuir significativamente a la reducción de emisiones, al desarrollo socioeconómico y a la seguridad energética. Destacan, atendiendo a su desarrollo comercial: biodiesel, biohidrógeno y, principalmente, bioetanol.

1.2. EL BIOETANOL DE SEGUNDA GENERACIÓN: UNA ALTERNATIVA A LOS COMBUSTIBLES FÓSILES

1.2.1. Definición y características generales del bioetanol de segunda generación

El bioetanol es alcohol etílico obtenido a partir de la fermentación de la biomasa azucarada, amilácea o lignocelulósica. Se trata del biocombustible más utilizado en el sector del transporte ya que se puede obtener en grandes cantidades mediante biorrefinería y utilizarse solo, como aditivo remplazando al éter metil tert-butílico (MTBE) o mezclarse con la gasolina convencional (Song *et al.,* 2006). La presencia de bioetanol mejora el índice de octano de la gasolina y su oxidación, favoreciéndose así un mayor rendimiento y la reducción de las emisiones de CO_2 y de otras partículas contaminantes a la atmósfera como los óxidos de azufre y de nitrógeno (Sánchez y Cardona, 2008; González-García *et al.,* 2009; Chen y Qiu, 2010; Balat, 2011).

Por otro lado, la mayor parte del bioetanol que se comercializa procede de la caña de azúcar, de la remolacha y de diferentes granos de cereales. El etanol obtenido a partir de estos cultivos que forman parte de la cadena alimentaria humana y animal se denomina bioetanol de primera generación o 1G. Para satisfacer las demandas energéticas, se llevaron a cabo deforestaciones y se destinó una parte importante de la superficie agraria a la plantación de cultivos energéticos, con el consecuente impacto negativo sobre el medioambiente (Mussatto *et al.,* 2010). Su uso contribuyó además, al aumento de los precios de los alimentos durante los años 2008, 2010 y 2011 y desató una fuerte controversia (Xavier *et al.,* 2010). Es por ello que en los últimos años, la investigación se ha centrado en la búsqueda de materias primas de tipo no alimentario, como es el caso de la biomasa lignocelulósica de origen residual procedente de actividades agrícolas, forestales o industriales (Reshamwala *et al.,* 1995; Bjerre *et al.,* 1996; Duff y Murray, 1996). Algunos autores como Singh *et al.* (2010) sugieren que el empleo de estos biocombustibles

de segunda generación o 2G, reducirían un 60% las emisiones de GEI con respecto a los combustibles fósiles según diferentes estudios de Análisis de ciclo de Vida (ACV).

1.2.2. Mercado y perspectivas del bioetanol

La producción mundial de etanol superó los 97.200 millones de litros en 2015. Además, la OCDE y la FAO preveen que esta aumentará hasta alcanzar los 168.000 millones de litros en 2022 (OCDE-FAO, 2012). Estados Unidos es el mayor productor mundial con el 57,7% del total de la producción que procede casi en exclusiva del maíz (Renewable Fuels Association, 2016). Existen diferentes iniciativas para fomentar la financiación y consumo de biocombustibles como la RFS2 (US Congress, 2007) que establece que el consumo de bioetanol debe alcanzar los 36.000 Mgal/año en 2022. La Environmental Protection Agency (EPA) permite la comercialización de E15 (mezcla al 15% de bioetanol y 85% de gasolina) capaz de ser usado por el 80% de los vehículos americanos. Además, el parque de vehículos Flexible-Fuel Vehicule (FFV) estadounidense cuenta con 20 millones de unidades (8% del total) que funcionan con mezclas de etanol en gasolina al 85% (E85) (Renewable Fuels Association, 2016).

Con el 27,6% de la producción mundial, Brasil ocupa el segundo puesto en la producción, mayoritariamente procedente de la caña de azúcar (Renewable Fuels Association, 2016). La mayor parte de los coches fabricados en Brasil tienen tecnología FFV y, desde marzo de 2016, es posible utilizar mezclas de hasta el 27% de bioetanol en gasolina (antes E25). De esta manera, se pretende impulsar el mercado del bioetanol en el país y reducir las importaciones de petróleo.

Europa está arrancando con fuerza en el uso de los biocombustibles y ya es el tercer productor mundial con más de 1,39 millones de litros de etanol fabricados a partir de trigo, maíz y subproductos de las industrias azucareras (Renewable Fuel Association, 2016). Para 2020, la Directiva 2009/28/CE (CE, 2009a) sobre energías renovables indica que al menos el 10% del combustible usado para el transporte debe

proceder de fuentes renovables y los proveedores de combustibles deben reducir la intensidad de las emisiones de GEI en un 6% según la Directiva 2009/30/CE (CE, 2009b) sobre la calidad de los combustibles. Por otro lado, desde el 2012 es posible obtener gasolina E10 en la Unión Europea que no requieren modificaciones en el motor de los vehículos cumpliendo las garantías del fabricante.

1.3. PROCESO DE OBTENCIÓN DE BIOETANOL 2G

1.3.1. Estructura de la biomasa lignocelulósica

Los materiales lignocelulósicos comprenden aproximadamente el 50% de la biomasa mundial (Claassen *et al.,* 1999) y su composición química y estructura determinan el rendimiento del proceso de obtención de bioetanol (Balat, 2011). La estructura de la biomasa lignocelulósica (Figura 1.1) se caracteriza por su elevada rigidez y complejidad en la que los polímeros de celulosa $(C_6H_{10}O_5)_n$ y hemicelulosa $(C_5H_8O_4)_n$ se encuentran fuertemente ligados a la lignina $[C_9H_{10}O_3(OCH_3)_{0,9\text{-}1,7}]_n$, compuesto recalcitrante que limita el acceso de las enzimas y los microorganismos (Pu *et al.,* 2013).

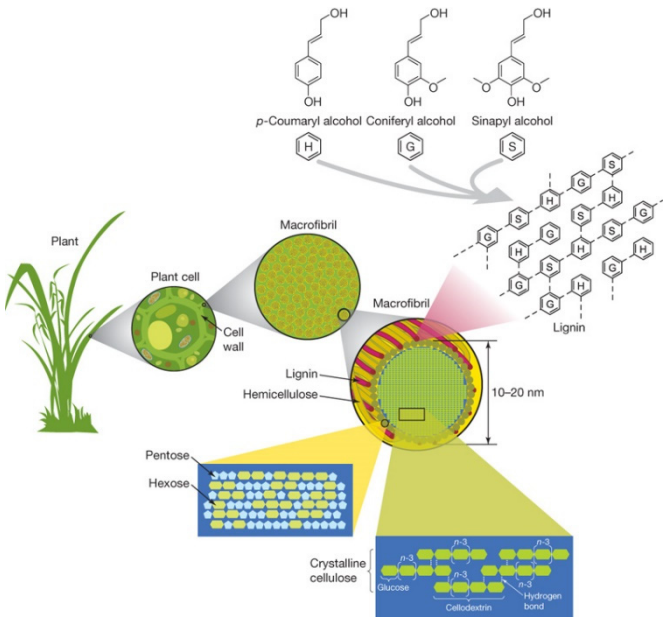


Figura 1.1. Estructura de la biomasa lignocelulósica (Rubin, 2008).

Las moléculas de celulosa, conocidas como microfibrillas, se organizan en largas cadenas lineales de D-glucosa, unidas por enlaces β-1,4 glucosídicos. Se trata del mayor constituyente de las plantas y es la molécula orgánica más abundante la tierra. Las microfibrillas están ensambladas entre sí por puentes de hidrógeno y fuerzas de van der Waals intramoleculares, formando una estructura cristalina resistente a la hidrólisis y regiones amorfas susceptibles a la degradación enzimática (Laureano-Perez *et al.,* 2005; Ovando y Waliszewski, 2005).

La hemicelulosa es más difícil de clasificar ya que es un polímero complejo de heteropolisacáridos formado por pentosas (D-xilosa y L-arabinosa) y hexosas (D-glucosa, D-manosa y D-galactosa) que forman una cadena ramificada con enlaces β-(1→4). Se puede clasificar la hemicelulosa según la cadena principal en xilanos, xiloglucanos, mananos, glucomananos, etc. (Scheller, 2010). Las ramificaciones de la hemicelulosa consisten en pequeñas cadenas de D-galactosa, D-manosa, L-arabinosa, D-xilosa, desoxihexosas (L-fucosa y L-ramnosa) y/o ácidos urónicos (D-ácido glucurónico, D-galacturónico y 4-O-metilglucorónico) fácilmente hidrolizables. La hemicelulosa sirve de conexión entre la lignina y las microfibrillas de celulosa, dando toda la rigidez a la red compleja que confeccionan estos tres polímeros (Laureano-Pérez *et al.,* 2005).

La lignina, por su parte, es un heteropolímero amorfo, tridimensional y ramificado que consta de varios ácidos y alcoholes fenilpropílicos (cumarílico, coniferílico y sinapílico) unidos entre sí por diferentes tipos de enlace (Harmsen *et al.,* 2010). La lignina se solubiliza en agua a temperaturas mayores de 180 °C en condiciones neutras lo que dificulta su degradación (Hendriks y Zeeman, 2009; Harsem *et al.,* 2010; Fengel y Wegener, 1984). De esta manera, actúa como material de soporte de la pared celular, aporta rigidez, impermeabilidad y protección al resto de la estructura. Además, desempeña funciones en el transporte de agua, nutrientes y metabolitos en el sistema vascular.

Los materiales lignocelulósicos también contienen pequeñas proporciones de pectina, proteínas y cenizas entre otros compuestos

(Kumar *et al.*, 2009). La proporción y composición de cada uno de los constituyentes de la biomasa lignocelulósica puede variar mucho según el material vegetal analizado.

1.3.2. Hidrólisis de la biomasa lignocelulósica

Para la obtención de bioetanol a partir de biomasa lignocelulósica son necesarias dos etapas fundamentales: la hidrólisis de la celulosa y hemicelulosa a mono y disacáridos y su fermentación posterior. Como se observa en la figura 1.2., existen diferentes configuraciones del proceso y alternativas en cada etapa. Este es el caso de la hidrólisis que puede ser ácida o enzimática y que a su vez puede necesitar de un pretratamiento para facilitar el acceso de las enzimas a la estructura (Galbe y Zacchi, 2002).

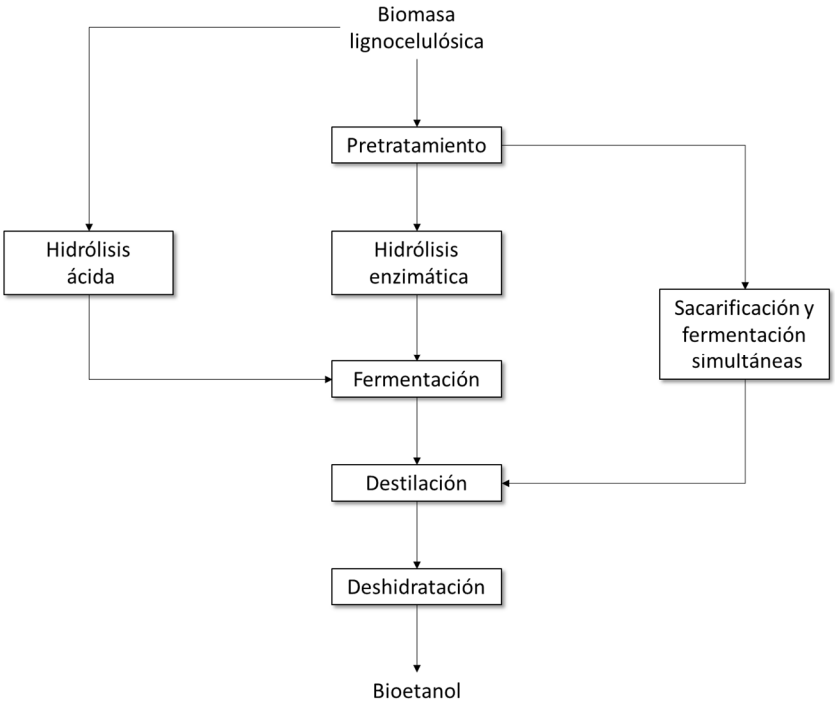


Figura 1.2. Proceso de obtención de bioetanol a partir de biomasa lignocelulósica

1.3.2.1. Hidrólisis ácida

Para este proceso, se pueden utilizar diferentes tipos de ácidos, tales como: sulfuroso, clorhídrico, fluorhídrico, fosfórico, nítrico, fórmico y sobre todo el sulfúrico y el hidroclorídrico (Galbe y Zacchi, 2002; Lenihan *et al.*, 2010). Estos ácidos penetran directamente en la lignina sin necesidad de pretratarla e hidrolizan la celulosa y hemicelulosa a mono y disacáridos fermentables.

La hidrólisis ácida concentrada se lleva a cabo con concentraciones de ácido en torno al 10-30% y a bajas temperaturas. Se obtienen altos rendimientos durante la hidrólisis (90% del rendimiento teórico en glucosa) pero pueden aparecer problemas de corrosión en los equipos y es necesaria una etapa posterior de recuperación y neutralización de los ácidos (Iranmahboob *et al.*, 2002).

En el caso de emplear ácidos diluidos (2-5%), es necesario utilizar altas temperaturas para que el proceso de hidrólisis alcance rendimientos aceptables. Estas temperaturas pueden degradar los azúcares, generar compuestos inhibidores de la posterior fermentación y aumentar el riesgo de corrosión de los equipos (Larsson *et al.*, 1999; Kootstra *et al.*, 2009; Jones y Semrau, 1984).

1.3.2.2. Hidrólisis enzimática

Las últimas tendencias se centran en la aplicación de enzimas hidrolíticas producidas por hongos filamentosos como: *Sclerotium rolfsii*, *Phanarichaete chrysosporium* y especies de *Trichoderma, Aspergillus, Schizophyllum* y *Penicilium* (Duff y Murray, 1996). Estos hongos filamentosos segregan dos tipos de complejos enzimáticos: las celulasas que hidrolizan la celulosa a pequeños oligosacáridos y posteriormente a glucosa, y las hemicelulasas que hidrolizan la hemicelulosa a azúcares monoméricos (Sehnem *et al.*, 2006; Wen *et al.*, 2005; Krogh *et al.*, 2004; Goyal *et al.*, 1991). Estas enzimas suelen presentar una mayor actividad enzimática a 50 ± 5 °C y a pH 4,5 – 5 (Taherdazeh y Karimi, 2007; Galbe y Zacchi, 2002).

La celulasa fúngica consta de tres grupos de enzimas: las endoglucanasas (EC 3.2.1.4), las celobiohidrolasas o exoglucanasas (EC 3.2.1.74) y las β-glucosidasas (EC 3.2.1.21) (Goyal *et al.*, 1991). El mecanismo enzimático de la hidrólisis de la celulosa consta de las siguientes etapas, como se muestra en la Figura 3 (Ting *et al.*, 2009):

- Las endoglucanasas hidrolizan al azar enlaces glicosídicos en las regiones amorfas de la celulosa generando oligosacáridos. Esto disminuye la longitud de las cadenas de celulosa e incrementa el contenido en azúcares reductores. Se producen cambios químicos (disminuye el grado de polimerización) y físicos (aumenta la superficie de contacto con respecto a la enzima) en la celulosa.
- Las celobiohidrolasas actúan, a continuación, sobre los extremos no-reductor y reductor de las cadenas de celulosa liberando glucosa y celobiosa. Este proceso es muy lento y es considerado como la primera fase de la hidrólisis enzimática.
- Las β-glucosidasas hidrolizan la celobiosa no fermentable a glucosa. Esta etapa, mucho más rápida que la anterior, se conoce como la segunda fase de la hidrólisis enzimática.

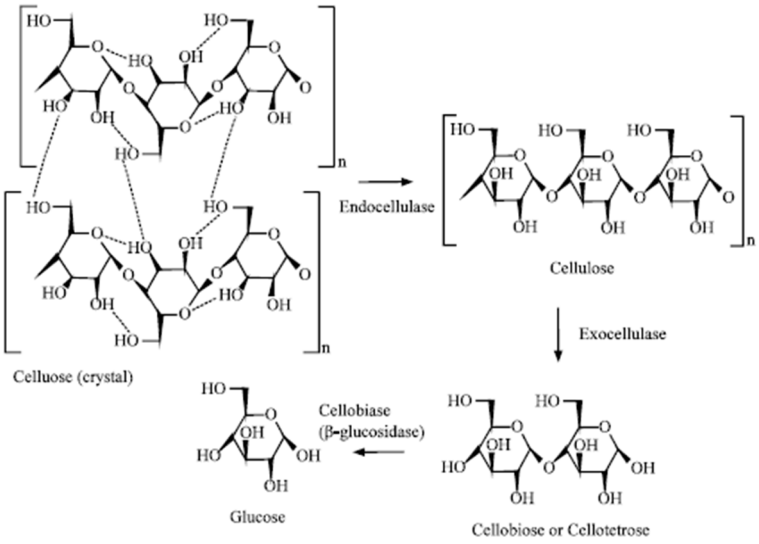


Figura 1.3. Modo de acción de la celulasa fúngica (Karmakar y Ray, 2011).

El mecanismo de acción de las hemicelulasas se describe en la Figura 1.4. y consta de:

- Las enzimas que hidrolizan la cadena principal. En el caso del xilano, se tratan de endo-1,4-β-xilanasas (endoxylanasas) (EC 3.2.1.8) y de 1,4-β-xilosidasas (EC 3.2.1.37) (Shallom y Shoham, 2003).
- Las enzimas responsables de la hidrólisis de las ramificaciones de la hemicelulosa. En función de la naturaleza de la ramificación, se encuentran entre otras: las α-L-arabinofuranosidasas (EC 3.2.1.55), las α-4-O-metil-D-glucuronosidasas (EC 3.2.1.39), las acetilxilano esterasas (EC 3.1.1.72) y las ferúlico esterasas (EC 3.1.1.73) (Saha *et al.,* 2005).

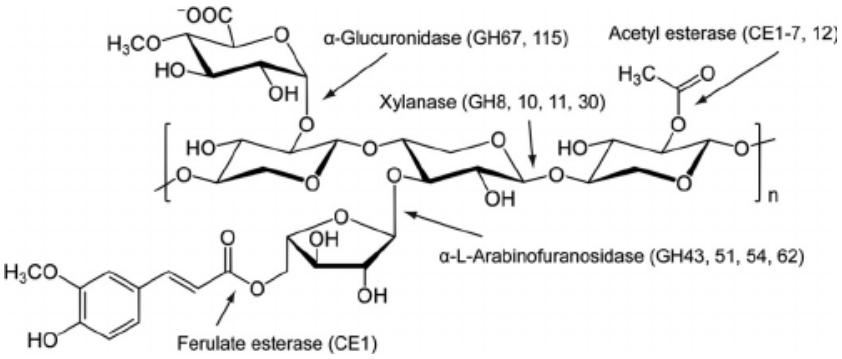


Figura 1.4. Mecanismo de acción de la hemicelulasa fúngica (Rogowski *et al.*, 2013).

La efectividad de la hidrólisis enzimática dependerá del uso adecuado de los complejos enzimáticos seleccionados y de las características de la materia prima: índice de cristalinidad y grado de polimerización de la celulosa, área superficial disponible, contenido y distribución de la lignina y la hemicelulosa, tamaño de partícula y porosidad (Alvira *et al.*, 2010). Pretratar la matriz lignocelulósica es un paso previo indispensable que complementa la hidrólisis enzimática ya que facilita el acceso de las enzimas dentro de la estructura y posibilita la obtención de altos rendimientos de azúcares (Sánchez *et al.*, 2010).

1.3.3. Pretratamiento de la biomasa lignocelulósica

En la actualidad, existen una gran variedad de pretratamientos físicos, fisicoquímicos, químicos y biológicos. En la tabla 1.1., se resumen las principales características de los tratamientos más destacados.

Los principales objetivos de un pretratamiento son: solubilizar y/o redistribuir la lignina, reducir la cristalinidad y el grado de polimerización de la celulosa y aumentar el contenido de la celulosa, incrementar el área superficial y disminuir la presencia de aquellas sustancias que dificulten la hidrólisis (Sánchez y Cardona, 2005; Hari Krishna *et al.,* 2000). Además, un pretratamiento eficaz debe tener un bajo consumo energético, bajos costes de inversión, reactivos baratos y recuperables y debe ser aplicable a diversos sustratos (Sun y Cheng, 2002).

Tabla 1.1. Principales características de los pretratamientos físicos, fisicoquímicos, químicos y biológicos más utilizados en la actualidad.

Tratamiento	Pretratamiento	Condiciones operativas	Ventajas	Inconvenientes
Físico	**Triturado (astillado y molienda)** (Cadoche y López, 1989)	Temperatura ambiente. Aporte de energía ≤ 30kW por tonelada de biomasa	Reduce la cristalinidad de la celulosa	Alto consumo energético
Fisicoquímico	**Explosión de vapor** (Duff y Murray, 1996)	Material lignocelulósico sometido a la acción de vapor saturado (160-290 °C, 20-50 bar) durante 1-10 min. Después rápida despresurización	Provoca la autohidrólisis de la hemicelulosa; permite extraer lignina que queda intacta; económico; apropiado para residuos y maderas duras	Destruye fracción de xilano; genera compuestos inhibidores de la fermentación
	Termohidrólis (Sousa *et al.*, 2009)	Agua caliente a presión (160-230 °C, 5 MPa) durante 20 min.	Elevadas tasas de recuperación de pentosas y no produce inhibidores	
	Explosión de fibra de amoniaco (AFEX) (Sun y Cheng, 2002)	Material es impregnado con amoniaco líquido (1 - 2 kg amoniaco/kg biomasa seca) a 60-90 °C entre 5 y 30 min, seguido de una rápida descompresión	No se producen compuestos inhibidores; no requiere pequeños tamaños de partícula para aumentar su eficacia	No hidroliza la hemicelulosa; No elimina la lignina; bajo rendimiento biomasa; Amoniaco debe de ser reciclado
	Explosión de CO_2 (Zheng *et al.,* 1998)	4 kg CO_2/kg de fibra a 160 bares durante 90 min a 50 °C. El CO_2 forma ácido carbónico	No produce compuestos inhibidores de la fermentación; Proceso más económico que AFEX	Rendimiento bajo comparado con la explosión de vapor y el proceso AFEX

Tabla 1.1. (continuación). Principales características de los pretratamientos físicos, fisicoquímicos, químicos y biológicos más utilizados en la actualidad.

Tratamiento	Pretratamiento	Condiciones operativas	Ventajas	Inconvenientes
Químico	**Pretratamiento alcalino** (Sun y Cheng 2002)	Tratamiento con NaOH a bajas temperaturas y tiempos altos de exposición. Requiere de una concentración alta de NaOH	Saponificación de los enlaces éster intramoleculares. Aumenta área, superficie interna, desciende cristalinidad; elimina la lignina	Sales residuales presentes en la biomasa
	Organosolventes (Thring *et al.,* 1990)	Mezcla de solventes orgánicos y ácidos inorgánicos que actúan como catalizadores a 150 – 200 °C	Hidroliza la lignina y la hemicelulosa	Coste elevado de los solventes
	Oxidación húmeda (Klinnke *et al.,* 2002)	Se somete a agua a 148 – 200 °C durante 30 min, en presencia de oxígeno	No genera compuestos inhibidores; baja demanda energética	Alto coste de oxígeno y catalizador alcalino
	Ozonolisis (Vidal y Molinier, 1988)	Temperatura ambiente	Eliminación de la lignina; no origina productos tóxicos	Hemicelulosa atacada; celulosa apenas afectada
Biológico	Hongos de la podredumbre blanca, parda o blanda (Fan *et al.*, 1987).	Degradan la lignina y la hemicelulosa; requiere de poca energía. No generan compuestos tóxicos.	Bajo rendimiento. Proceso lento que limita su aplicación industrial	

1.3.3.1. Pretratamientos con microondas

Una alternativa a estos pretratamientos convencionales es el calentamiento por microondas (Hu y Wen, 2008). Las microondas son radiaciones electromagnéticas no ionizantes que se hallan dentro de una gama de frecuencias de 300 MHz (λ = 1 m) a 300 GHz (λ = 1 mm). Las telecomunicaciones y los radares de microondas ocupan la mayoría de las frecuencias de banda en esta región. Para evitar interferencias, la longitud de onda de los microondas domésticos e industriales está fijada a 12,2 cm, es decir a 2,450 (± 0,050) GHz.

Al incidir sobre un cuerpo la radiación por microondas, se ve afectada la rotación de las moléculas de la sustancia que lo forman sin que su estructura molecular se vea alterada. La radiación por microondas se puede dividir en un componente de campo eléctrico y un componente de campo magnético, aunque solamente el campo eléctrico transfiere calor a la sustancia con la que dicha radiación entra en contacto mediante dos mecanismos:

- La orientación e inducción dipolar: Se trata de una interacción entre las moléculas polares que intentan alinearse sobre sí mismas a medida que el campo eléctrico de la radiación microondas cambia. El movimiento rotacional de las moléculas para orientarse en la dirección del campo genera fricción y colisiones moleculares, dando lugar a un calentamiento (Lidström et al, 2001).
- La conducción iónica: Tiene lugar cuando hay especies iónicas libres o iones libres presentes en la disolución. El campo eléctrico genera un movimiento iónico mediante el cual las especies intentan orientarse al cambio del campo eléctrico, y de forma análoga a dipolar, se produce un supercalentamiento (Lidström et al, 2001).

A diferencia de los mecanismos de calentamiento convencionales, las microondas permiten un calentamiento volumétrico, selectivo y más rápido del producto ya que existe un contacto directo entre este y el campo electromagnético generado por el microondas. Esto se traduce

en una mayor efectividad energética y facilidad de manejo con respecto a los pretratamientos convencionales (De la Hoz *et al.,* 2005). Además, Xiong *et al.* (2000) demostraron que el uso de las microondas puede cambiar la ultraestructura de la celulosa, degradar la lignina y la hemicelulosa y facilitar la penetración de las enzimas hidrolíticas en el sustrato lignocelulósico (Kitchaiya *et al*. 2003; Zhu *et al.* 2006). Por otro lado, las microondas puede combinarse con otros tratamientos químicos para aumentar la eficacia y la velocidad de estos últimos (Zhu *et al.,* 2006; Hu y Wen, 2008; Binod *et al*., 2012).

1.3.3.2. Productos inhibidores de la fermentación

Las condiciones de los pretratamientos empleados (temperatura, presión, tiempo), de la materia prima (maderas duras, blandas o residuos agroindustriales) y de la presencia de catalizadores ácidos, pueden favorecer la aparición de los compuestos inhibitorios de la fermentación. Estos pueden clasificarse en función de su origen o de su estructura química (Parajó *et al.*, 1998; Mussatto *et al.*, 2010):

1) Productos de la degradación de los azúcares (furaldehídos):

Destacan el Furfural (F) y el 5-hidroximetilfurfural (HMF), productos de la degradación de las pentosas y hexosas, respectivamente. Respecto al F, Sárvári Horváth *et al.* (2003) y Gorsich *et al.* (2006) demostraron que este compuesto inhibe la actividad glucolítica y el ciclo de Krebs. También, produce estrés celular e inhibe la alcohol deshidrogenasa, induciendo la formación de acetaldehído (Modig *et al.*, 2002; Allen *et al.*, 2010). Es por ello que su presencia disminuye el crecimiento celular de las levaduras etalogénicas reduciendo la producción de biomasa y etanol, puede generar daños en la membrana plasmática celular e inhibir la acción enzimática (Palmqvist y Hahn-Hägerdal, 2000). El HMF produce los mismos efectos que el F, pero en menor medida (Delegenes *et al.*, 1996; Palmqvist y Hahn-Hägerdal, 2000). Mussatto *et al.* (2010) demostraron que se produce un efecto sinérgico cuando estos compuestos se combinan con otros formados durante la degradación de la lignina.

2) *Productos de la degradación de la lignina (compuestos aromáticos, poliaromáticos, fenólicos y aldehídos):*

Los compuestos fenólicos (4-hidroxibenzaldehído, vainillina, sinringaldehído, acetosiringona, ácido vainíllico y ácido siríngico) presentes en los hidrolizados son los productos más tóxicos para los microorganismos. Generan una pérdida de integridad de las membranas biológicas, afectando su capacidad como barrera selectiva y como matrices enzimáticas y disminuyendo el crecimiento celular y la asimilación de azúcares de los microorganismos (Parajó *et al.*, 1998; Palmqvist y Hahn-Hägerdal, 2000).

3) *Productos de degradación de la hemicelulosa:*

Los ácidos alifáticos (principalmente ácido acético) se generan a partir de los restos acetilo de las hemicelulosas (Almeida *et al.*, 2007). El ácido acético es un conocido agente antimicrobiano. A pH bajo, en la forma no disociada, puede difundirse a través de la membrana celular, promoviendo la disminución de la actividad de las células citoplasmáticas e incluso causando la muerte celular (Lawford *et al.*, 1998; Mussatto *et al.*, 2010). Combinado con el F, produce un efecto sinérgico (Oliva, 2003). Otros ácidos alifáticos son el ácido fórmico y levulínico, procedentes de la degradación del F y HMF (Almeida *et al.*, 2007).

4) *Compuestos derivados de los agentes de extracción (resinas ácidas, tánicas y ácidos terpénicos):*

En términos de toxicidad, los extractos se consideran menos tóxicos para el crecimiento microbiano, que los derivados de la lignina o ácido acético (Mussatto *et al.*, 2010). El ácido gálico y pirogálico (pirogalol). Son compuestos fenólicos de bajo peso molecular, normalmente formados a partir de los taninos hidrolizables (Marques *et al.*, 2009), de los que algunos autores han demostrado propiedades antifúngicas (Dix, 1979; Panizzi *et al.*, 2002; Upadhyay *et al.*, 2010).

5) *Iones de metales pesados (principalmente, hierro, cromo, níquel y cobre):*

Proceden de la corrosión de los reactores durante el tratamiento ácido. Su toxicidad actúa a nivel de las vías metabólicas, inhibiendo la actividad enzimática.

Pueden ser eliminados mediante lavado o detoxificación con carbonato cálcico o carbón activo (Kuhad *et al.*, 2010; Arslan y Eken-Saraçoglu, 2010; Cantarella *et al.*, 2004).

1.3.4. Fermentación de la biomasa lignocelulósica

La fermentación y la hidrólisis de la biomasa lignocelulósica pueden ser simultáneas (Simultaneous Saccharification and Fermentation, SSF) o consecutivas (Separate Hydrolysis and Fermentation, SHF). La SSF muestra mayores rendimientos de etanol y menores consumos energéticos que la SHF, pero las temperaturas de operación no son óptimas para la hidrólisis y se requiere de mayores dosis de enzimas (Öhgren *et al.*, 2007).

En la actualidad, existen diferentes especies de bacterias (*Zymomonas mobilis, Escherichia coli* y *Klebsiella oxytoca*), hongos (*Mucor indicus y Rhizopus oryzae*) y levaduras (*Pichia stipitis, Candida shehatae, Pachysolen tannophilus* y *Saccharomyces cerevisiae*) capaces de fermentar los azúcares a etanol (Karimi *et al.,* 2006; Huang *et al.*, 2009; Fernandes *et al.,* 2012; Krull *et al.,* 2013). Algunos de ellos sólo pueden metabolizar las hexosas mientras que otros también fermentan las pentosas, aunque con rendimientos mucho más bajos (Hahn-Hägerdal *et al.*, 2007).

Las levaduras son las más empleadas en la fermentación, ya que aunque son más lentas en la ejecución del proceso de fermentación, poseen una alto rendimiento, baja producción de inhibidores y mayor facilidad de separación tras la fermentación (Sánchez *et al.*, 2010). En concreto, la levadura *Saccharomyces cerevisiae*, capaz de fermentar la glucosa, fructosa y sacarosa, es el microorganismo más utilizado para la fermentación alcohólica. *S. cerevisiae* se emplea con éxito a escala

industrial debido a sus altos rendimientos y a su elevada tolerancia al etanol y otros inhibidores usualmente presentes en los residuos lignocelulósicos (Hahn-Hägerdal *et al.*, 2007; Matsushika *et al.*, 2009; Balat, 2011), además de ser capaz de trabajar a temperaturas superiores a los 40 °C (Hari Krishna *et al.,* 2000). Puesto que *S. cerevisiae* no es capaz de fermentar de forma natural las pentosas, (Karagöz *et al.*, 2012) se han modificado algunas cepas, como la TMB3400, para tal fin (Öhgren *et al.*, 2006). Por otro lado, existen cepas naturales de *P. stipitis, C. shehatae* y *P. tannophilus* capaces de convertir las pentosas a etanol pero presentan una tolerancia más baja al alcohol y peor rendimiento que *S. cerevisiae* (Girio *et al.,* 2010; Balat, 2011).

Algunos estudios utilizan la co-fermentación que se basa en añadir dos microorganismos diferentes al medio, cada uno de los cuales fermenta las hexosas o las pentosas (Bellido Díez, 2013). Este es el caso de los co-cultivos de *Saccharomyces cerevisae* y *Pichia stipitis* (Grootjen *et al.*, 1990; Rudolf *et al.*, 2007), *Zymomonas mobilis* y *Pichia stipitis* (Laplace *et al.*, 1993; Fu *et al.*, 2009) o *Saccharomyces cerevisae* y *Candida shehatae* (Lebeau *et al.*, 2007).

Finalmente, el bioetanol obtenido se destila a presión atmósferica, obteniéndose una concentración máxima de etanol del 90-95%. Esto se debe a que el etanol forma con el agua un azeótropo binario de mínimo punto de ebullición a 78,2 °C (1 atm) con un contenido en peso de alcohol del 95,6% (Pedraza Berenguer, 2012). Es por ello que es necesario deshidratar el etanol concentrado para conseguir un alcohol anhídrico al 99,5% que pueda ser mezclado con gas. Esto puede realizarse mediante destilación azeotrópica o extractiva, extracción supercrítica o mediante disolventes orgánicos, tecnologías de membrana y adsorción sobre tamices moleculares o agentes sólidos (Huang *et al.,* 2008; Hatti-Kaul, 2010). Debido al alto consumo de energía durante esta etapa, se están buscando alternativas más sostenibles a la deshidratación convecional (Kreis y Gorak, 2006; del Pozo-Gómez *et al.,* 2007; Bakhsni *et al.,* 2008). No obstante, en la

actualidad la destilación azeotrópica sigue siendo la más empleada a nivel industrial (Kahr *et al.,* 2012).

1.4. MONITORIZACIÓN DEL PROCESO DE OBTENCIÓN DE BIOETANOL 2G

La fermentación y la sacarificación enzimática son etapas complejas cuyos rendimientos dependen de las condiciones del proceso (pH, temperatura, contenido en oxígeno para la fermentación, etc.), de las propiedades de la materia prima a fermentar (características de la fracción lignocelulósica, contenido en azúcares inicial y liberados, nutrientes en el medio, etc.) y de los propios microorganismos utilizados (enzimas, cepas etalogénicas y viabilidad de éstas) (Taherzadeh y Karimi, 2007; Cesaro *et al.*, 2015). Por lo tanto, monitorizar ambos procesos resulta de importancia capital para optimizar el proceso de obtención de bioetanol de 2G.

En la actualidad, existen diferentes técnicas cromatográficas consideradas de referencia para la identificación y cuantificación precisas de azúcares y etanol en un medio. Este es el caso de la cromatografía líquida de alta eficacia (HPLC) (Raessler, 2011; Stefansson y Westerlund, 1996), la cromatografía de gases-espectrometría de masas (GC-MS) (Sanz *et al.*, 2004; Terrab *et al.*, 2001) y la cromatografía de intercambio aniónico de alta resolución con detector de pulso amperométrico (HPAEC-PAD) (LaCourse, 2002) para los azúcares y la cromatografía de gases (GC) y el HPLC (Mohammed Al-Mhanna y Huebner, 2011) para el etanol. Estas técnicas de laboratorio se consideran de referencia pero son destructivas, lentas, caras y complejas ya que requieren de un tratamiento previo de las muestras (destilación, pervaporación, etc.) y de la adición de compuestos químicos. Por otro lado, los métodos enzimáticos resultan mucho más sencillos pero permiten determinar un solo tipo de azúcar y requieren de medidas espectrofotométricas que no los hacen recomendables para medios complejos debido a la posible presencia de sustancias interferentes (Sánchez-Mata *et al.*, 2002; Karkacier *et al.*, 2003; Azevedo *et al.*, 2005).

Por el contrario, las técnicas electroquímicas (conductimetría, potenciometría, voltametría, culombimetría y espectroscopía de impedancias) se están revelando como una alternativa a los métodos tradicionales de medida. Permiten identificar compuestos químicos diferentes de una manera rápida, fácil, no destructiva y en línea durante el proceso, ya que son capaces de medir la corriente o el voltaje generado por la actividad de las especies electroactivas presentes en el medio.

1.4.1. Fundamentos de la espectroscopía de impedancias electroquímica

La espectroscopía de impedancias electroquímica (Electrochemical Impedance Spectroscopy, EIS) está experimentando en la actualidad un gran auge debido a los avances informáticos y a la aparición de sistemas electrónicos de gran precisión y alta velocidad de procesamiento que hacen posibles las exigencias de esta técnica analítica. La EIS es un método no destructivo, particularmente sensible a pequeños cambios en las variables del sistema, que permite la caracterización de las propiedades de las muestras sólidas o líquidas y de sus interfaces con los electrodos incluso en medios poco conductores (Alcañiz, 2011). La EIS consiste en aplicar señales eléctricas senoidales (tensión o corriente) de frecuencia variable y posteriormente registrar la respuesta (corriente o tensión) dentro de una celda electroquímica (Bard y Faulkner, 2001; Barsoukov y Macdonald, 2005). Así, la impedancia eléctrica del sistema se corresponde con la relación entre la perturbación y la respuesta (Figura 1.5.).

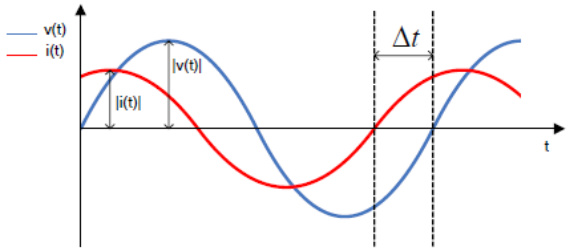


Figura 1.5. Relación entre las señales de tensión aplicada y corriente medida (Masot, 2010).

A partir de las mediciones del desfase (φ) entre las señales y de amplitud de la respuesta, es posible obtener la impedancia del material estudiado (Ecuación 1).

$$Z = |Z|e^{\varphi j} \begin{cases} |Z| = \frac{|v(t)|}{|i(t)|} Módulo \\ \varphi = 2\pi f \Delta t \; Fase \end{cases} \qquad \textbf{Ecuación 1}$$

Por lo tanto, la impedancia eléctrica es un valor complejo cuya representación en coordenadas polares (módulo y fase en función de la frecuencia) recibe el nombre de Diagrama de Bode (Figura 1.6.). Se trata de una herramienta ampliamente utilizada en electrónica pues permite representar de manera simple y eficaz un amplio espectro de frecuencias en un reducido espacio.

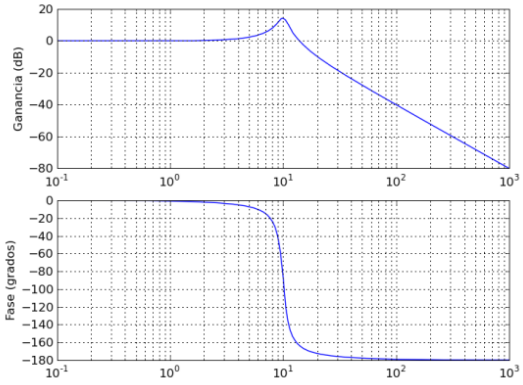


Figura 1.6. Diagramas de Bode representando el módulo y la fase de un espectro de impedancias. (Masot, 2010)

En la actualidad, se dispone de varios métodos para la obtención del espectro de impedancias. La técnica multiseno se aplica cuando se requieren de tiempos de medida cortos y permite hallar todos los valores de impedancia del espectro con un solo ensayo. Esta técnica consiste en aplicar al electrodo una señal compuesta por la suma de varias señales senoidales de distintas frecuencias. Las señales obtenidas son sometidas a un análisis Transformada Rápida de Fourier (TRF) para calcular el módulo y la fase de cada una de sus componentes frecuenciales (Masot, 2010).

1.4.2. Impedancia en los tejidos celulares

En los sistemas biológicos, la impedancia varía con la frecuencia del potencial aplicado y está relacionada con las propiedades dieléctricas de los materiales (conductividad y permitividad) y de las cualidades y factores geométricos de los electrodos determinados por la constante de celda (Ecuación 2). La permitividad (ε) se define como la tendencia de un material a polarizarse cuando se somete a un campo eléctrico mientras que la conductividad (σ), es la capacidad de permitir el paso de corriente a través de él.

$$Z = K_{cell} \frac{1}{\sigma + j\omega\varepsilon} \qquad \textbf{Ecuación 2}$$

Donde K_{cell} es la constante de celda (m^{-1}) y se calcula como el cociente entre la distancia a los electrodos y la superficie de los mismos y ω es la frecuencia angular.

Respecto a las propiedades dieléctricas, Schwan (1994) define tres regiones frecuenciales o dispersiones: α, β y γ en las que éstas cambian significativamente de valor (Figura 1.7).

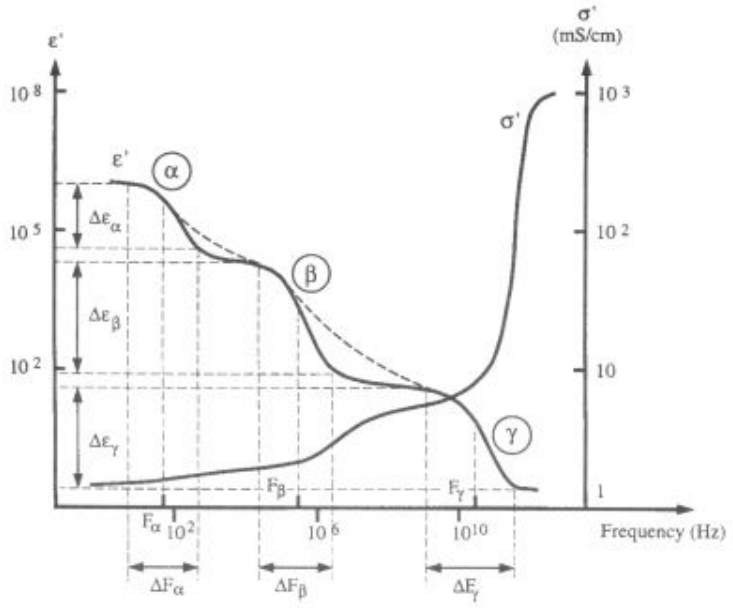


Figura 1.7. Permitividad y reactividad de los tejidos biológicos en función de la frecuencia. Las dispersiones α, β y γ están caracterizadas por la frecuencia de relajación central (F) y las variaciones en la permitividad (Δε). (Rigaud *et al.*, 1996).

La dispersión α se produce a bajas frecuencias (Hz-kHz) y se asocia a fenómenos de orientación de los iones en un medio fluido. También se la conoce con el nombre de counterion o ion contra ion porque cuando los iones de una carga se orientan pueden producir una movilidad contraria de los de signo opuesto. Gabriel *et al.* (1996) y Foster y Schwan (1996) sugieren que esta dispersión permite el equilibrio eléctrico de las cargas que constituyen la doble capa lipídica de las membranas en las células. Además, Kuang y Nelson (1998) señalan que las medidas en α permiten predecir las reacciones electroquímicas siempre que se utilicen electrodos polarizados y se controlen a lo largo del tiempo.

La dispersión β comprende un amplio rango de frecuencias desde el kHz hasta el MHz y permite describir todas las interacciones con cargas fijas o de baja movilidad. A frecuencias en torno al kHz, predominan las interacciones con cargas procedentes de las macromoléculas

estructurales como son las proteínas. No obstante, a frecuencias mayores, los fenómenos de relajación son causados por el efecto Maxwell-Wagner que provoca la polarización de la interfase. Es la relajación de la cual se puede extraer mayor información de la estructura de la suspensión celular y es por ello que se la conoce como relajación estructural.

La dispersión γ (> 1 MHz) está asociada a la orientación dipolar de los dipolos de pequeño tamaño molecular como las moléculas de agua predominantes en los tejidos biológicos.

La interpretación del espectro de impedancias requiere en muchos casos de la selección de un modelo eléctrico apropiado que se ajuste a los datos experimentales obtenidos. Para ello se recurre a la ecuación de Debye o a la ecuación empírica de la permitividad de Cole-Cole. Del mismo modo, es posible utilizar circuitos eléctricos análogos al sistema físico estudiado, también llamados circuitos equivalentes o modelos de parámetros concentrados, como es el modelo de Fricke.

No obstante, la complejidad de las muestras analizadas y las geometrías de los electrodos utilizados en esta tesis hacen extremadamente difícil la obtención de un modelo teórico fiable. Por lo tanto, el control de cada una de las etapas involucradas en la obtención de bioetanol 2G se llevará a cabo en esta tesis mediante la aplicación de potentes herramientas estadísticas, tales como: los métodos multivariantes y las Redes Neuronales Artificiales (RNA).

1.4.3. Herramientas para el análisis de datos de la EIS

La quimiometría es una disciplina química que, mediante la aplicación de conocimientos matemáticos y estadísticos, extrae la mayor cantidad posible de información de los datos experimentales. Esta disciplina, que nace en la década de los 70 gracias al desarrollo de la computación y de las técnicas analíticas, permite diseñar y optimizar experimentos y realizar un análisis descriptivo y/o predictivo a partir de los datos químicos (Mongay Fernández, 2005).

La EIS, al igual que otras técnicas electroquímicas de medida tales como la voltametría y la potenciometría, generan un gran volumen de información. De esta manera, se obtiene un gran número de variables independientes para cada variable dependiente. Por lo tanto, para su correcto tratamiento estadístico, es necesario recurrir a métodos multivariantes o a redes neuronales artificiales (Masot, 2010).

1.4.3.1. Métodos multivariantes

Los métodos de análisis multivariante son un conjunto de herramientas estadísticas cuya finalidad es estudiar, analizar, representar e interpretar simultáneamente conjuntos de datos multivariantes, es decir aquellos que presentan diversas variables para cada elemento estudiado.

El objetivo principal de los métodos multivariantes es reducir la dimensionalidad de los datos sin pérdida de información relevante. Además permiten comprender la relación existente entre varios grupos de variables, minimizar o eliminar el ruido de la señal y trabajar con datos faltantes (correspondientes en su mayoría a fallos en los sensores) o erróneos.

Gracias a la evolución de los equipos informáticos, existen en la actualidad un gran número de técnicas de análisis multivariante. Esta Tesis Doctoral se centra en:

1) *Análisis de componentes principales*

El análisis de componentes principales (Principal Component Analysis, PCA) permite que un número elevado de variables más o menos correlacionadas sean transformadas en un número inferior de variables ortogonales (no correlacionadas) llamadas componentes principales que explican la máxima variabilidad del sistema (Porcel Gracía, 2001).

Las componentes principales son utilizadas como nuevos ejes de coordenadas. Así, la primera componente principal (PC1) es la dirección que explica la máxima variablidad de los datos; la segunda

componente principal (PC2) se elige de forma que contenga el máximo de la varianza restante y sea ortogonal a la primera componente y así sucesivamente (Brezmes, 2001).

Una vez construido el nuevo sistema de referencia, la representación de las muestras proyectadas nos permitirá determinar agrupaciones espontáneas (clasificación) e identificar de manera visual los datos anómalos (outliers). Por último, el PCA se utiliza en muchos casos como paso previo de otros métodos multivariantes.

2) Regresión por mínimos cuadrados parciales

El objetivo de la regresión por mínimos cuadrados parciales (Partial Least Squares, PLS) es obtener un modelo lineal en un espacio de menor dimensión que maximice la covarianza entre una matriz independiente X (matriz de predicción generalmente compuesta por las variables del proceso) y otra, dependiente Y (matriz predicha, a partir de las variables respuesta). Se trata de una de las herramientas estadísticas más usadas en quimiometría, química e ingeniería para la obtención de modelos predictivos (Wold *et al.*, 2001).

Para ello, se generan nuevos ejes ortogonales llamados variables latentes que, a diferencia de los componentes principales de las PCA, maximizan la correlación entre los datos de ambas matrices. La primera variable latente (LV1) posee la mayor covarianza posible entre las variables dependientes e independientes mientras que el resto de variables latentes se construyen a partir de la covarianza residual que queda en cada proceso. Así las primeras variables latentes poseen la información relevante existente entre la matrices X e Y.

A continuación, es necesario determinar el número de variables latentes necesarias para la construcción del modelo. Si éste es muy elevado, el modelo se ajustará perfectamente a las muestras de la calibración pero fallará en la predicción de nuevos datos. Este fenómeno se le conoce con el nombre de sobreajuste o sobreentrenamiento. Para evitarlo se recurre a la validación cruzada (Cross Validation, CV), metodología que permite determinar el número

necesario de variables latentes para la obtención de un modelo que minimice el error de predicción. Para ello y en primer lugar, se construyen modelos con una sola variable latente a partir de los datos de una serie de subconjuntos de muestras y se validan con las muestras no incluidas en estos subconjuntos. A continuación, se representan gráficamente los valores conocidos del parámetro estudiado frente a los predichos. Se repite el procedimiento seleccionando para la validación de una serie distinta de subconjuntos y así sucesivamente hasta haberlos utilizado todos. Los valores predichos por todos estos modelos se representan en un gráfico de valores conocidos frente a predichos y se calcula el error cuadrático medio de validación cruzada (Root Main Squared Error of Cross-Validation, RMSECV) (Ecuación 3). Este parámetro indica cual es el error promedio de nuestra predicción con respecto a los valores reales.

$$RMSECV = \sqrt{\frac{\sum_i^M (y_{OBSi} - y_{PREDCVi})^2}{M}} \qquad \textbf{Ecuación 3}$$

Siendo y_{OBSi}, el valor del parámetro y conocido para la muestra i; y_{PREDi}, valor del parámetro y predicho para la muestra i; M, el número de muestras.

A continuación, se repite todo el procedimiento para cada una de las variables latentes. Se representa el valor de cada uno de los RMSECV calculados en función de cada variable latente. El número óptimo de variables latentes se corresponderá con el primer mínimo que presente la gráfica.

Existen en la actualidad diferentes modalidades de validación cruzada, en esta tesis se ha utilizado la validación cruzada dejando uno fuera (Leave-one-out cross-validation, LOOCV) que se basa en separar los datos de forma que para cada iteración tengamos una sola muestra para validar y todo el resto conformando los datos para la construcción del modelo.

Finalmente, para la validación del modelo se utilizan muestras no utilizadas previamente para la calibración (construcción) del modelo.

Se aplica sobre ellas el modelo de predicción obtenido y se representan gráficamente los valores conocidos frente a los predichos. La precisión del modelo viene determinada por los siguientes parámetros estadísticos:

- Coeficiente de determinación (R^2): es el cuadrado del coeficiente de regresión de la recta de tendencia.
- Error cuadrático medio de predicción (Root Main Squared Error of Prediction, RMSEP): indica cual es en promedio la diferencia entre los valores predichos y los observados (ecuación 4).

$$RMSEP = \sqrt{\frac{\sum_i^M (y_{OBSi} - y_{PREDi})^2}{M}} \qquad \textbf{Ecuación 4}$$

Siendo y_{OBSi}, el valor del parámetro y conocido para la muestra i; y_{PREDi}, valor del parámetro y predicho para la muestra i; M, el número de muestras.

1.4.3.2. Redes neuronales artificiales

1) Fundamentos de redes neuronales artificiales

Las redes neuronales artificiales (RNA) son modelos computacionales inspirados en el funcionamiento del sistema biológico humano. Kohonen (1989) las define como elementos de cálculo simples, usualmente adaptativos, interconectados masivamente en paralelo y con una organización jerárquica que le permite interactuar con algún sistema del mismo modo que lo hace un sistema nervioso biológico.

En 1943, los neurólogos Warren McCulloch y Walter Pitts propusieron el primer modelo de neurona artificial que modelizaba de manera simplificada la estructura y el funcionamiento de las neuronas cerebrales. Consideraron la neurona como una función lógica con un umbral prefijado y utilizaron circuitos electrónicos para crearlas (McCulloch y Pitts, 1943). Este modelo simplificado y bastante inexacto ha servido de base para el estudio y desarrollo de posteriores RNA. Unos pocos años más tarde, Donald Hebb propuso una ley matemática sobre el aprendizaje neuronal, conocida como la "regla de Hebb": si dos neuronas se activan simultáneamente, su relación se

refuerza; los caminos que llevaron a la respuesta correcta se potencian. Para lograrlo, se varían los pesos a la entrada de las neuronas artificiales de manera que se refuerzan las conexiones que más se acercan al resultado correcto y así, los sistemas neuronales aprenden (Hebb, 1949). En 1958, Rosenblatt desarrolló el Perceptrón con un algoritmo de aprendizaje mejor que el de Hebb mediante ajuste iterativo de los pesos (Rosenblatt, 1958). En 1960, Widrow y Hoff desarrollaron el Adaptative Linear System (ADALINE), que se comercializó para la eliminación de los ecos en las líneas telefónicas. Se basaba en la regla de aprendizaje Delta Rule que fue la precursora del algoritmo de retropropagación (Widrow y Hoff, 1960). En los años posteriores, la investigación en redes neuronales se redujo debido a la falta de algoritmos de aprendizaje y a las limitaciones del Perceptrón según los estudios realizados por Minsky y Papert (1969). A partir de 1980 y hasta la fecha, las RNA han vivido un claro resurgimiento gracias a los estudios realizados por John Hopfield que utilizó técnicas estadísticas para analizar el almacenamiento y optimización de las redes y, sobre todo, a Rumelhart, Hinton y William creadores del algoritmo de aprendizaje de retropropagación para las redes multicapa (Rumelhart *et al.*, 1986).

Las RNA ofrecen numerosas ventajas, entre las que destacan (Rich y Knight, 1991; Gestal Pose, 2009):

- Aprendizaje autoadaptativo: las RNA tienen la capacidad de aprender a realizar tareas mediante algoritmos de aprendizaje adaptativos. Para ello deben pasar por una etapa previa de entrenamiento para actuar sobre los pesos de sus conexiones.
- Autoorganización: las RNA pueden crear su propia representación de la información que reciben durante la etapa de aprendizaje.
- Tolerancia a fallos: gracias a poseer la información de manera redundante, las RNA pueden seguir funcionando de manera aceptable incluso tras haber sufrido daños considerables.
- Operación en tiempo real: la estructura paralela de las RNA (usan un gran número de nodos de procesado muy interconectados) favorece que puedan ser implementadas en ordenadores o

dispositivos electrónicos, favoreciéndose así respuestas en tiempo real.

- Procesado no Lineal: aumenta la capacidad de las RNA para aproximar funciones, clasificar patrones y aumenta su inmunidad frente al ruido.
- Flexibilidad: las RNA son flexibles ante pequeños cambios en la información de entrada.

Gracias a ello, en la actualidad, las RNA se aplican con éxito en campos tan diversos como la medicina, biología, ingenierías, finanzas, así como en el ámbito militar entre otros (Hilera y Martínez, 1995). Se utilizan, por ejemplo, para reconocer patrones, optimizar y controlar procesos y predecir comportamientos en dinámicas complejas (Nascimento *et al.*, 2000; Narendra *et al.*, 2008; Garrigues, 2013).

Para entender el funcionamiento de las RNA, es necesario explicar previamente el de la neurona biológica. Esta recibe estímulos a través de sus dendritas, los procesa en el interior de su cuerpo celular y emite una respuesta a través de su axón a las dendritas de las neuronas adyacentes a través de una estructura llamada sinapsis (Figura 1.8). En concreto, el estímulo se corresponde con una diferencia de potencial eléctrico que si supera un determinado umbral de activación, conocido como potencial de acción, el soma (cuerpo celular) de la neurona emite un estímulo de salida. Se dice, por lo tanto, que la neurona actúa como una función escalón (Guerra Fernández *et al.*, 2013).

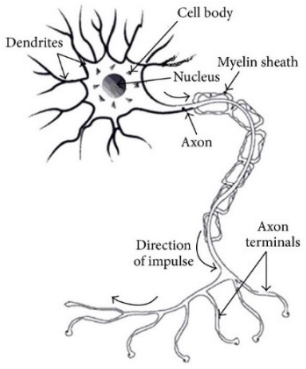
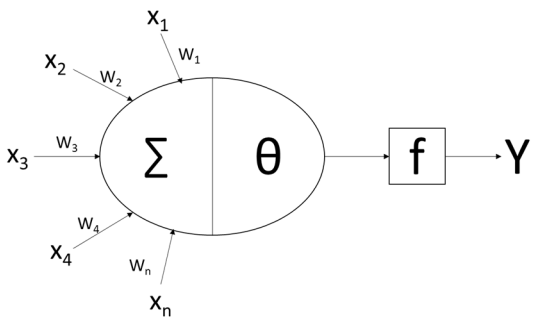


Figura 1.8. Esquema de una neurona biológica (Koo *et al.*, 2013).

Paralelamente, el nodo es la unidad básica de las RNA y consta de un cierto número de entradas (x_i) que pueden proceder de otras unidades o de una fuente externa de datos (Figura 1.9). Cada entrada proviene de una conexión que tiene una cierto peso (W_i). Estos pesos equivaldrían a la intensidad de la sinapsis que interconecta dos neuronas. Además, cada nodo posee un cierto valor umbral (θ). Si el la suma de productos de las entradas por sus pesos (valor de activación o de transferencia, η) supera el valor umbral, el nodo se activa y la función de activación (o de transferencia, *f*) dará lugar al valor *Y* de salida (Haykin, 1994).



$$\eta = \sum_{i=1}^{n} x_i W_i$$

Figura 1.9. Esquema de una neurona artificial. Modificado de Garrigues (2013).

Existen dos fases en toda modelización de RNA:

- Fase de aprendizaje o entrenamiento: se usa un conjunto de datos para determinar los pesos que definen el modelo de RNA. Se pueden calcular de una vez o de manera iterativa con el fin de minimizar el error cometido entre la salida obtenida por la red neuronal y la salida deseada.
- Fase de prueba o reconocimiento: el modelo entrenado se utiliza en esta fase en la que se procesan patrones de prueba reales para dar los resultados finales. Para evitar el sobreentrenamiento, es aconsejable utilizar para la fase de validación un segundo grupo

de datos diferentes a los de entrenamiento. De esta manera, se podrá controlar el proceso de aprendizaje.

Por otro lado, es importante destacar que una RNA se diferencia por tres aspectos fundamentales: la arquitectura y las funciones de aprendizaje y activación.

Las neuronas se organizan por capas en función de su comportamiento (función de activación y patrones de interconexión). Así, como se muestra en la Figura 1.10, según el número de capas se distinguen entre:

- Redes monocapa: existe una capa de neuronas que recibe las señales de entrada (Capa de entrada) y las1 envía a una capa de neuronas de salida (Capa de salida) donde se realizan diferentes cálculos. Al no realizar ningún cálculo, la capa de entrada no se contabiliza.
- Redes multicapa: son aquellas que contienen un conjunto de capas intermedias (capas ocultas) entre la capa de entrada y salida y pueden estar total o parcialmente conectadas.

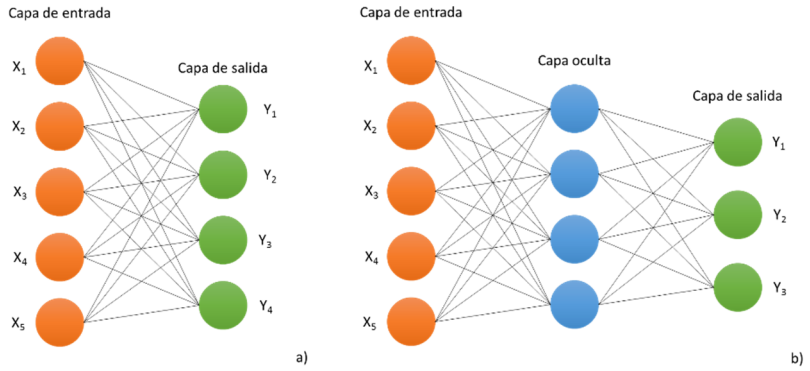


Figura 1.10. RNA monocapa a) y multicapa b)

En función del tipo de conexión, se encuentran:

- RNA no recurrentes: en las que la información fluye de la capa de entrada a la de salida en una única dirección

- RNA recurrentes: en las que se encuentran interconexiones de cada neurona consigo misma. De esta manera, la red es competitiva y totalmente interconectada

Desde el punto de vista del esquema de conexión, se hallan (Tabla 1.2.):

- Conexiones hacia adelante (feedforward): las salidas de las neuronas de una capa se propagan sólo hacia las neuronas que pertenecen a la siguiente capa. En el caso concreto del perceptrón multicapa y a pesar de ser una red feedforward, el gradiente de error de la fase de aprendizaje se propaga hacia atrás (backpropagation) conforme se verá más adelante.
- Conexiones hacia atrás (feedback): se encuentran en las redes recurrentes y en algunas dinámicas y se caracterizan por que los datos circulan de una capa superior a una inferior.
- Conexiones laterales: donde las salidas de las neuronas de una capa son entradas a las neuronas de la misma capa.
- Conexiones de retardo: se utilizan para el reconocimiento de patrones temporales.

Tabla 1.2. Clasificación de las principales en función del tipo de aprendizaje y sus conexiones (Garrigues, 2013).

Aprendizaje supervisado	
Feedforward	Perceptron (Rosenblatt, 1958) Perceptron multicapa (Minsky y Papert, 1969) Adaline (Widrow y Hoff, 1960)
Feedback	Recurrent backpropagation (Pineda, 1987) Máquina de Boltzman (Ackley *et al.*, 1985) Fuzzy Artmap (Carpenter *et al.*, 1992)
Aprendizaje no supervisado	
Feedforward	Learning Vector Quantization (Kohonen, 1988)
Feedback	Hopfield Net (Hopfield, 1982) Kohonen Feature Map (Kohonen, 1982) ART1 (Carpenter y Grossberg, 1987a) ART2 (Carpenter y Grossberg, 1987b)

En una RNA, la fase de aprendizaje consiste en ajustar los valores de los pesos y tiene lugar durante el entrenamiento de la red. En función de cómo se realiza este aprendizaje, se distingue entre (Guerra Fernández *et al.*, 2013):

- Supervisado: es el más popular y para ello es necesario poseer un conjunto de pares conocidos (datos de entrada y salida). Si cuando se utiliza uno de los pares de entrada, la salida calculada no coincide con la deseada, entonces los pesos de las conexiones de red y el resto de los parámetros se modifican para acercarse más a la salida conocida. Las iteraciones se repiten para todos los pares de entrenamiento, y así se obtiene una función de error que permite realizar los reajustes necesarios, hasta que la salida de la red para cada entrada se aproxime a la deseada. Fuente y Calonge (1999) sugieren que para un ajuste adecuado, es necesario que el conjunto de patrones de entrenamiento sea lo suficientemente grande para contener el máximo de información
- Aprendizaje no supervisado: son autoorganizativas y se basan sólo en la información de entrada. En este caso los parámetros de la red modifican únicamente los datos de entrada.

La función de activación de una neurona permite relacionar la información de entrada de una neurona con su siguiente estado de activación. Existe un gran número de funciones de activación (f). En general, para las capas de entrada se emplea la función identidad (lineal) mientras que para el resto, las funciones no lineales tales como: escalones y las sigmoides (Garrigues, 2013).

A continuación, se resumen las principales características de las RNA utilizadas en este trabajo.

2) El perceptrón multicapa

Un perceptrón multicapa, también conocido como redes multilayer feedforward (MLP), es un tipo concreto de RNA que se caracteriza por su aprendizaje supervisado, conexiones hacia adelante (feedforward)

y por estar compuesta de varias capas de neuronas ocultas entre la entrada y la salida (Figura 1.11).

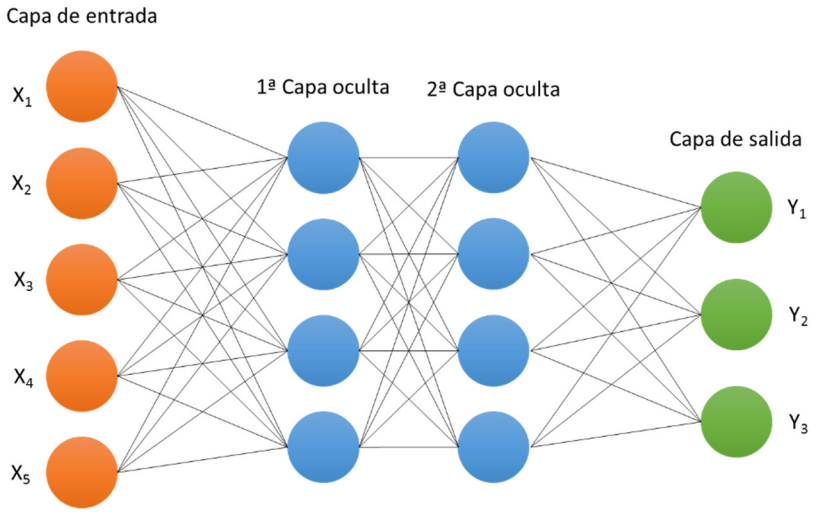


Figura 1.11. Perceptrón multicapa con dos capas ocultas

Las MLP son entrenadas habitualmente con el algoritmo de retropropagación (backpropagation, BP). Para ello, se introduce un dato en la red y se calcula el dato de salida. Éste se compara con el valor deseado y se obtiene un error en cada neurona de salida que se transmite hacia las capas ocultas (Fritsch, 1996). En función de dicho error se ajustan de manera iterativa los pesos sinápticos de cada neurona. Así, en las MLP cada neurona oculta o de salida emite dos tipos de señales:

- La señal de función que se propaga de la entrada a la salida (feedforward)
- La señal de error que es la generada por las neuronas de salida y que se retropropaga en forma de ajuste de las conexiones sinápticas hacia la capa de entrada con el fin de ajustar la salida obtenida.

Como en las PLS, para evitar el sobreentrenamiento se recurre a la validación cruzada utilizando un nuevo conjunto de pares de valores (conjunto de test). De esta manera, para cada x número de iteraciones,

la red se evaluará con el conjunto de test mediante el cálculo del error cuadrático medio.

Las MLP son las RMA más utilizadas. Entre sus diversas aplicaciones, destacan por su capacidad para clasificar y reconocer patrones y modelizar. No obstante, presenta algunos inconvenientes conocidos como el dilema de plasticidad-estabilidad. Durante el entrenamiento de una MLP, se utilizan una serie de datos de entrada con los que se fijan los pesos de la red. Si con el tiempo, los vectores de entrada varían, la red puede perder precisión por lo que la MLP tiene poca plasticidad. Para remediarlo, es posible reentrenar la red para fijar los pesos de nuevo. En este caso, se perderá la información de los vectores antiguos y por lo tanto la MLP dejará de ser estable.

3) Redes Fuzzy Artmap

Para poder solucionar los dilemas de plasticidad y estabilidad, Stephen Grossberg y posteriormente Gail Carpenter desarrollaron la Teoría de la Resonancia Adaptativa (Adaptative Resonance Theory, ART) que intenta emular la manera de procesar la información de un cerebro humano. A partir de esta teoría, se han desarrollado una serie de algoritmos neuronales que permiten un aprendizaje rápido y estable, tales como: ART1 (Carpenter y Grossberg, 1987), ART2 (Carpenter y Grossberg, 1987), ART3 y Fuzzy ART (Carpenter *et al.*, 1991) para la realización del aprendizaje no supervisado, y Artmap (Carpenter *et al.*, 1991) y Fuzzy Artmap (Carpenter *et al.*, 1992), para el supervisado.

En concreto, la red Fuzzy Artmap es una red de clasificación que constituye una generalización a vectores analógicos de la red binaria Artmap. Como se muestra en la figura 1.12, una red Fuzzy Artmap está compuesta por dos redes, ARTa y ARTb, relacionadas entre sí por un vector denominado "mapfield". La red ARTa se encarga de procesar los vectores de entrada (V) mientras que la ARTb, recibe los vectores que clasifican los datos de entrenamiento en su categoría correspondiente (C).

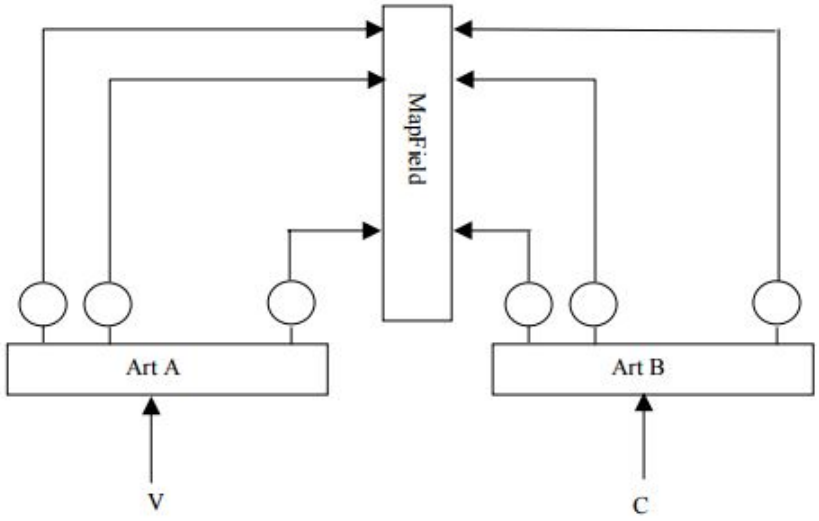


Figura 1.12. Esquema general de una red Fuzzy Artmap. (Brezmes, 2001).

Para cada medida de entrenamiento, la red ARTa activa un nodo y la red ARTb, otro. A continuación se establece una relación entre ambos nodos a través del mapfield. Así, cuando un nodo en ARTa es activado debido a una nueva entrada de datos, se comprueba que la imagen del mapfield creada para este nodo coincida con el nodo activado en ARTb (Brezmes, 2001). El siguiente paso en el caso de no coincidir es aumentar el valor del parámetro de vigilancia tratando de conseguir que las neuronas activadas en ARTa y ARTb coincidan. Sólo en el caso en el que no se consiga dicha coincidencia, se creará una nueva neurona en ARTa que será relacionada por el mapfield con el nodo en ARTb activado.

Entre las principales ventajas de este tipo de red, destacan (Gardner *et al.*, 1996; Llobet *et al.*, 1999):

- Aprendizaje rápido, lo que facilita su programación en dispositivos portátiles de bajo coste.
- No requiere de un número elevado de medidas para su entrenamiento.

- Aprendizaje continuo, que le permite adaptarse a los cambios en las propiedades del sistema a medir (plasticidad y estabilidad).
- Determinación automática de las neuronas de su capa oculta.

Estas características hacen de las Redes Fuzzy Artmap una herramienta potente para la clasificación de los datos electroquímicos procedentes de la EIS.

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2. OBJETIVOS

CONTENIDOS

2.1. OBJETIVO GENERAL

La presente Tesis Doctoral tiene un doble objetivo. Por un lado, avanzar en la revalorización de los residuos agroindustriales de frutas como la piña y el caqui mediante la mejora del proceso de obtención de bioetanol. Por otra parte, conseguir monitorizar en línea las etapas de sacarificación enzimática y fermentación mediante el uso de la espectroscopía de impedancias electroquímica (EIS).

2.2. OBJETIVOS PARTICULARES

Con el fin de cumplir con el objetivo general de la Tesis Doctoral se proponen los siguientes objetivos particulares:

1) Evaluar la acción hidrolítica de las celulasas comeciales producidas por *Aspergillus niger* y *Trichoderma reesei* en los residuos industriales de piña.
2) Investigar la aplicación de microondas como pretratamiento de los residuos industriales de piña, teniendo en cuenta su efecto sobre el rendimiento de la sacarificación, la liberación de compuestos inhibidores de la fermentación y los cambios estructurales observados.
3) Estudiar la idoneidad del uso de microondas en medio alcalino como pretratamiento de los residuos industriales de piña, analizando los azúcares y compuestos inhibidores liberados al medio y los cambios en la estructura de las muestras.
4) Evaluar el potencial de revalorización del residuo industrial de caqui de la variedad “Rojo Brillante”.
5) Estudiar la validez de las técnicas basadas en la EIS para identificar y cuantificar los azúcares fermentables obtenidos durante el proceso de hidrólisis enzimática de los residuos industriales de piña.
6) Comprobar que las metodologías que aplican la EIS son capaces de monitorizar con precisión la sacarificación enzimática de los residuos industriales de piña.

7) Validar los sistemas basados en la EIS para cuantificar el etanol añadido en residuos reales de piña mediante modelos que puedan ser implementados en dispositivos programables.

3. RESULTADOS

CONTENIDOS

3.1. ARTÍCULO 1

HYDROLYTIC PERFORMANCE OF *ASPERGILLUS NIGER* AND *TRICHODERMA REESEI* CELLULASES ON LIGNOCELLULOSIC INDUSTRIAL PINEAPPLE WASTE INTENDED FOR BIOETHANOL PRODUCTION

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Hydrolytic performance of *Aspergillus niger* and *Trichoderma reesei* cellulases on lignocellulosic industrial pineapple waste intended for bioethanol production

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ABSTRACT

Purpose: The hydrolytic action of *Aspergillus niger* and *Trichoderma reesei* commercial cellulases, alone or combined with A. niger hemicellulase, against industrial pineapple waste as a previous step to produce bioethanol was investigated.

Methods: Enzymatic hydrolysis experiments were conducted in static conditions in an incubation oven, by adding the corresponding enzyme mixture to the pineapple waste (combinations of 0, 0.1, 0.2 and 0.4 (w/w) of cellulase from *A. niger* or *T. reesei* and hemicellulase from *A. niger*). pH and total soluble solids were examined along the treatments, and the sugar profile in the final hydrolysates was evaluated by High-Performance Anion-Exchange Chromatography.

Results: *T. reesei* cellulase exhibited a significantly faster initial hydrolysing rate than *A. niger* cellulase (0.258±0.004 vs. 0.15±0.07, for the maximum enzyme concentrations assayed), although differences regarding soluble sugars increments were not significant at the end of the treatment (0.349±0.009 vs. 0.34±0.05). Glucose, fructose, sucrose, arabinose, xylose and cellobiose were identified in the hydrolysates. Increasing enzyme concentration (cellulase or hemicellulase) produced an increase in total and fermentable sugars released (17 and 11%, respectively, for the maximum enzymatic concentration assayed); besides, a synergistic effect of combining hemicellulase and cellulase was identified. Accumulation of cellobiose (up to 4.4 g/L),

which may slow down hydrolysis, evidenced the weaker β-glucosidase activity of *T. reesei* cellulase. Due to its performance and the lower cost of the enzyme, *A. niger* cellulase was chosen as an alternative.

Conclusions: Commercial *A. niger* cellulase represents an efficient alternative to *T. reesei* cellulase for the saccharification of industrial pineapple waste, especially when combined with a hemicellulase. Total sugars present in the final hydrolysates indicated that *A. niger* cellulase performed similarly at a lower cost, with no cellobiose accumulation. However, if processing time is a limiting factor, *T. reesei* cellulase could be the one preferred.

Keywords: pineapple waste, lignocellulosic biomass, enzymatic hydrolysis, cellulases, bioethanol.

INTRODUCTION

At present, the search for renewable, sustainable and environmentally-friendly energy sources is encouraged so as to face the need for energy supply and in response to climate change (EC, 2009). In this context, bioethanol is recognized worldwide as an alternative to petroleum-derived transport fuels (Demirbas, 2011). However, competition for food resources has made first generation bioethanol production a controversial issue, for which much of recent research has been focused on second generation bioethanol. Among the raw materials available to produce the so-called second generation of biofuels, those obtained from agricultural, forestry or industrial wastes are characterized for being low-cost raw materials that do not compete with food supplies or threaten biodiversity. The use of residues or waste feedstocks for energy production continues to be an interesting issue due to growing energy demand, depletion of fossil fuels, greenhouse emissions and climate change concern, among other reasons. On the other hand, residual biomass needs to be properly disposed in order to reduce its environmental impact, for which there is a great interest in giving added value to these waste materials. The agro-food industry is particularly characterized by generating

significant amounts of organic residues. This is the case of the pineapple industry since industrial pineapple waste (peel, core and crown) generally accounts for at least 50% of the total pineapple weight (Ketnawa *et al.,* 2012), 20 to 40% in the form of peel and core (Nga and Trang, 2015). Disposal of this waste is of capital importance for the industry due to its high biochemical and chemical oxygen demand (Abdullah and Mat, 2008); therefore, valorisation of these residues would contribute both to facilitate their disposal and to obtain a benefit from an otherwise non-valuable material.

Residual biomass mostly consists of lignocellulose, which contains large quantities of sugar polymers such as cellulose and hemicellulose (Abdullah and Mat, 2008). According to Roda *et al.* (2014), pineapple peel waste is made of 35-50% cellulose, 20-35% hemicellulose, and 5-30% lignin. Khedkar *et al.* (2016) have recently reported a content of 35% cellulose, 19% hemicellulose, and 16% lignin on dry basis. Therefore, industrial pineapple waste is a good candidate for biofuels or other metabolites production, since cellulose and hemicellulose are polymers potentially hydrolysable to simple fermentable sugars.

Producing ethanol from lignocellulosic biomass requires the hydrolysis of part of the cellulose and hemicellulose to fermentable sugars (Galbe and Zacchi, 2002). Among the hydrolysis treatments, enzymatic hydrolysis has a lower utility cost than acid or alkaline hydrolysis, since the hydrolysis is conducted at milder conditions (Sun and Cheng, 2002). Together with sulphuric acid, cellulolytic enzymes are the major hydrolysers of cellulose and hemicelluloses, and present fewer disadvantages than the use of chemicals. Filamentous fungi segregate two types of enzymatic complexes that can hydrolyse the lignocellulosic matrix: cellulases that hydrolyse the crystalline cellulose into small oligosaccharides and then into glucose; and hemicellulases, which hydrolyse the hemicellulose into monomeric sugars. Fungal cellulase is made up of three major groups of cellulases: endoglucanases, cellobiohydrolases or exoglucanases and β-glucosidases (Fujii *et al.,* 2009). Among the enzymes produced by

fungi, the enzymes derived from *Trichoderma reesei* represent the best characterized and have been assayed for the enzymatic saccharification of lignocellulosic materials (Rosgaard *et al.,* 2007). *T. reesei* cellulase has been said to be the most productive and powerful destroyer of crystalline cellulose; however, it has also demonstrated a relatively weak β-glucosidase activity for which the reaction from cellobiose to glucose has been shown to be slow (Sternberg *et al.,* 1997; Balat, 2011). *Aspergillus niger* cellulase has also been tested in some lignocellulosic raw materials (Sternberg *et al.,* 1997; Aguiar, 2001; Park *et al.,* 2002; Chootnut *et al.,* 2014). Although not such hydrolytic power have been attributed to it, it could represent an efficient alternative to *T. reesei* cellulase. In addition, enzyme costs can be significantly reduced with the use of *A. niger* cellulase, since some of the commercial options available are less expensive. On the other hand, the combined use of a cellulase and hemicellulase could potentially increase the final amount of sugars available for fermentation, since hemicellulase would partially cleave hemicellulose bonds to yield monomeric sugars such as glucose or xylose.

Therefore, the aim of the present study was to analyse and compare the hydrolytic action of both cellulases, *A. niger* and *T. reesei*, against industrial pineapple waste as a previous step to produce bioethanol. Additionally, the synergistic effect of combining them with *A. niger* hemicellulase is discussed. Finally, optimum condition of *A. niger* cellulase hydrolysis were determined.

MATERIALS AND METHODS

Raw material and pre-treatment of pineapple waste

Pineapple fruits (*Ananas comosus* [L.] Merr., MD-2 cv.) were selected based on external factors such as the absence of injuries, ripeness and weight. The crown was first removed, and the pulp was separated from the rest of the fruit (peel and core) by using a pineapple cutter. The waste material (crown, peel and core) was cut into smaller pieces and then physically treated by grinding it in a blender in order to decrease particle size and increase cellulose and hemicellulose

accessibility. After grinding, pH was adjusted to 5 by adding $Ca(OH)_2$. The resulting product was then thermally treated in an autoclave at 121 °C for 20 min.The grinded and sterilized material was finally frozen and kept at -22 °C until the experiments were conducted.

Characterization of the pineapple waste liquid phase

Pineapple waste was characterized in terms of pH, total soluble solids (TSS) and sugars present in the waste liquid phase. The pH was measured with a digital pH meter (Mettler Toledo Inlab) and the TSS (°Brix) were measured by refractrometry (table-top ABBE-Atago refractometer thermostated to 20 °C). The sugars present in the liquid phase of the grounded waste were measured by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detector (HPAEC-PAD), using a Metrohm IC chromatograph system equipped with a 716 Compact module and an ICnet 2.0 software program. A three-step PAD setting was used with the following path intervals (ms) and potentials (V): t_1: 400/E_1 = +0.05 (detection); t_2: 200/E_2 = +0.75 (cleaning); t_3: 400/E_3 = -0.15 (regeneration). The column used was a Metrosep Carb 1 250/4.6 column (250 mmL x 4.6 mmID) coupled to a guard column. Analyses were done at 32 °C, 8.8 MPa, injection volume: 20 µL and using sodium hydroxide 0.1 M as the mobile phase (1 mL/min). Liquid phase was previous filtered (0.45 µm nylon filter) and diluted (1:2000 v/v in bidistilled water). Glucose, fructose and sucrose standards (Sigma-Aldrich, Co.; purity ≥ 99.5%) were used to prepare standard calibration curves (2.5, 5, 10, 15, 25 and 50 ppm). Determinations were carried out in triplicate.

Enzymatic hydrolysis experiments

Cellulase from *Aspergillus niger* (1.08 U/mg solid; Sigma-Aldrich, Co.) and *Trichoderma reesei* ATTC 26921 (6 U/mg solid; Sigma-Aldrich, Co.) were combined with *A. niger* hemicellulase (5 U/ mg solid; Sigma-Aldrich, Co.) at the following concentrations: 0, 0.1, 0.2 and 0.4% (w/w). The corresponding enzyme mixture was added to 50 g of thawed pineapple waste in a 100 mL beaker, and samples were placed in an incubation oven at 40 °C, where enzymatic hydrolysis was

performed in static conditions for 24 h. Conditions of pH and temperature were fixed according to information sheet of supplier, whereas duration was based on preliminary studies. Experiments were conducted in triplicate.

The evolution of the TSS and pH values was registered at hourly intervals for the first seven hours and at the end of the experiment (24 h). Sugars present in the final hydrolysate (24 h) were determined by HPAEC-PAD as stated previously. Arabinose, glucose, xylose, fructose, sucrose and cellobiose standards (Sigma-Aldrich, Co; purity ≥99.5%) were used in order to identify and quantify the sugars released. Standard curves were prepared from dilutions of these standards (2.5, 5, 10, 15, 25 and 50 ppm). Determinations were performed in duplicate.

Optimum conditions for *A. niger* cellulase performance against pineapple waste

After the enzymatic hydrolysis experiments, *A. niger* cellulase was selected for further investigations, with the aim of determining the optimum conditions for *A. niger*. An experimental design with two independent variables (pH and temperature) at three levels (pH = 4, 5 and 6; T = 40, 50 and 60 °C) was performed. Pineapple blended waste was saccharified with 0.4% (w/w) of cellulase and 0.2% (w/w) of hemicellulase from *A. niger* at the above mentioned pH and temperatures. Sugar profile was measured at the end of the saccharification process (24 h).

The data were analyzed by multiple regressions to fit second-order polynomial regression models for total sugars containing the coefficient of linear, quadratic and two factor interaction effects (Equation 1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j + e \quad \textbf{Equation 1}$$

Where, Y is the response parameter (total sugar yield), β_0 is the intercept value, β_i (i = 1, 2, …, k) is the first order model coefficient, β_{ij}

is the interaction effect and β_{ii} represents the quadratic coefficient of x_i. x_i and x_j are the independent variables that influence the response parameter (pH and temperature) and e represents the random error.

Statistical analysis

Statgraphics Centurion XVI (Manugistics Inc.; Rockville, MD, USA) was used for statistical analyses. One-way and multifactor analyses of variance (ANOVA, 95% confidence level) were performed on the data obtained.

RESULTS AND DISCUSSION

Characterization of the raw material (industrial pineapple waste)

Glucose (27 ± 2 g/L), fructose (22 ± 2 g/L), and sucrose (20 ± 3 g/L), were the sugars identified in the liquid phase of pineapple waste. The amount of total simple sugars obtained (69 ± 1 g/L) was similar to the reported by Abdullah and Mat (2008), and lower than the reported by Ban-Koffi and Han (1990). Differences were probably due to processing and/or raw material characteristics. Total soluble solids, measured as Brix degrees, were 10.6 ± 0.2. Pineapple waste had an acid pH (3.63 ± 0.01) as a result of the different organic acids present in the liquid phase (Roda *et al.,* 2014).

Kinetics of the hydrolysis

Kinetics of saccharification were studied by measuring the increase in total soluble solids present in the liquid phase (Δz_{ss}) during the treatments. Results are given in figure 3.1.1. Each graph (A, B, C, D) corresponds to a different concentration of hemicellulase, while different concentrations and origin of cellulase are plotted within a graph. As it is deduced from the plots, the rate of enzymatic hydrolysis was faster during the first two hours of saccharification, fell in the following hours and was negligible at 24 h of enzymatic treatment, indicating the end of the process. Increasing enzyme concentration (cellulase and hemicellulase) increased the hydrolysis rate as well as the final result of hydrolysis.

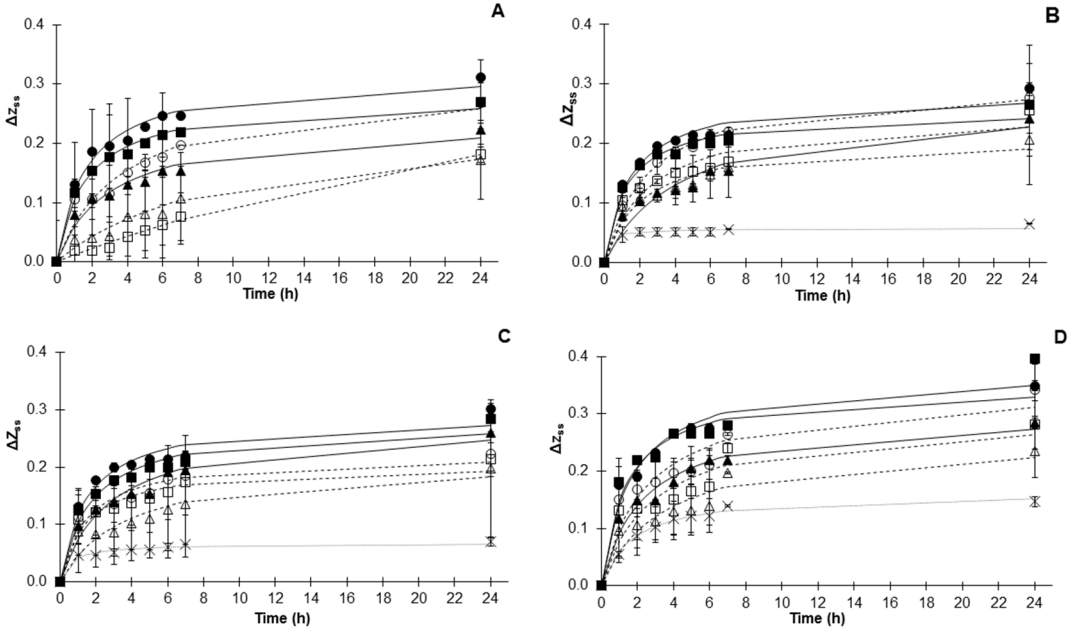

Figure 3.1.1. Total soluble solids increase (Δz_{ss}) in the pineapple waste as a result of the different enzyme mixtures used for saccharification. A: 0% hemicellulase, B: 0.1% hemicellulase, C: 0.2% hemicellulase and D: 0.4% hemicellulase. Symbols: x: 0% cellulose; ▲, 0.1% cellulase; ■, 0.2% cellulase; ●, 0.4% cellulase. The lines represent the model fitted to experimental data: dotted line and empty point: cellulase from *A. niger*; solid line and full point: cellulase from *T. reesei*. Error bars indicate the standard deviation from the mean of two replicates.

As can be deduced from the plots, *T. reesei* cellulase implied a faster initial hydrolysis rate, although significant differences between *T. reesei* and *A. niger* cellulases were not as manifested at the end of the treatment. Variation in the soluble solids content of the liquid phase followed a hyperbolic type pattern (figure 3.1.1). In order to determine the effect of the different enzyme combinations and particularly the cellulase source in the initial rate of hydrolysis, an adaptation of the empirical model proposed by Peleg (1988) was used. This model has been used to describe different processes that respond to this type of curves and it has not been deduced from any law or physical principle (Cárcel *et al.,* 2010). Thus, the evolution of the increment in total soluble solids in the liquid phase (Δz_{ss}) along the enzymatic process was fitted to equation 2, in which the inverse of the constant K_1 corresponds to the initial rate of the reaction (R_0), and the inverse of the constant K_2 represents the asymptotic value of the curve ($\Delta z_{ss\ assymptote}$). Data were fitted to the model by non-linear regression using Statgraphics Centurion XVI. Results (R_0, $\Delta z_{ss\ Pred.}$, R^2) as well as the experimental value at 24 h ($\Delta z_{ss\ Exp.24\ h}$) are shown in table 3.1.1.

$$\Delta z_{ss} = \frac{t}{K_1 + K_2 \times t} \qquad \textbf{Equation 2}$$

Where $R_0 = 1/K_1$ and $\Delta z_{ss\ predicted} = 1/K_2$

Table 3.1.1. Results of fitting TSS increments along saccharification (Δz_{ss}) to Eq. 1. Initial reaction rate (R_0), predicted asymptotic value ($\Delta z_{ss\ Pred.}$), R^2, and experimental value at 24 h are given ($\Delta z_{ss\ Exp.24h}$).

Cellulase (%) (w/w)	Hemicellulase from *A. niger* (%)(w/w)	R_0	$\Delta z_{ss\ Pred.}$	$\Delta z_{ss\ Exp.\ 24h}$	R^2
0	**0.1**	0.06 (0.02)^{abcd}	0.11 (0.07)^{ab}	0.120 (0.014)^{ab}	0.9309
0	**0.2**	0.05 (0.05)^{abc}	0.10 (0.04)^{ab}	0.10 (0.02)^{a}	0.9673
0	**0.4**	0.10 (0.04)^{abcdefg}	0.160 (0.011)^{bc}	0.147 (0.009)^{abc}	0.9520
0.1	**0**	0.03 (0.03)^{ab}	0.2 (0.9)^{bc}	0.17 (0.07)^{abcd}	0.9674
0.1	**0.1**	0.11 (0.03)^{abcdefg}	0.21 (0.09)^{bc}	0.21 (0.07)^{bcdefg}	0.9500
0.1	**0.2**	0.06 (0.05)^{abc}	0.21 (0.11)^{bc}	0.20 (0.12)^{bcdef}	0.9032
0.1	**0.4**	0.09 (0.05)^{abce}	0.250 (0.008)^{bc}	0.24 (0.05)^{defghi}	0.8733
0.2	**0**	0.013 (0.011)^{a}	-0.1 (0.6) ^{a}	0.181 (0.013)^{abcde}	0.9818
0.2	**0.1**	0.11 (0.04)^{abcdefg}	0.3 (0.1)^{bc}	0.26 (0.08)^{defghij}	0.9227
0.2	**0.2**	0.16 (0.12)^{cdefghi}	0.26 (0.02)^{bc}	0.21 (0.03)^{cdefg}	0.9359
0.2	**0.4**	0.17 (0.17)^{defghi}	0.21 (0.04)^{bc}	0.28 (0.04)^{fghijk}	0.8399
0.4	**0**	0.09 (0.04)^{abcdef}	0.300 (0.007)^{bc}	0.27 (0.03)^{efghijk}	0.9226
0.4	**0.1**	0.12 (0.05)^{abcdefgh}	0.32 (0.09)^{bc}	0.28 (0.09)^{fghijk}	0.9650
0.4	**0.2**	0.15 (0.11)^{cdefghi}	0.23 (0.03)^{bc}	0.22 (0.03)^{cdefgh}	0.9402
0.4	**0.4**	0.15 (0.07)^{cdefghi}	0.340 (0.003)^{bc}	0.34 (0.05)^{jkl}	0.8950

A. niger

^{a,b,c} Similar superscripts indicate statistically homogenous groups with a confidence level of 95%.

Table 3.1.1. (Cont.) Results of fitting TSS increments along saccharification (Δz_{ss}) to Eq. 1. Initial reaction rate (R_0), predicted asymptotic value ($\Delta z_{ss\ Pred.}$), R^2, and experimental value at 24 h are given ($\Delta z_{ss\ Exp.24h}$).

Cellulase (%) (w/w)		Hemicellulase from *A. niger* (%)(w/w)	R_0	$\Delta z_{ss\ Pred.}$	$\Delta z_{ss\ Exp.\ 24h}$	R^2
T. reesei	**0.1**	**0**	0.082 (0.014)^{abcde}	0.21 (0.04)^{bc}	0.2233 (0.0015)^{cdefgh}	0.9626
	0.1	**0.1**	0.07 (0.02)^{abcd}	0.27 (0.05)^{bc}	0.24 (0.02)^{defghi}	0.9447
	0.1	**0.2**	0.099 (0.011)^{abcdefg}	0.280 (0.004)^{bc}	0.3 (0)^{defghij}	0.9620
	0.1	**0.4**	0.132 (0.005)^{bcdefgh}	0.300 (0.011)^{bc}	0.284 (0.008)^{fghijk}	0.9719
	0.2	**0**	0.165 (0.017)^{cdefghi}	0.28000 (0.00001)^{bc}	0 (0)^{fghijk}	0.9876
	0.2	**0.1**	0.20 (0.02)^{ghi}	0.260 (0.005)^{bc}	0.265 (0.008)^{efghijk}	0.9732
	0.2	**0.2**	0.161 (0.010)^{cdefghi}	0.280 (0.007)^{bc}	0.284 (0.008)^{fghijk}	0.9615
	0.2	**0.4**	0.227 (0.013)^{hi}	0.370 (0.005)^{c}	0.395 (0.009)^{l}	0.9831
	0.4	**0**	0.19 (0.06)^{efghi}	0.316 (0.013)^{bc}	0.312 (0.009)^{ijkl}	0.9816
	0.4	**0.1**	0.195 (0.007)^{fghi}	0.284 (0.007)^{bc}	0.293 (0.009)^{ghijk}	0.9696
	0.4	**0.2**	0.196 (0.004)^{fghi}	0.290 (0.009)^{bc}	0.302 (0.009)^{hijk}	0.9613
	0.4	**0.4**	0.258 (0.004)^{i}	0.350 (0.009)^{bc}	0.349 (0.009)^{kl}	0.9344

^{a,b,c} Similar superscripts indicate statistically homogenous groups with a confidence level of 95%.

The determination coefficients (table 3.1.1) indicate a good agreement between experimental ($\Delta z_{ss\ experimental}$) and predicted ($\Delta z_{ss\ predicted}$) values. The coefficients were generally greater when cellulase from *T. reesei* was employed, for which it was deduced that the model fitted better these data. Multifactor ANOVA indicated that *T. reesei* and *A. niger* cellulases have a statistical significant effect on the initial rate of the hydrolysis (p-value < 0.05). R_0 values were higher when cellulase from T. reesei was used, indicating a faster initial reaction rate of this enzyme, which is in line with the greater hydrolytic power attributed to this enzyme in the literature (Bayer *et al.,* 1998). However, differences between both cellulase types were not maintained at the end of the experiments according to the experimental values after 24 h of hydrolysis ($\Delta z_{ss\ Exp.24h}$). Therefore, the present results suggest that both enzyme combinations may lead to a similar increase in the TSS content after 24 h of hydrolisis, despite the lower initial saccharification rate of *A. niger* cellulase.

As for pH, it decreased as the enzymatic hydrolysis proceeded, this reduction being faster in the first two hours of saccharification, concurrently to sugar release. The action of both cellulases, *A. niger* and *T. reesei*, had a statistically significant effect on pH (p-value < 0.00); nevertheless, lower pH values were obtained when *A. niger* cellulase was used (Figure 3.1.2.).

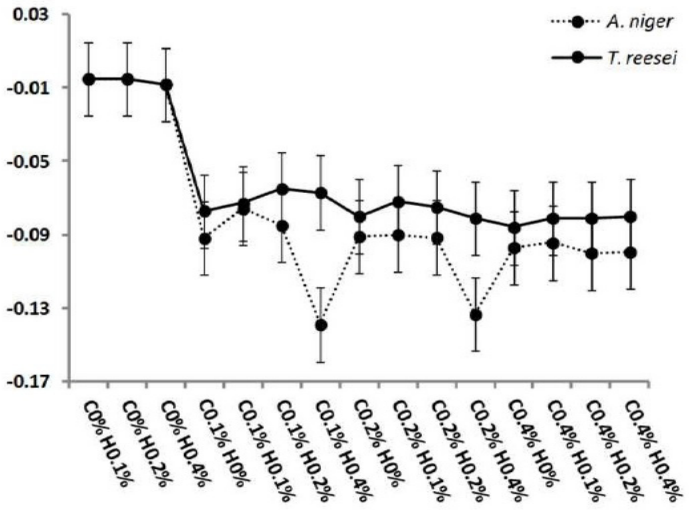

Figure 3.1.2. Interaction plot and LSD intervals for pH decreases for factors 'enzymatic treatment' and 'cellulase origin'. Solid line: cellulase from *T. reesei*. Dotted line: cellulase from *A. niger*.

Sugar profile of the hydrolysates

Total soluble solids is an indirect measure of the amount of sugars present in the hydrolysate; however, in order to obtain more accurate results, the specific sugars present in the final hydrolysate (24 h) were identified and quantified by ion exchange chromatography. Results of this analysis are summarized in table 3.1.2.

Table 3.1.2. Sugar profile and sugar content (g/L) in the hydrolysed waste, for each combination of cellulases (*A. niger* or *T. reesei*) and hemicellulase (*A. niger*).

Cellulase (%) (w/w)	Hemicellulase from *A. niger* (%)(w/w)	Glucose	Fructose	Sucrose	Fermentable sugars	Arabinose	Xylose	Cellobiose	Total Sugars
0	**0**	26 (2)^{a}	22.4 (1.4)^{a}	20.8 (1.8)^{i}	69.6 (1.5)^{abcdefgh}	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	69.4 (1.5)^{a}
0	**0.1**	27 (3)^{ab}	22.6 (1.8)^{a}	20.5 (1.6)^{hi}	70 (4)^{abcdefg}	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	70 (4)^{a}
0	**0.2**	28 (2)^{abc}	23.5 (1.4)^{abcd}	19.2 (1.5)^{ghi}	70.4 (1.2)^{bcdefg}	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	70.4 (1.2)^{a}
0	**0.4**	28 (2)^{abc}	23.6 (0.9)^{abcd}	18.2 (1.0)^{g}	70 (3)^{abcdefg}	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	70 (3)^{a}

^{a,b,c} Similar superscripts indicate statistically homogenous groups with a confidence level of 95%

Table 3.1.2. (Cont.) Sugar profile and sugar content (g/L) in the hydrolysed waste, for each combination of cellulases (*A. niger* or *T. reesei*) and hemicellulase (*A. niger*).

Cellulase (%) (w/w)		Hemicellulase from *A. niger* (%)(w/w)	Glucose	Fructose	Sucrose	Fermentable sugars	Arabinose	Xylose	Cellobiose	Total Sugars
From *A. niger*	**0.1**	**0**	32 (3)^{defghi}	24 (4)^{abcde}	7.9 (0.6)^{d}	64 (7)^{a}	0.9 (0.2)^{cdefgh}	1.3 (0.3)^{b}	4.3 (0.9)^{h}	64 (7)^{ab}
	0.1	**0.1**	33 (1)^{fghij}	24.5 (1.0)^{abcdef}	7.5 (0.6)^{cd}	65 (3)^{ab}	1.0 (0.2)^{defghi}	1.3 (0.4)^{b}	4.3 (1.0)^{h}	65 (3)^{abc}
	0.1	**0.2**	32.9 (0.8)^{efghi}	25.4 (0.9)^{bcdefg}	7.3 (0.3)^{bcd}	65.6 (1.7)^{abc}	1.1 (0.2)^{fghijk}	1.4 (0.3)^{bc}	4.1 (0.8)^{fgh}	65.6 (1.7)^{abcd}
	0.1	**0.4**	34(1)^{ghijk}	25.8 (1.6)^{cdefgh}	7.1 (0.8)^{bcd}	67 (3)^{abcde}	1.1 (0.1)^{ghijkl}	1.5 (0.3)^{bcd}	3.7 (0.5)^{efgh}	67 (3)^{abcde}
	0.2	**0**	34 (2)^{ijk}	26.0 (1.1)^{defgh}	5.7 (0.9)^{abc}	66.1 (1.9)^{abcd}	1.2 (0.3)^{hijkl}	1.5 (0.2)^{bcde}	3.6 (0.6) ^{efgh}	66.1 (1.9)^{abcd}
	0.2	**0.1**	37 (2)^{jkl}	26.5 (1.4)^{efgh}	5.3 (0.4)^{abc}	69 (4)^{abcdef}	1.2 (0.1)^{ijklm}	1.6 (0.2)^{bcde}	3.5 (0.7) ^{efgh}	69 (4)^{abcdefg}
	0.2	**0.2**	37 (2)^{kl}	26.5 (1.6)^{efgh}	5.2 (0.8)^{ab}	69 (3)^{abcdef}	1.2 (0.2)^{ijklm}	1.6 (0.3)^{bcde}	3.3 (1.0)^{defg}	69 (3)^{abcdefg}
	0.2	**0.4**	37 (3)^{kl}	27 (3)^{fgh}	4.3 (0.9)^{a}	68 (5)^{abcdef}	1.2 (0.1)^{jklm}	1.6 (0.2)^{bcde}	3.3 (1.0)^{def}	68 (5)^{abcdef}
	0.4	**0**	40 (5)^{lm}	27 (2)^{fgh}	4.5 (0.7)^{a}	71 (8)^{cdefg}	1.2 (0.3)^{jklm}	1.6 (0.3)^{bcde}	1.8 (0.8)^{bc}	71 (8)^{bcdefgh}
	0.4	**0.1**	40 (5)^{lm}	28.0 (1.5)^{gh}	4.4 (0.5)^{a}	72 (7)^{fg}	1.3 (0.1)^{klm}	1.7 (0.3)^{bcde}	1.0 (0.5)^{b}	72 (7)^{cdefgh}
	0.4	**0.2**	42 (4)^{m}	28 (4)^{gh}	4.2 (0.6)^{a}	74 (7)^{fg}	1.3 (0.2)^{klm}	1.7 (0.1)^{bcde}	1.0 (0.4)^{ab}	74 (7)^{efgh}
	0.4	**0.4**	42 (4)^{m}	28 (2)^{h}	3.9 (0.7)^{a}	75 (6)^{g}	1.4 (0.1)^{lm}	1.8 (0.4)^{bcdef}	0 (0)^{a}	75 (6)^{efgh}

^{a,b,c} Similar superscripts indicate statistically homogenous groups with a confidence level of 95%

Table 3.1.2. (Cont.) Sugar profile and sugar content (g/L) in the hydrolysed waste, for each combination of cellulases (*A. niger* or *T. reesei*) and hemicellulase (*A. niger*).

Cellulase (%) (w/w)		Hemicellulase from *A. niger* (%)(w/w)	Glucose	Fructose	Sucrose	Fermentable sugars	Arabinose	Xylose	Cellobiose	Total Sugars
From *T. reesei*	**0.1**	**0**	29 (1)^{abcd}	24 (3)^{ab}	19.1 (0.2)^{ghi}	72 (3)^{cdefg}	0.5 (0.2)^{b}	1.8 (0.8)^{bcdef}	3.9 (1.0)^{efgh}	78 (3)^{defgh}
	0.1	**0.1**	29.2 (0.5)^{abcd}	24 (2)^{ab}	19 (3)^{ghi}	72 (4)^{cdefg}	0.7 (0.1)^{bc}	1. 9 (0.5)^{bcdefg}	4.4 (0.5)^{h}	79 (4)^{fgh}
	0.1	**0.2**	29 (2)^{abcd}	24.2 (0.2)^{ab}	18.8 (1.1)^{ghi}	72 (3)^{defg}	0.7 (0.3)^{bcd}	2.0 (0.3)^{cdefgh}	4.4 (0.5)^{h}	79 (3)^{fgh}
	0.1	**0.4**	29.3 (0.4)^{abcde}	24.3 (1.4)^{abc}	18.7 (1.1)^{ghi}	72.3 (1.4)^{defg}	0.74 (0.07)^{bcd}	2.2 (0.6)^{defgh}	4.2 (0.5)^{fgh}	79 (1)$^{fgh\ g}$
	0.2	**0**	29.7 (0.8)^{bcdef}	24.5 (1.4)^{abcd}	19 (3)^{gh}	73 (2)^{efg}	0.74 (0.08)^{bcd}	2.2 (0. 5)^{efghi}	4.3 (0.8)^{gh}	80 (2)^{fgh}
	0.2	**0.1**	29.9 (0.6)^{cdefg}	25 (2)^{abcd}	18.2 (1.1)^{gh}	73 (4)^{defg}	0.76 (0.04)^{bcd}	2.4 (0.9)^{fghij}	3.6 (0.6)^{efgh}	79 (3)^{fgh}
	0.2	**0.2**	30.0 (0.4)^{cdefg}	25 (4)^{abcd}	17.9 (1.1)^{gi}	73 (4)^{defg}	0.8 (0.2)^{bcde}	2.6 (0.8)^{ghijk}	3.5 (1.1)^{efgh}	79 (4)^{fgh}
	0.2	**0.4**	30 (1)^{cdefg}	26 (2)^{abcde}	18 (2)^{g}	73 (4)^{fg}	0.8 (0.3)^{cdef}	2.7 (0.9)^{hijk}	3.2 (1.3)^{def}	80 (2)^{fgh}
	0.4	**0**	31 (1)^{cdefgh}	26 (2)^{abcdef}	18 (2)^{fg}	74 (5)^{fg}	0.9 (0.3)^{cdefg}	2.8 (0.6)^{ijk}	3.0 (0.3)^{de}	81 (5)^{gh}
	0.4	**0.1**	31.3 (0.5)^{defghi}	25.9 (1.4)^{abcdef}	17.4 (1.4)^{fg}	75 (3)^{fg}	0.9 (0.3)^{cdefgh}	2.9 (1.1)^{jk}	2.5 (0.3)^{cd}	81 (2)^{gh}
	0.4	**0.2**	32 (3)^{defghi}	26.2 (1.4)^{abcdef}	16 (2)^{ef}	74 (2)^{fg}	0.9 (0.2)^{cdefgh}	2.9 (0.7)^{jk}	1.9 (0.2)^{bc}	80 (3)^{fgh}
	0.4	**0.4**	33.7 (0.6)^{hijk}	27.4 (1.4)^{defgh}	15 (4)^{e}	76 (4)^{g}	1.0 (0.3)^{efghij}	3.2 (0.7)^{k}	1.9 (0.9)^{bc}	82 (4)^{h}

^{a,b,c} Similar superscripts indicate statistically homogenous groups with a confidence level of 95%

Lignocellulosic hydrolysates are characterized by presenting a variety of sugars. Among them, six-carbon sugars (hexoses) such as glucose or fructose and five-carbon sugars (pentoses) such as xylose and arabinose are usually present if both cellulose and hemicellulose have been hydrolysed (Balat, 2011). In this particular case, and apart from the sugars identified in the pineapple waste (glucose, fructose and sucrose), arabinose, xylose and cellobiose were present in the hydrolysates.

The yeast *Sacharomyces cerevisiae*, which is naturally able to ferment sucrose, fructose and glucose, is one of the most effective and well-known ethanol producing microorganisms. It is successfully employed at industrial scale, allowing for high ethanol productivity, since it has high tolerance to ethanol and to inhibitors normally present in lignocellulosic residues (Balat, 2011; Hahn-Hagerdal *et al.,* 2007; Matsushika *et al.,* 2009). Since sucrose, fructose and glucose are naturally fermented by *S. cerevisiae* and other ethanologenic microorganisms such as *Zymmomonas mobilis*, they are frequently grouped under the concept of fermentable sugars. Fermentable sugars significantly increased when increasing both enzymes concentration (cellullase and hemicellulase) to the pineapple waste (Table 3.1.2). In particular, *T. reesei* cellulase exhibited a higher hydrolytic power at lower enzyme concentrations. In contrast, enzyme concentration effect was more significant for *A. niger* cellulase so that increasing the enzyme concentration up to 0.4 resulted in a similar fermentable sugars content in both hydrolysates (*T. reesei* or *A. niger*). In all cases, the addition of hemicellulase had a positive impact in fermentable sugars release.

Addition of both cellulases, *A. niger* or *T. reesei*, had a statistically significant effect on the release of glucose (p-value < 0.05), although glucose content in the hydrolysates was significantly higher when *A. niger* cellulase was used for saccharification. This is evidenced in figure 3.1.3., were main differences between *A. niger* and *T. reesei* performance have been plotted. During cellulose hydrolysis glucose is

released due to a synergistic action of three fungal cellulases (Rosgaard *et al.,* 2007; Balat, 2011): 1) the endoglucanases, which act by randomly hydrolysing the internal glycosidic linkages of the cellulose chain; 2) the cellobiohydrolases, also known as exoglucanases, which act on the ends of the chains, releasing glucose monomers, cellobiose and oligosaccharides with a low molecular weight; and 3) the β-glucosidases, which convert cellobiose into glucose (Fujii *et al.,* 2009). In addition, enzymatic hydrolysis of hemicellulose also releases glucose, among other sugars.

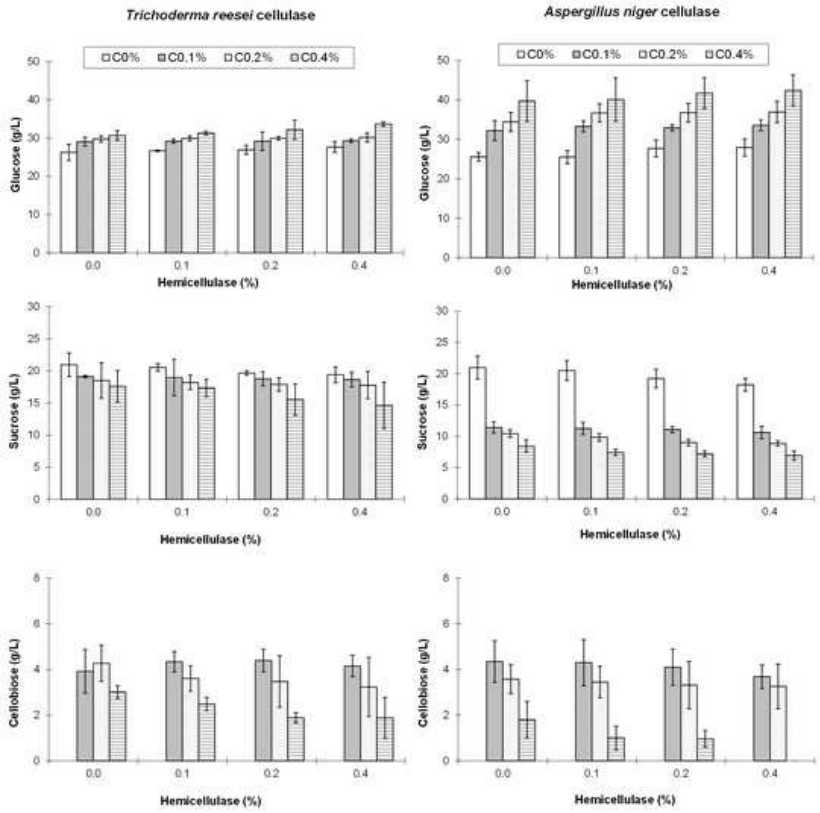

Figure 3.1.3. Comparison of glucose, sucrose and cellobiose contents (0, 0.1, 0.2, and 0.4 g/L) after 24 h of hydrolysis with *T. reesei* and *A. niger* cellulase, combined with *A. niger* hemicellulase (0, 0.1, 0.2, and 0.4 g/L).

Apart from the hydrolysis phenomenon, a decrease in sucrose concentration together with an increase in fructose and glucose contents was also observed, which suggested the inversion of this dimmer. As shown in figure 3.1.3., sucrose decrease was more evident for *A. niger* cellulase as compared to *T. reesei* cellulase, this contributing to a more significant increase in the glucose content. Since the enzymes used in the present work are not expected to promote sucrose inversion, it is postulated that acid pH was responsible for this inversion (Ban-Koffi and Han, 1990; Abdullaha and Mat, 2008). This is reinforced by the fact that lower pH values were registered when *A. niger* cellulase was used (Figure 3.1.2.).

T. reesei cellulase has been claimed to be the most productive and powerful destroyers of crystalline cellulose (Balat, 2011). However, *T. reesei* cellulases present a relatively weak β-glucosidase activity and the reaction from cellobiose to glucose has been shown to be slow (Medve *et al.,* 1998; Sun y Cheng, 2002; Verardi *et al.,* 2012). In addition, cellobiose has a strong inhibition towards endo and exoglucanases so that the accumulation of this dimmer significantly slows down the hydrolysis process, leading to a non-desirable result. In fact, supplementation of β-glucosidase is sometimes recommended due to its insufficient amount in *T. reesei*, in order to avoid cellulases inhibition due to cellobiose accumulation (Balat, 2011). Therefore, cellobiose accumulation is an important phenomenon to evaluate. The enzymes assayed in the present work had a statistically significant effect on cellobiose yield (p-value < 0.05). It was observed that if low cellulose concentrations were used, (0.1% for both cellulases), cellobiose started to accumulate in the hydrolysates (figure 3.1.3.). Then, including hemicellulase in the mixture or increasing the amount of cellulase added to the medium promoted cellobiose depletion. The latter phenomenon was more noticeable in the case of *A. niger* cellulase, cellobiose completely dissapearing at the higher cellulase concentration assayed. In line with the literature, the amount of β-glucosidase in *T. reesei* cellulase was lower than the needed for the efficient hydrolysis of cellobiose into glucose, as compared to *A. niger*

cellulase. The fact that *T. reesei* cellulase tends to accumulate more cellobiose could be responsible for a slowdown of the hydrolytic process, due to inhibition of endo and exoglucanases, despite the faster initial hydrolysis rate of this cellulase.

As said, xylose and arabinose are pentoses that may appear in the hydrolysates as a result of hemicellulose hydrolysis. Although pentoses are not fermentable by *S. cerevisiae*, some yeast have been reported to be efficient in xylose conversion into ethanol, such as *Pichia stipitis*, *Candida shehatae*, *Candida parapsilosis* and *Pachysolen tannophilus* (Huang *et al.,* 2009; Balat, 2011). In addition, several genetic engineered strains of *S. cerevisiae* capable of metabolizing pentoses have already been developed (Hahn-Hagerdal *et al.,* 2007; Mussatto *et al.,* 2010). Among bacteria, *Klebsiella oxytoca* is able to grow either on hexoses or pentoses, as well as on cellobiose and cellotriose (Cardona and Sánchez, 2007). Likewise, *Escherichia coli* is naturally able to use a variety of sugars, for which work has been focused on selectively produce ethanol and increase ethanol tolerance (Dien *et al.,* 2003). As for *Zymomonas mobilis*, it is naturally able to ferment glucose, fructose and sucrose, producing ethanol at high yields. It has also been engineered to successfully co-ferment xylose and arabinose (Zhang *et al.,* 1995; Deanda *et al.,* 1996; Dien *et al.,* 2003).

As compared to fermentable sugars, xylose and arabinose were present at significant lower concentrations in the hydrolysates. On the other hand, although it is hemicellulase that hydrolyses hemicellulose into monomeric sugars, results indicated that the addition of cellulases from *A. niger* or *T. reesei* (p-value < 0.05) had a statistically significant effect on pentose release. Moreover, when cellulase was not added to the ground material neither arabinose nor xylose were detected, suggesting that cellulose hydrolysis promoted hemicellulase action. This was also confirmed the other way around, since hemicelluloses hydrolysis also enhanced cellulose accessibility. This synergistic effect of both enzymes, was probably a consequence of the increased

accessibility of the structure thanks to the combined action of both enzymes. Some differences in the amount of xylose and arabinose released to the medium were also found depending on cellulase origin. On the other hand, xylose beings the main constituent of the xylan linear chain, more xylose than arabinose was obtained in all cases.

As for total sugars, which included the six sugars identified, both cellulases and hemicellulase had a significant positive effect on total sugars release (p-value < 0.05). Again, substantial differences between both cellulases are found at low enzyme concentrations, whereas increasing the amount of cellulase up to 0.4 (w/w) and combining it with hemicellulase, led to not significant differences.

Optimum conditions for *A. niger* cellulase performance against pineapple waste

According to the present results, no significant differences are obtained after 24 h of hydrolysis when *A. niger* or *T. reesei* cellulases are used, mainly if combined with hemicellulase. In addition, cellobiose accumulation was more evident when using *T. reesei* cellulase. This, together with the fact that the commercial *T. reesei* cellulase used in the experiments was significantly more expensive than the *A. niger* cellulase assayed, *A. niger* cellulase was chosen as the best option to perform the enzymatic hydrolysis step of pineapple waste for bioethanol production. Therefore, the optimum conditions (pH and T) for its performance were assayed as explained in the materials and methods section.

Individual sugars were identified and quantified at the different conditions assayed in order to obtain the total sugars values. Total sugars released to the medium were fitted to equation 1 which yielded the following equation (eq. 3). Statistical analysis of the fitting (table 3.1.3.) indicated that both factors (T and pH), as well as their interactions were significant (p-value < 0.05). The determination coefficient indicates that the model obtained explains 83.6% of the variability in total sugars yield. All coefficients had a positive effect on total sugar release (figure 3.1.4.). Enzyme action was significantly

more affected by pH than temperature, since pH x pH and pH were the most important effects, followed by pH, Temperature, Temperature x pH and Temperature x Temperature. In order to estimate the optimum conditions for the pineapple waste saccharification by *A. niger* cellulase, three dimensional response surface curves (Figure 3.1.4.) were used. Results indicated that enzyme performance is enhanced at the 4-5 pH interval, as compared to pH 5-6, where a significant reduction in total sugar's release is observed. Increasing temperature up to 50 °C also yielded better results. According to the surface curves analysis, 50 °C and pH = 4.9 were the optimum condition for *A. niger* cellulase, which is in the range of most fungal cellulases (Jahangeer *et al.,* 2005; Taherzadeh and Karimi, 2007; Qin *et al.,* 2008).

$$Total\ sugars\ release\ (g/L) = -168.351 - 5.42571 \cdot Temperature\ (°C) + 139.256 \cdot pH + 0.0371494 \cdot Temperature\ (°C)^2 + 0.656445 \cdot Temperature\ (°C) \cdot pH - 17.5523 \cdot pH^2 \qquad \textbf{Equation 3}$$

Table 3.1.3. Analysis of variance (ANOVA) results of fitting experimental data (total sugars released) to equation 1.

Source	Sum of Squares	df	Mean Square	F-ratio	P-value
A:Temperature (°C)	3294.57	1	3294.57	75.56	0.0000
B:pH	4808.66	1	4808.66	110.29	0.0000
AA	220.813	1	220.813	5.06	0.0278
AB	1378.95	1	1378.95	31.63	0.0000
BB	4929.35	1	4929.35	113.06	0.0000
Total Error	2877.54	66	43.5991		
Total (Corr.)	17509.9	71			

R^2 = 83.5662

Standard Error of Est. = 6.6029

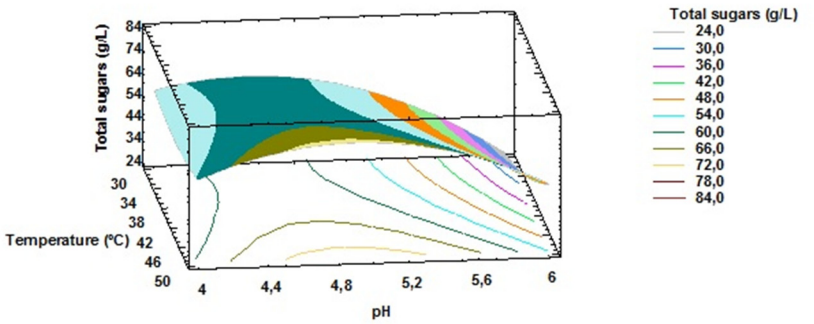

Figure 3.1.4. Estimated contour and response surface for total sugars released (g/L), for factors temperature and pH.

CONCLUSIONS

Pineapple processing generate significant amounts of residues that need to be properly disposed in order to meet environmental requirements. The use of this waste material for the production of bioethanol is a good opportunity to give added value to this residue of the pineapple industry, in the context of the second generation of biofuels. In order to obtain fermentable sugars from this residual biomass, two different commercial cellulases (*Trichoderma reesei* and *Aspergillus niger*) have been assayed, either alone or combined with hemicellulase from *A. niger*. The increase in the total soluble solids present in the hydrolysate indicate that, in spite of exhibiting a significant slower initial hydrolytic rate than *T. reesei* cellulase, *A. niger* cellulase may lead to a similar amount of soluble solids present in the final hydrolysate. This trend has been also confirmed when analysing the specific sugars released to the medium, especially when combined with the enzyme hemicellulase. The fact that *T. reesei* cellulase tend to accumulate more cellobiose, could be responsible for a slowdown of the hydrolytic process, despite the faster initial rate. On the other hand, a synergistic effect of combining both cellulase and hemicellulase enzymes has been proven, since the addition of one enzyme conditioned the action of the other one.

In conclusion, this study shows that commercial *A. niger* cellulase represents an efficient alternative to *T. reesei* cellulase for the saccharification of industrial pineapple waste, especially when combined with a hemicellulase. Total sugars present in the final hydrolysates indicated that *A. niger* cellulase performed similarly at a lower cost, with no cellobiose accumulation. However, if processing time is a limiting factor, *T. reesei* cellulase could be the one preferred.

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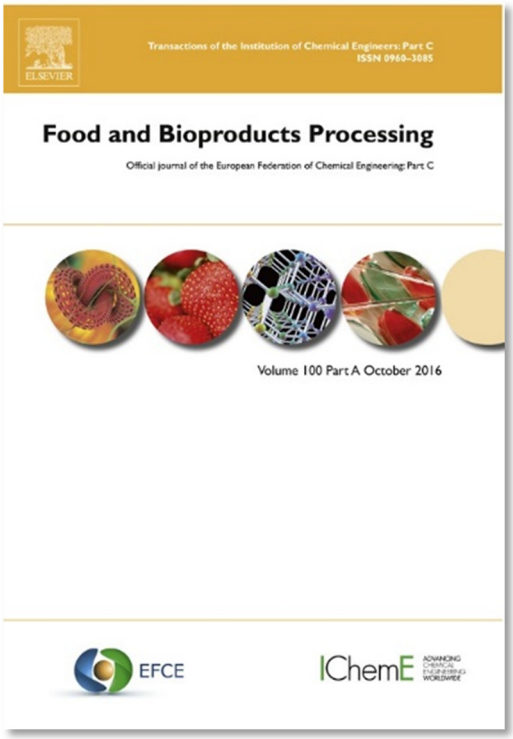

3.2. ARTÍCULO 2

MICROWAVES AS A PRETREATMENT FOR ENHANCING ENZYMATIC HYDROLYSIS OF PINEAPPLE INDUSTRIAL WASTE FOR BIOETHANOL PRODUCTION

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Microwaves as a pretreatment for enhancing enzymatic hydrolysis of pineapple industrial waste for bioethanol production

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ABSTRACT: The pineapple industry generates significant amounts of residues which are classified as lignocellulosic residual biomass. In the present paper, microwaves are studied as a pretreatment to improve pineapple waste saccharification. Different microwave (MW) powers (10.625, 8.5, 6.375, 4.25 and 2.125 W/g) and exposure times (1–20 min) were applied to the solid part of the waste before enzymatic hydrolysis. Infrared thermography was used to assess temperature evolution and structural modifications were evaluated by Cryo-SEM. Sugar content and fermentation inhibitors (phenols, furfural and hydroxymethylfurfural) were also determined. MW increased sugar yield as long as intermediate powers were used (up to 6.375 W/g). However, high powers and longer treatments resulted in sugar degradation and/or a decrease in the efficiency of the enzymatic hydrolysis process. Temperature records indicated that thermal sugar degradation may occur in those cases. The presence of fermentation inhibitors have been confirmed and related to prolonged MW treatments. Microscopic observations suggested that mild microwave pretreatments may promote microstructural changes that enhance enzyme performance, whereas harsher treatments could increase tissue compactness and reduce the effectiveness of the enzymatic treatment. It is concluded that microwave pretreatments using the appropriate energy supply and exposure time enhances

saccharification efficiency, potentially improving further bioethanol yield.

KEYWORDS: Lignocellulose pretreatments; Microwaves; Pineapple industrial waste; Saccharification; Enzymatic hydrolysis; Bioethanol

INTRODUCTION

The global energy problem and the search for solutions based on sustainable and environmentally friendly renewable energies (Sun and Cheng, 2002) such as biomass and others (EC, 2009) has promoted bioethanol to be a clear alternative to fossil fuels. In this scenario, second-generation bioethanol, i.e. the produced from the fermentation of lignocellulosic biomass (waste and energy crops), deserves especial attention. Unlike first-generation bioethanol, second-generation of this biofuel helps diversify energy supplies without competing in the global food market (Rutz and Janssen, 2008 and Bacovsky, 2010). Furthermore, the use of waste as a source for bioethanol production would also add up value to the whole manufacturing process.

The food industry generates significant amounts of residues which are a potential source for bioethanol production. In particular, pineapple production increases annually, and reached 24.79 million tons in 2013 (FAOSTAT, 2016). In addition, the industrialization of these fruit (juice, cannery, minimally processed) generates significant amounts of residues which consist mainly of the peel, core and crown of the pineapple. Pineapple waste usually represents about 50% (w/w) of the total processed fruit (Ketnawa *et al.,* 2012), although some authors have even suggested values up to 80% (Ban-Koffi and Han, 1990). On the one hand, the liquid phase of this residue contains a high content of fermentable sugars (glucose, fructose, and sucrose) (Nigam, 1999). On the other hand, the solid phase is a lignocellulosic material which, apart from lignin, consists of cellulose and hemicellulose, polymers which are potentially hydrolyzable into fermentable mono- and disaccharides (Abdullah and Mat, 2008). Consequently, pineapple

industrial waste has been investigated as an interesting source for ethanol (Ban-Koffi and Han, 1990; Nigam, 1999; Tanaka *et al.,* 1999 and Ruangviriyachai *et al.,* 2010) and other metabolites production such as citric acid (Imandi *et al.,* 2008).

However, bioethanol production from lignocellulosic biomass continues to be a challenge due to the complexity of this material in which cellulose and hemicellulose are densely coated by a hard-to-degrade lignin cover (Taherzadeh and Karimi, 2007). Hydrolysis of cellulose (polymer of d-glucose units linked by β-1,4-glycosidic bonds) and hemicellulose (complex heteropolysaccharide polymer that consists of pentoses, hexoses and uronic acids) could yield fermentable sugars to be used in bioethanol production (Scheller and Ulvskov, 2010). Lignocellulose would need to be disrupted in order to expose cellulose and hemicellulose to further chemical or enzymatic hydrolysis. Therefore, a pretreated lignocellulosic matrix becomes an essential prerequisite to obtain ethanol.

Nowadays, different physical, physicochemical, chemical and biological pretreatments, as well as a combination of all of them, are being assayed for pretreating lignocellulose (Sun and Cheng, 2002). Most of the conventional pretreatments require high temperatures usually reached by convection or conduction heating (Liu and Wyman, 2005). This creates a high energy cost that reduces the efficiency of the process. Therefore, there is a need for alternative methods to conventional pretreatments, among which microwaves have been suggested (Hu and Wen, 2008). The use of microwaves enables a volumetric, targeted and faster heating of the product than conventional heating, since there is direct contact between the product and the electromagnetic field generated by the microwave (De la Hoz *et al.,* 2005). Furthermore, Xiong *et al.* (2000) showed that the use of microwaves could change the ultrastructure of cellulose, degrade lignin and hemicellulose and facilitate hydrolytic enzymes to access the lignocellulosic substrate (Kitchaiya *et al.,* 2003 and Zhu *et al.,* 2005).

In this context, the aim of the present study was to investigate microwaves as an alternative pretreatment in order to improve the enzymatic hydrolysis yield of pineapple industrial waste.

MATERIALS AND METHODS

Sample preparation

A total of 20 pineapple fruits (*Ananas comosus* [L.] Merr., MD-2 cv.) were obtained from a tropical fruit importer and selected on the basis of their external factors such as the absence of injuries, ripeness and weight. Pineapples were first washed in a sodium hypochlorite solution (0.1%) for 5 min. Next, a pineapple cutter was used to remove the crown and separate the pulp. The resulting waste, consisting of the peel and core, were cut into smaller pieces and pressed in a screw press at 2.5 bar (Vincent Corporation model CP-4), the resulting press ratio being 0.49 (kg pressing cake/kg liquid phase). Liquid phase was removed from the original pineapple waste since it already contains simple sugars which would be directly fermentable (Nigam, 1999). In addition, sugar degradation can take place during microwave heating, for which only the solid part or press cake was subjected to subsequent microwave (MW) pretreatment and hydrolysis. Thus, the resulting press cake (*solid pineapple waste*) was grinded in a blender (Solac Inox Professional 1000 W Mixer), introduced in glass flasks (40 g each) and kept frozen (−22 °C) until the experiments were conducted. The resulting product was named *grinded solid pineapple waste*.

Experiments were performed on 40 g of thawed grinded solid pineapple waste to which distilled water had been added in 1:1 (w/w) proportion, resulting in the final sample identified as *reconstituted pineapple waste*. Distilled water was added to the press cake in order to enhance the microwave pretreatment effect as well as to avoid calcinations, as suggested by some authors (Azuma *et al.,* 1984 and Ooshima *et al.,* 1984).

Microwave pretreatment

Microwave (MW) pretreatment was carried out in a microwave oven provided with a turntable plate (LG MH63340F/MH6340FS) with a frequency of 2.45 GHz. Samples were introduced in microwave intended plastic containers. The samples were treated at the following nominal powers: 170, 340, 510, 680 and 850 W, which resulted in the applied nominal powers: 2.125, 4.25, 6.375, 8.5 and 10.625 W/g; and exposure times from 1 up to 6, 8, 10, 14 and 20 min, respectively. Time exposure limits were defined by the appearance of calcinations or scorching. The power absorbed by the sample at these nominal power levels was estimated by heating 1 kg of distilled water from 10 °C up to 20 °C at 170, 340, 510, 680 and 850 W, according to the international standard IEC 60705 (1999). A thermocouple (HIBOK-14, sensor type K, sensitivity 39 μV °C−1, accuracy ±0.1 °C) was used for temperature measurements. Experiments were performed in triplicate and results showed an average (and standard deviation) of 129 (3) W for the 170 W, 247.4 (1.2) W for the 340 W, 336 (2) W for the 510 W, 485.5 (1.3) W for the 680 W and 602.0 (0.9) W for the 850 W. Corresponding absorbed powers in W/g were then estimated as 1.61 (0.04), 3.09 (0.02), 4.2 (0.03), 6.07 (0.02) and 7.53 (0.01). Finally, the pH of the samples was adjusted to 5 by adding NaOH 1 N (Panreac Química, S.L.U.). Water loss due to microwave processing was determined by weight difference and restored before proceeding with saccharification. Experiments were conducted in triplicate.

Evolution of microwave heating by infrared thermography

In order to estimate the temperatures reached during MW pretreatments, an infrared thermocamera Testo 870-1 (Testo AG) with a spectral infrared range of wavelength from 7.5 to 14 mm, 9 Hz frame rate and detector with 160 × 120 pixels, was used. In order to compare the difference in the heating undergone by the pineapple waste due to the different MW powers and exposure times applied, an image of the bottom surface of the container was taken just after each microwave pretreatment. Testo IRSoft software was used for image

analyses, which allowed the study of microwave heating evolution, and the registration of hot spots location as well as maximum, minimum and average temperatures.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out by adding 0.4% (w/v) of cellulase (1.13 U/mg solid) and 0.1% (w/v) of hemicellulase (1.5 U/mg solid) from *Aspergillus niger* (both enzymes purchased to Sigma–Aldrich Química SL (Tres Cantos, Madrid, Spain)). In each experiment, the reconstituted pineapple solid waste was introduced in a 100 mL glass beaker and placed in an incubation oven at 50 °C (PSelecta, model Incudigt) during 24 h. Concentration of the enzymes as well as optimum conditions of the saccharification process were based on previous experiments performed at the same laboratory. Saccharification was undergone on microwave pretreated and non-pretreated samples, which were used as a reference. Experiments were performed in triplicate.

Physicochemical determinations

The following physicochemical determinations were performed on the reconstituted pineapple waste before pretreatment and saccharification, as well as after saccharification, on samples subjected to MW pretreatments or saccharified without having been pretreated.

pH, moisture content (x_w) and total soluble solids (TSS)

pH and moisture content (x_w) were determined as a control measure, using a digital pH-meter and the 20.013 AOAC gravimetric method (AOAC, 1980), respectively. pH can be modified during hydrolysis as a consequence of the different species released to the medium, whereas moisture content is not expected to change significantly. Total soluble solids (TSS) present in the liquid phase were estimated by obtaining the Brix degrees (°Brix) values after saccharification, which were considered a good approximation of the simple sugars released as a consequence of cellulose and hemicellulose hydrolysis.

Sugar profile

Identification and quantification of specific sugars present in the liquid phase of the reconstituted pineapple waste was measured by High-Performance Anion-Exchange Chromatography with a Pulsed Amperometric Detector (HPAEC-PAD), using a Metrohm IC chromatograph system equipped with a 716 Compact module and an ICnet 2.0 software program for interpreting the results. A three-step PAD setting was used with the following path intervals (ms) and potentials (V): t_1: 400/E_1 = +0.05 (detection); t_2: 200/E_2 = +0.75 (cleaning); t_3: 400/E_3 = −0.15 (regeneration). The column used was a Metrosep Carb 1 250/4.6 column (250 mm L × 4.6 mm ID) coupled to a guard column. Analyses were done at 32 °C, 8.8 MPa, injection volume: 20 µL and using sodium hydroxide 0.1 M as the mobile phase (1 mL/min). Chromatographic measurements required filtration of the liquid (0.45 µm nylon filter) and dilution of the resulting filtered sample (1:2000, v/v, in bidistilled water). High-purity standards (Sigma–Aldrich Química SL, Tres Cantos, Madrid, Spain; purity ≥99%) of glucose, fructose, sucrose, arabinose and xylose were used to prepare standard calibration curves (2.5, 5, 10, 15, 25 and 50 ppm) and proceed to identification and quantification of the sugars present in the hydrolizates. All the determinations were carried out in triplicate. Sugar content was expressed in g/kg of pineapple waste.

Determination of fermentation inhibitory compounds

Inhibitory components present on the saccharified samples was evaluated in terms of phenolic content and the presence of furfural (F) and hydroxymethylfurfural (HMF) on the liquid phase of pretreated and non-pretreated samples.

Phenolic content

The Folin–Ciocalteau method was used in order to determine total phenols present in the samples (Singleton *et al.,* 1999). Monohydrate gallic acid was used as a standard and results expressed in mg equivalent of gallic acid per mL (mg GAE/mL). All reagents used were

of analytical grade (Sigma–Aldrich Química SL, Tres Cantos, Madrid, Spain).

Determination of furfural (F) and hydroxymethylfurfural (HMF)

High performance liquid chromatography (HPLC) was used in order to determine the content of furfural and hydroxymethylfurfural, according to the methodology developed by Blanco-Gomis *et al.* (1991). An Alliance® System (Water Co., Mildford, Mass, USA) equipped with a degasser and a 2695 separation module, coupled to a diode array detector (DAD 2996, Waters Co.) was used. A Kromasil® 100 C-18 column (3 mm × 250 × 4.6 mm ID) was used for chromatographic separation. The analyses were performed isocratically at 25 °C using acetonitrile/water (8:92, v/v) as the mobile phase (1 mL/min) and an injection volume of 10 µL. UV detection was fixed at 280 nm. Quantification was based on external calibration by using standard solutions of F (0–5 µg/mL) and HMF (1–100 µg/mL) in 10% methanol–water (v/v). All reagents were purchased from Sigma–Aldrich Química SL (Tres Cantos, Madrid, Spain) and of analytical grade. Purity of standards was ≥ 98%.

Analysis of microstructural changes by low temperature scanning electron microscopy – Cryo-SEM

Low-temperature scanning electron microscopy (Cryo-SEM) was used in order to evaluate the microstructural changes undergone by the reconstituted pineapple waste as a consequence of microwave pretreatments. A Cryostage CT-1500C (Oxford Instruments) coupled to an electronic scanning microscope Jeol JSM-5410 was used for this purpose. First, samples were sublimated in the microscope stage during 20 min at −90 °C and −5 kV; subsequently, these were moved to another stage and metalized with gold during 1.5 min, at vacuum conditions. Then, sample observation was performed at 15 kV and 15 mm of working distance. Micrographs of the reconstituted pineapple waste before and after microwave pretreatments were taken.

Statistical analysis

Statgraphics Centurion XVI® (Manugistics Inc.; Rockville, MD, USA) was used for statistical analyses. Statistically significant differences across the different analyzed results were determined by using one-way or multiple analyses of variance (ANOVA) at 95% confidence level (p-value < 0.05).

RESULTS AND DISCUSSION

Enzymatic hydrolysis of the reconstituted pineapple waste: sugar profile

Pineapple waste saccharification resulted in an increase in the amount of sugars present in the samples. Specific changes in the sugar content of the liquid phase were studied by examining the sugar profile before and after saccharification (Table 3.2.1.). Before saccharification, glucose, fructose and sucrose were the only sugars identified in the samples, whereas the pentoses xylose and arabinose were also present in the enzymatically hydrolyzed ones. The enzymatic treatment produced a statistically significant increase in the glucose and fructose contents, whereas sucrose content slightly decreased after saccharification. The action of the fungal cellulase complex that consists of three groups of enzymes: endoglucanases (EC 3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21) (Goyal *et al.,* 1991) is most probably the main responsible for glucose increase; On the contrary, fructose increase would not be the result of the enzymatic action but of sucrose inversion (Ban-Koffi and Han, 1990), considering the acid pH (which reduces from 5.0 to 4.28 ± 0.07 during saccharification) of the medium and the fact that selected enzymes are not potentially capable of reversing sucrose. Hemicellulase action led to xylose and arabinose release from hemicellulase. This enzyme complex consists of enzymes that hydrolyze the main chain: xylanases and β-xylosidases (Shallom and Shoham, 2003) to xylan; as well as those responsible for the hydrolysis of hemicellulose branches: α-l-arabinofuranosidases to arabinose (Saha *et al.,* 2005). Nevertheless, xylose and arabinose contents in the

saccharified samples were minimal, significantly lower than the content of the other sugars identified (glucose, fructose, and sucrose).

Table 3.2.1. Sugar profile of pineapple waste before and after saccharification.[1]

Sample	Glucose (g/kg)	Fructose (g/kg)	Sucrose (g/kg)	Arabinose (g/kg)	Xylose (g/kg)	Total Sugars (g/kg)
Non-saccharified waste	21.3 (1.4)[a]	13.3 (0.4)[a]	4.8 (0.5)[a]	0 (0)[a]	0 (0)[a]	39.4 (0.5)[a]
Saccharified waste	25.82 (0.11)[b]	16.71 (0.20)[b]	4.02 (0.2)[a]	0.590 (0.016)[b]	0.525 (0.012)[b]	47.67 (0.12)[b]

(abc) Similar letters indicate statistically homogenous groups with a confidence level of 95% (p-value <0.05).

[1] Values correspond to the average of three replicates (standard deviation).

Regarding total sugars content, enzymatic saccharification produced a statistically significant sugar increase in the pineapple waste. However, due to the slight increase obtained, pretreatment is recommended in order to disrupt the lignocellulosic structure and facilitate the action of hydrolytic enzymes, thereby increasing the efficiency of enzymatic hydrolysis and the saccharification yield.

Effect of microwave (MW) pretreatments on pineapple waste saccharification

MW pretreatments significantly modified the result of enzymatic hydrolysis of pineapple waste as it was deduced from the analysis of the total soluble solids of pretreated samples after saccharification (Figure 3.2.1.). Generally, the application of a MW pretreatment produced an increase in the TSS content when powers up to 6.375 W/g were applied. However, higher powers resulted in a decrease in TSS content, the power being statistically significant. Besides, all the powers applied showed a critical time at which TSS content was reduced indicating that not only high powers but also longer treatments could result in sugar degradation and/or a decrease in the efficiency of the enzymatic hydrolysis process. In fact, both factors and their interaction appeared to be statistically significant.

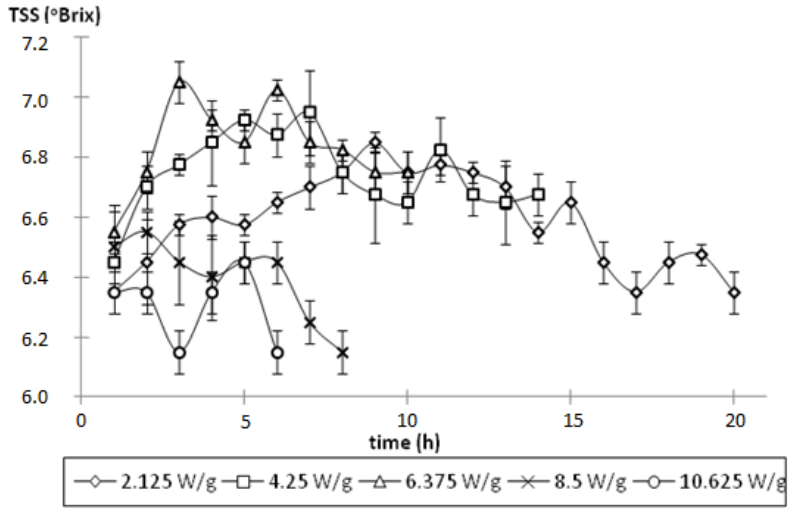

Figure 3.2.1. Total soluble solids (TSS) content of MW pretreated pineapple waste at different powers and exposure times.

As for pH and moisture content, significant differences were obtained when comparing the results before and after saccharification; however, none of these parameters were significantly affected by the different MW pretreatments. In particular, pH decreased from the adjusted 5.0 to an average of 4.3 (0.3), as a result of the different species released to the medium during enzymatic hydrolysis, whereas xw decreased from 92.2 (0.5) to an average of 91.3 (0.3).

In order to go into detail, HPAEC-PAD was used to analyze individual sugars in each pretreated pineapple waste, and compared them to the obtained in the non-pretreated one. All reported values correspond to sugar content after saccharification (Figure 3.2.2.).

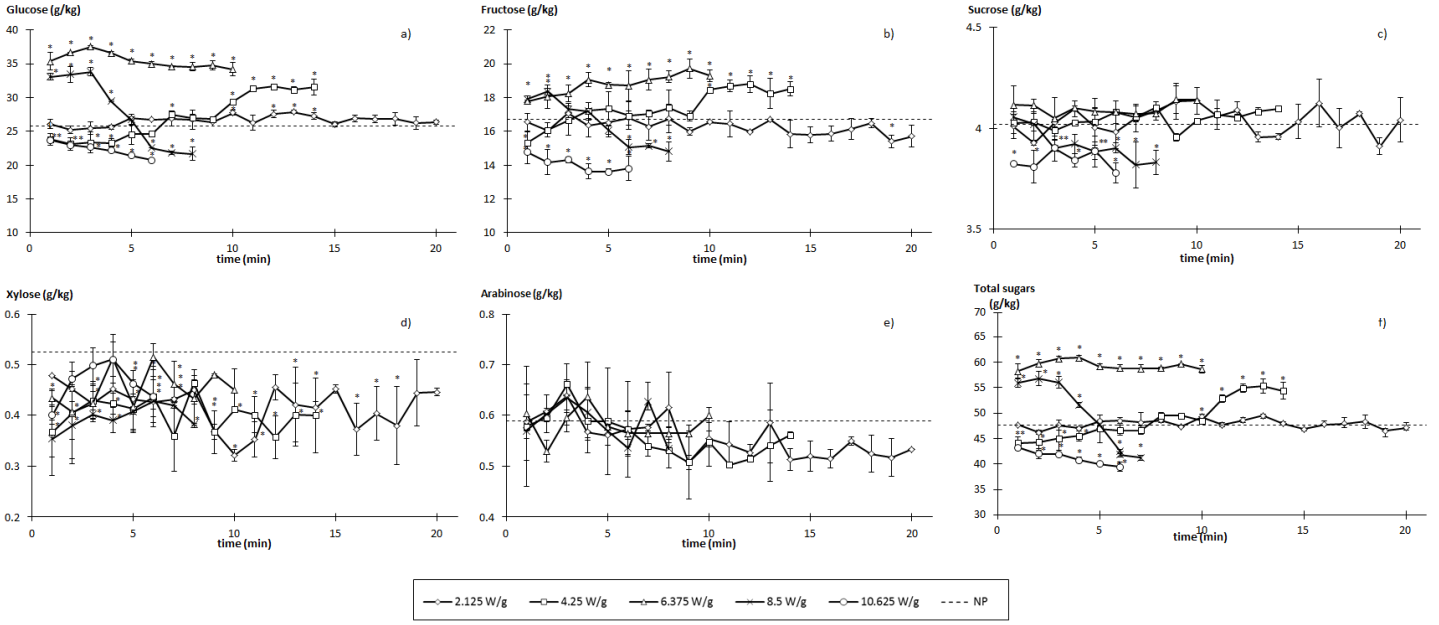

Figure 3.2.2. Sugar content of MW pretreated waste at different powers and exposure times: (a) glucose, (b) fructose, (c) sucrose, (d) xylose, (e) arabinose and (f) total sugars. *Indicates statistical differences at the 95% confidence level between MW pretreated and non-pretreated samples (p-value < 0.05).

Microwave pretreatments at the lowest power assayed (2.125 W/g) did not lead to any statistically significant modification in glucose content when compared to the non-pretreated waste (Figure 3.2.2a.). Nevertheless, glucose experienced a significant increase when higher powers were used (4.25 W/g) at exposure times from 10 min. Increasing the applied power to 6.375 W/g led to a statistically significant increase in the glucose yield for any exposure time, as occurred when 8.5 W/g were applied at exposure times up to 4 min. On the contrary, lengthening treatment beyond 4 min at the latter power led to a decrease in the sugar content to that of the reference treatment (NP) or even to lower values in some cases. Finally, the highest MW power applied (10.625 W/g) led to a statistically significant reduction in glucose release in all cases.

Fructose content followed a similar pattern to that of glucose yield but variations in fructose content were more moderate (Figure 3.2.2b.). Likewise, the greatest fructose yield was observed at intermediate MW powers, specifically at 6.375 W/g. At the lowest microwave power (2.125 W/g) fructose content did not change for any exposure time, whereas a statistically significant reduction in fructose release was observed at the highest microwave power applied (10.625 W/g).

On the contrary, as shown in Figures 3.2.2c. and 3.2.2d., MW pretreatment did not have a significant effect on sucrose and arabinose yields whereas xylose content markedly decreased at almost every power and exposure time applied (Figure 3.2.2e.). When investigating MW-alkali pretreatments, Hu and Wen (2008) also found out that the yields of xylose dropped significantly when the pretreatment (190 °C) extended to 40 min. Xylose have been reported to be especially sensitive to hydrothermal treatments as compared to other monosaccharides such as glucose and fructose (Tsubaki *et al.,* 2015). However, xylose was not present in the original biomass, for which a lower concentration of this sugar in the hydrolyzed sample should be a consequence of the effect of MW on hemicelluloses, which contain the xylose monomers. In line with this, depletion of

hemicellulose due to the high temperatures reached during MW treatments has also been reported, it completely disappearing at temperatures above 190 °C (Hendriks and Zeeman, 2009). Therefore, solubilization of hemicellulose and further degradation of xylose, or either direct degradation of hemicelluloses when even higher temperatures are reached during the MW pretreatments, would be responsible for the lower xylose yields.

The effect of MW pretreatment in total sugars content is summarized in Figure 3.2.2.f. As it can be observed, when very low microwave powers are applied (2.125 W/g), insufficient energy is provided in order to disrupt the lignocellulosic structure and therefore promote the subsequent action of the enzymes. On the contrary, increasing microwave power up to 10.625 W/g produces a decrease in the total sugar yield, with respect to the non-pretreated waste, which could be a consequence of sugar degradation due to the high temperatures reached in the microwave (Zhu *et al.,* 2005). This phenomenon has also been observed by other authors such as Binod *et al.* (2012) that reported sample charring at 3 min of treatment when 850 W were applied, whereas at 100 W power charring did not occur even after 30 min. Liu and Wyman (2005) also indicated that temperatures above 190 °C might trigger sugar degradation. Hydrothermal degradation of sugars and its resulting formation of fermentation inhibitors such as furfural and hydroxymethylfurfural (HMF) have been extensively documented (Palmqvist and Hahn-Hagerdal, 2000 and Carvalho *et al.*, 2004). Kim and Pan (2010) noted that organosolvent biomass fractionation have shown that xylose dehydration to furfural mostly occurs at 190–205 °C, and the molar conversion of hexose sugars to HMF is higher at high temperatures (up to 200 °C), low pH and longer pretreatment times.

Nevertheless, intermediate powers and exposure times did have a positive effect on further enzymatic action. It has been reported that MW pretreatments enhances the saponification of intermolecular ester bonds cross-linking xylan hemicelluloses, as well as other

components such as lignin and other hemicelluloses (Jin *et al.,* 1999). Hu and Wen (2008), by using scanning electron microscope images, showed that when switchgrass was treated by MW, many granules appeared on the surface, indicating partial breakdown of the lignin structure. Thus, these structural changes would facilitate the enzymes' access to the potentially hydrolysable components. In our case, intermediate applied powers (6.375 W/g) did always imply a significant improvement in the saccharification yield. In particular, microwave pretreatment at 6.375 W/g–4 min produced an increase in total sugars of 27% compared to the non-pretreated waste. Similar results were obtained at low-intermediate powers (4.25 W/g) and long exposure times (≥10 min) and high-intermediate powers (8.5 W/g) and short exposure times (≤4 min); therefore, increasing the microwave power could result in shorter treatments. These results are in line with those obtained by Binod *et al.* (2012).

As for the thermal effect of microwaves, Hu and Wen (2008) reported that when lower temperature levels are reached (70–130 °C), the increase in the sugar yield obtained is largely due to biomass de-lignification. However, when temperatures exceed 130 °C, the improvement in sugar yield might be caused by the disruption of the crystalline cellulose instead of by lignin removal. Higher temperatures (>160 °C) may also induce the solubilization of lignin (Jackowiak *et al.,* 2011). However, this is a non-desired phenomenon since it results in the release of phenolic compounds which have an inhibitory toxic effect on bacteria and yeast (Hendriks and Zeeman, 2009). High temperatures also promote hemicellulose depletion, which is complete at temperatures above 190 °C (Hendriks and Zeeman, 2009).

Analysis of microwave induced heating of pineapple waste by thermography

Some effects of MW on the lignocellulosic biomass may be attributed to the high temperatures reached during the irradiation treatment. Therefore, an infrared camera was used to estimate any temperature changes produced as a consequence of MW treatments, as explained

in Materials and methods section. Since thermographs were taken immediately after MW pretreatments, temperature losses were considered negligible, as suggested by different authors (Liu *et al.,* 2014, Pitchai *et al.,* 2012, Wang *et al.,* 2001 and Zhou *et al.,* 1995).

Temperature distribution during MW heating is summarized in Figure 3.2.3., where thermographs corresponding to the different applied powers and exposure times are shown. In each thermography, the cross indicates the point exhibiting the maximum temperature or hot spot (HS); in addition, the temperature scale is given next to the corresponding image.

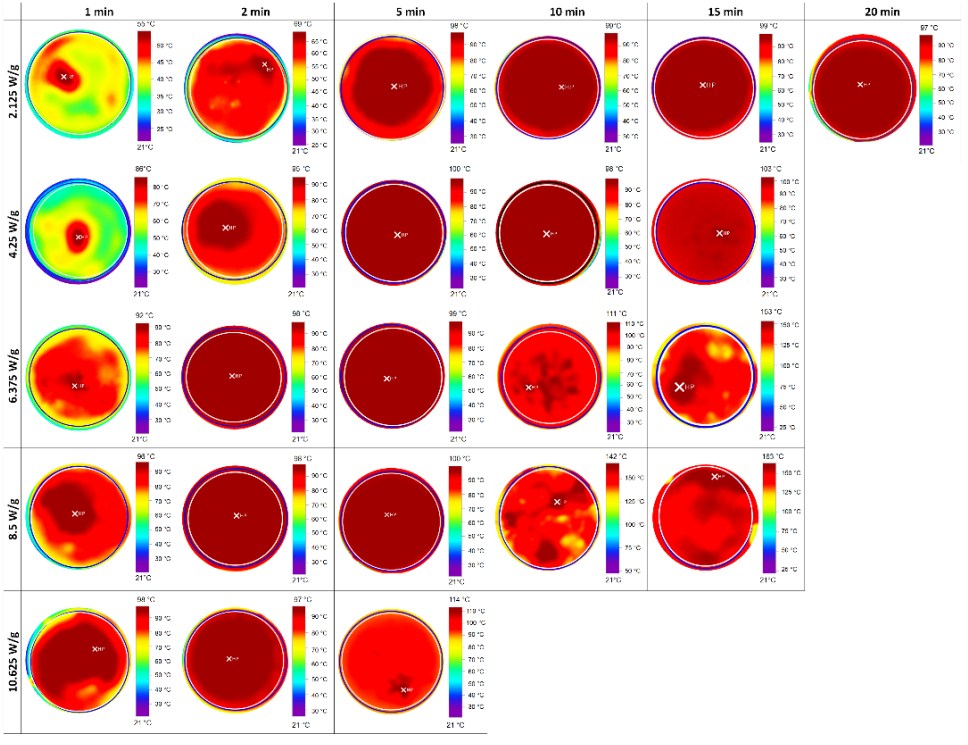

Figure 3.2.3. Thermographs corresponding to the bottom surface of the plastic container containing MW pretreated pineapple waste at different power: 2.125, 4.25, 6.375, 8.5 and 10.625 W/g, and exposure times: 1, 2, 5, 10 and 15 min. HS: hot spot.

The analysis of temperature evolution during MW pretreatment reveals that increasing the power and exposure time results in higher temperatures. However, different heating patterns are observed at the different powers applied.

MW irradiation heated from the inside to the outside of the sample surface which implied a non-homogeneous heating process, at least during the first minutes of treatment; this is a consequence of the polar part of the lignocellulosic material being selectively heated by microwaves. During this period, hot spots are created in the heterogeneous material. Some authors have discussed that the recalcitrant lignocellulosic structure may be disrupted by the generated "explosion effect" at the hot spots (Hu and Wen, 2008). Later, homogeneity is reached at different times depending on the power applied; the higher the power the earlier the uniformity is reached. These results are similar to the obtained by Kumar *et al.* (2014), who showed that lower powers required longer exposure times so as to reach a homogenous heating (150 s for 100 W power in their case). During the non-homogeneous heating of the waste, the registered temperatures are below the boiling point of water, whereas temperature approaches this point when uniformity is reached, as deduced from the scales next to the images. Lengthening the treatment implied reaching temperatures up to 160 °C, leading again to a loss of temperature homogeneity in the residue.

The location of the hot spot also depended on the heating period; it being centered during the homogeneous temperature period, and displaced at the beginning of the process, as well as once temperature uniformity has been lost. The latter case, as explained by several authors, could be due to the loss of water in the central part of the sample as a consequence of water evaporation; this modifying the maximum energy point which would then locate in the regions where there is still some moisture content (Liu *et al.,* 2014, Pitchai *et al*., 2012, Wang *et al.*, 2001 and Zhou *et al.,* 1995). In fact, scorching of the

surface sample was visually confirmed in the samples subjected to high MW powers (6.375 W/g and higher), at long exposure times.

In Figure 3.2.4., the registered maximum (T_{max}), minimum (T_{min}) and average (T_{ave}) temperatures are given, as a function of time for the different applied powers. In all cases, as long as power and time increase, there is an increase in all registered temperatures; this being in line with the results reported by Liu *et al.* (2014), Pitchai *et al.* (2012), Wang *et al.* (2001) and Zhou *et al.* (1995). The statistical analysis of the results indicated that both factors, power and time, as well as the interaction between them, were statistically significant.

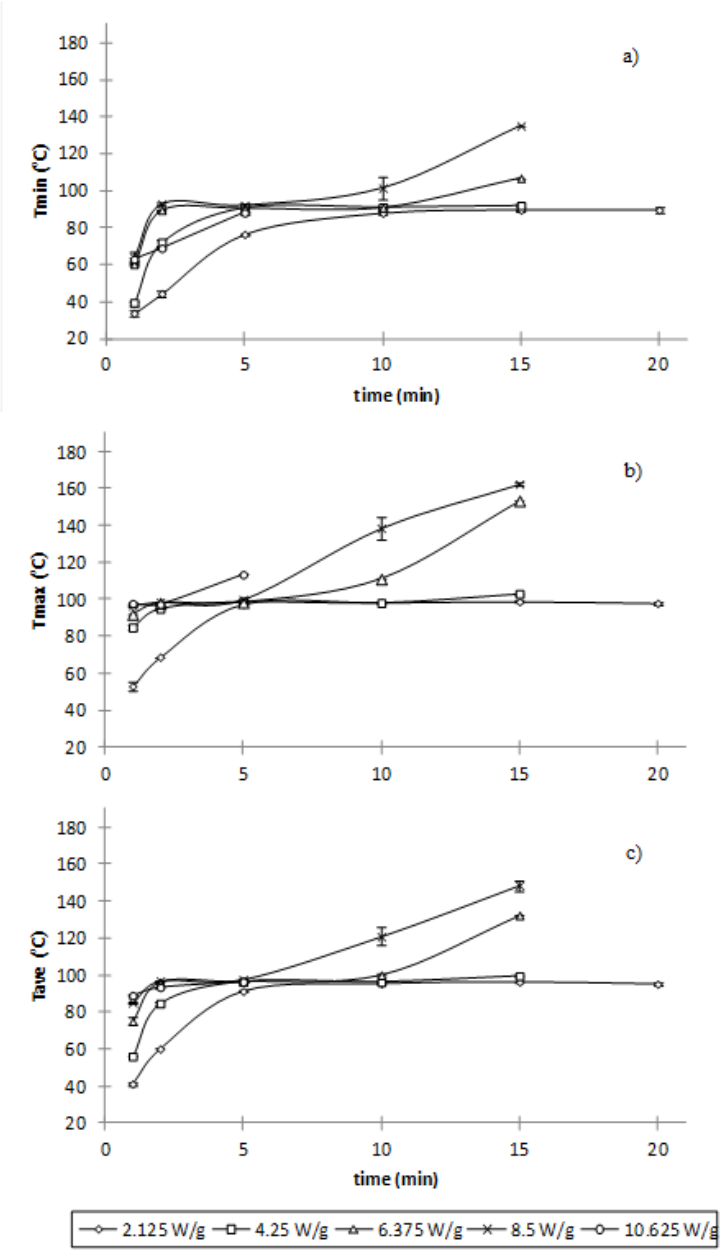

Figure 3.2.4. Temperature evolution graphs: (a) minimum temperature (T_{min}), (b) maximum temperature (T_{max}) and (c) average temperature (T_{ave}) of MW pretreated pineapple waste at different power: 2.125, 4.25, 6.375, 8.5 and 10.625 W/g, and exposure times: 1, 2, 5, 10 and 15 min.

The registered graphs confirmed the existence of different periods during MW heating, as it had been deduced from the thermographs: an initial or induction period, when temperature is below the boiling point of water; a constant period, in which temperature is around 100 °C; and a final one, when temperature reaches values above 100 °C. The exact temperatures reached, as well as the length of each of these periods, depended on the power applied, as discussed below for the maximum and average temperatures.

As for the maximum temperature, which would correspond to the temperature of the hot spot for each power and time, the registered value is slightly below 100 °C in all cases when MW start to act on the sample: the lower the power applied, the further from 100 °C the temperature is, approaching the boiling point of water as heating proceeds (2–5 min, depending on the applied power). Then, except for the maximum power applied, temperature remains constant for a specific period, it being coincident with the homogeneous heating period of the samples (Figure 3.2.3.). Next, when heating stops being homogeneous (Figure 3.2.3.), there is an increase in the maximum temperature registered, at intermediate and high powers, for which temperatures higher than 100 °C are reached. Temperatures above the water boiling point would suggest the presence of dry regions in the sample and the concentration of moisture in specific points, which could lead to overheating of some specific regions. This has also been confirmed when analyzing temperature distributions in Figure 3.2.3. and by visual inspection of the samples, since scorching of the sample surface was visually observed after some specific treatments. On the other hand, when lower powers were applied (2.125 and 4.25 W/g), all registered values were around 100 °C until the end of the treatment, which implies a homogenous heating until 20 and 15 min, respectively; the hot spot still being centered in the sample.

Evolution of minimum and average temperature was quite similar in all cases. Therefore, average temperature will be used as a reference, since it can be considered representative of the sample. Average

temperature increase is sharper during the first 2 min, 5 min in the case of the lowest power. Then, as occurred with the maximum temperature evolution, average temperature remains constant during a specific period, and increases later above 100 °C, except in the case of the two lowest powers applied, where temperature does not significantly change until the end of the treatment. The phenomena responsible for this evolution are the same as exposed above since, again, average temperature remains constant while the registered heating is homogeneous, and increases when the hot spot displaces as a result of dry regions appearing in the sample.

As explained by Kumar *et al.* (2014), MW power would be more efficient at short times, since more significant temperature changes are then registered. It agrees with the results of the present work (not taking into account the temperatures that produce scorching, since these are a cause for sugar degradation). However, it should be reminded that heating is not the only effect that MW may exert on the residue structure, since the electromagnetic field applied might create non-thermal effects that also accelerate the destruction of the crystalline structures (De la Hoz *et al.,* 2005).

Presence of inhibitory compounds on the saccharified samples

Phenolic content

As it can be deduced from the results (Figure 3.2.5a), microwave pretreatment causes an increase in the phenolic content of the saccharified samples. This increase would be a consequence of lignin solubilization (Jackowiak *et al.,* 2011) as a result of the microwaves acting on the lignocellulose matrix. Both power and time were statistically significant (p-value < 0.05), treatments longer than 10 min causing a particularly significant increase in the phenolic content. This phenomenon would be related to the high temperatures reached during the treatments; in fact, the evolution of the sample's average temperature (Figure 3.2.4c.) shows a quite similar pattern to that of total phenols (Figure 3.2.5a.). Hu and Wen (2008) reported similar results as for the generation of phenolic components due to

temperature-induced lignin degradation. Nevertheless, in spite of the significant increase in the phenolic content registered, values are still far from the concentrations that have been reported to be detrimental for ethanologenic microorganisms (Palmqvist and Hahn-Hagerdal 2000; Ando *et al.,* 1986).

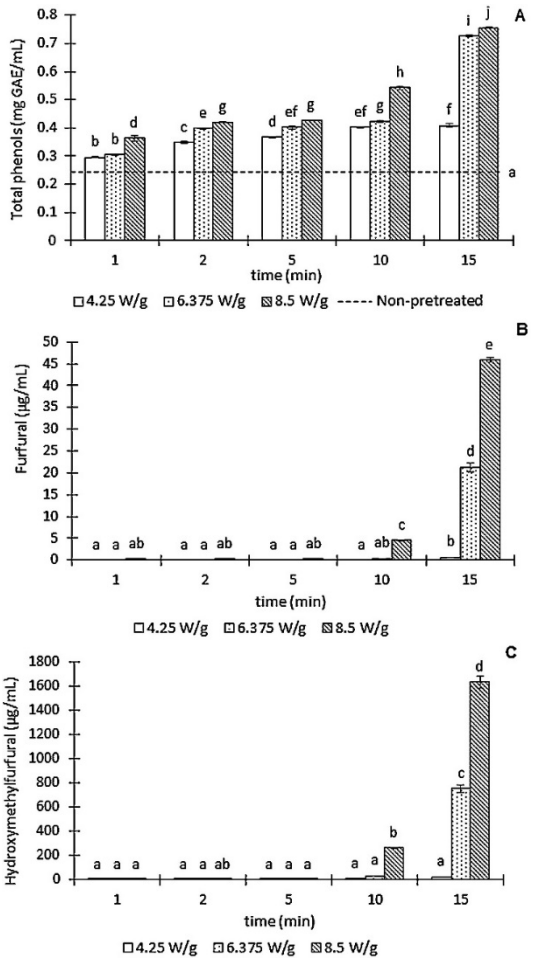

Figure 3.2.5. Generation of fermentation inhibitors during MW pretreatments. Total phenols (mg GAE/mL), furfural (µg/mL) and hydroxymethylfurfural (µg/mL) content in the liquid phase of the microwave pretreated pineapple industrial waste. [a,b,c] Different superscript letters indicate statistically significant differences at the 95% confidence level (p-value < 0.05).

It should be pointed out that that reducing sugars may interfere in the Folin–Ciocalteau assay, for which an increased phenolic content could also correspond to an increase in theses sugars' content. Nevertheless, according to Sánchez-Rangel *et al.* (2013), the presence of reducing sugars is only a problem when they are present and the total phenolic content is low. Therefore, phenolics would be the main components reacting with the Folin–Ciocaltau reagent in samples in which harsher MW treatments have been applied, i.e. when a significant increase has been registered, since sugar depletion is observed in those cases.

Furfural and hydroxymethylfurfural

Furfural and hydroxymethylfurfural can appear in the saccharified samples due to hydrothermal sugar degradation. Therefore, this would be a consequence of sugar release from the celluloses and hemicelluloses chain during MW treatments and subsequent thermal degradation due to a prolonged treatment. This phenomenon is evidenced in Figures 3.2.5b. and 3.2.5c., were the presence of F and HMF is confirmed when duration of MW treatments is ≥10 min. This increase being especially significant when treatments continued up to 15 min. MW power had also a significant effect, since F and HMF contents were almost negligible at the lowest power applied, independently on the exposure time. Several authors confirm that F and HMF do not inhibit Sacharomyces cerevisiae when these components are below 2 g/L (Banerjee *et al.,* 1981, Cantarella *et al.,* 2004, Gu *et al.,* 2014, Klinke *et al.,* 2004, Palmqvist *et al.,* 1999, Taherzadeh *et al.,* 1999 and Taherzadeh *et al.,* 2000), for which it is deduced that the concentrations obtained in the present study, F ≤0.0458 (0.6) g/L and HMF ≤1.63 (0.05) g/L, would not negatively affect the fermentation microorganisms. The increase in the F and HMF contents when increasing power and time confirms the hydrothermal degradation of sugars due to the temperatures reached when harsher MW pretreatments are applied, this being in line with temperature records (Figure 3.2.4.), as well as with sugars content (Figure 3.2.2.).

Analysis of microstructural changes by low temperature scanning electron microscopy – Cryo-SEM

Microstructural changes due to MW pretreatments were studied by Cryo-SEM, as explained in Materials and methods section. Some representative pretreatments were chosen and compared to a non-pretreated sample in order to identify any changes promoted by MW irradiation. Micrographs of a non-pretreated sample (Figure 3.2.6a.); a sample pretreated at 6.375 W/g – 5 min, which exhibited high sugar yield (Figure 3.2.6b.); and a sample subjected to 8.5 W – 8 min, in which sugar degradation had been identified (Figure 3.2.6c.), are shown.

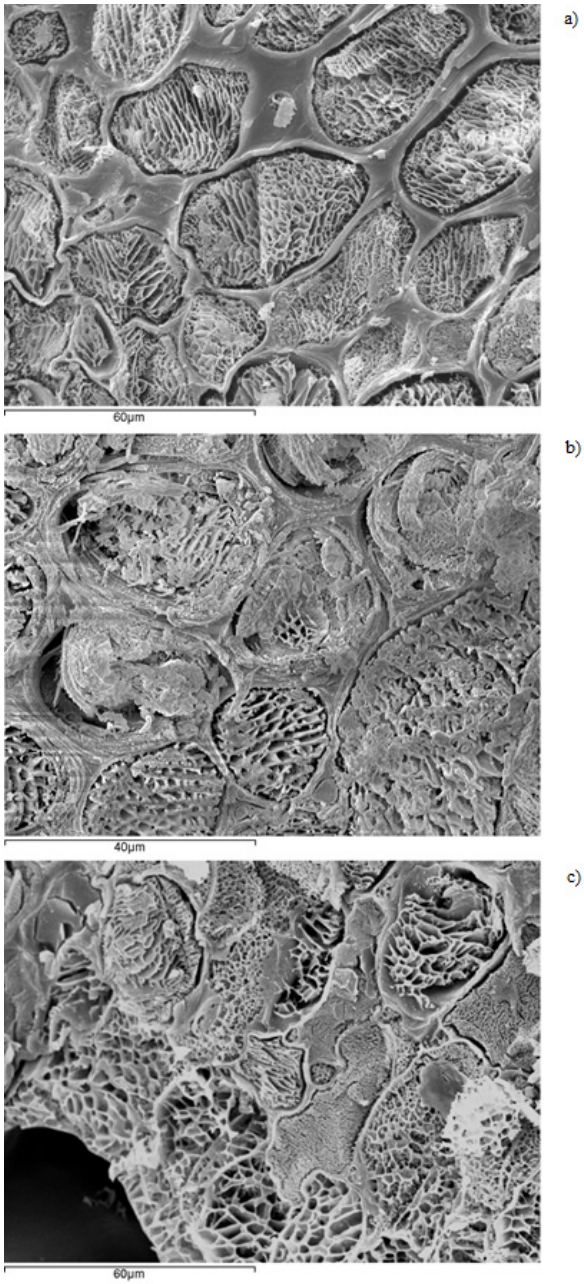

Figure 3.2.6. Scanning electron microscopic images of: (a) grinded solid pineapple waste (without any pretreatment); (b) MW pretreated waste at 6.375 W/g – 5 min and (c) MW pretreated at 8.5 W/g – 8 min.

In Figure 3.2.6a. (non-pretreated), rounded packed cells, typical from a parenchymatic tissue, are observed; cell walls and protoplasts are clearly identified using this technique. In addition, certain degree of cell degradation was observed when comparing the pretreated samples with the non-pretreated ones. Less rounded, more irregular cell walls, as well as the separation of the plasmatic membrane from the cell wall were identified (Figure 3.2.6b.). In spite of not being easily observable using this technique, the different signs of disruption identified suggest that cell degradation is taking place during pretreatments. Therefore, the microstructural changes described evidence that MW pretreatment is affecting the waste microstructure and, therefore, this is going to determine further enzyme action.

In the case of applying intermediate powers and times (Figure 3.2.6b.), structure would be affected in a way that enzyme action in the following stage is promoted, as deduced from the results of sugars released to the medium. On the contrary, increasing the power and time applied would cause more significant structural changes, which could result in reduced enzyme efficiency. In fact, in Figure 3.2.6c., cell wall roughness is more obvious and suggestive of tissue dehydration. In addition, protoplast plasmolysis is not observed in this case, but rather a significant cell shrinkage which would result in increased tissue compactness. This breakdown of the tissue would be negatively affecting further saccharification, since it would represent a decrease in the available surface area and, therefore, a lower effectiveness of the enzymatic treatment (Arantes and Saddler, 2011). Furthermore, it is known that dehydration has a negative effect on enzyme's accessibility to the substrate due to smaller pore sizes and narrowed pore size distribution in cellulose fibers (Laivins and Scallan, 1996).

Results are in line with other published studies in which cell structural modifications, especially cell membrane integrity, as a consequence of MW treatments have been reported (Binod *et al.,* 2012 and Hu and Wen, 2008). Sugar yield and structure modifications uphold the beneficial effect of a MW pretreatment in order to improve enzymatic

hydrolysis (Hu and Wen, 2008), since the exposed surface is increased in a way that cellulose results more accessible to hydrolytic enzymes (Binod *et al.,* 2012).

CONCLUSIONS

In the context of biofuel production pretreatments of lignocellulosic biomass are being currently studied in order to improve the saccharification step; in particular, microwaves have been suggested as an alternative pretreatment of this residual biomass. On the other hand, pineapple industry produces significant amounts of residues which need to be properly managed. Obtaining added value from these residues would not only reduce environmental impact but would also represent a benefit for the manufacturing industry.

In the present work microwaves have been studied as a pretreatment for improving pineapple waste saccharification. Results of applying different powers and exposure times to the pineapple waste material indicate that microwave pretreatment may increase saccharification performance as long as mild treatments are used. However, low powers and short exposure times do not modify sugar content whereas higher powers and/or exposure times may result in sugar decrease. Infrared thermography and Cryo-SEM microscopy observations indicated that both thermal sugar degradation and increased tissue compactness may be responsible for the lower yield when harsher microwave conditions are used. The presence of phenolic components as a result of lignin solubilization as well as sugar degradation to furfural and hydroxymethylfurfural have also been confirmed when lengthening the treatment, especially when higher powers are used. At milder conditions, however, explosion in hot spots and resulting tissue modifications facilitate enzyme action in the subsequent saccharification step.

The use of infrared thermography for the study of temperature profiles also allowed the identification of different periods during MW heating: a first period, characterized by a heterogeneous heating, the

existence of a hot spot in the central region of the sample and temperatures below 100 °C; a second one, in which a homogeneous heating is reached and temperature remains around 100 °C; and a final period, when sample overheating have been identified (temperatures > 100 °C) and new hot spots appear.

In conclusion, microwave pretreatments using the appropriate energy supply and exposure time allows to enhance the efficiency of lignocellulosics saccharification and, therefore, it may improve bioethanol yield in a subsequent step. This has been proved for industrial pineapple waste, although it could be potentially applicable to other food industry residues.

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3.3. ARTÍCULO 3

MICROWAVE-ASSISTED ALKALI PRETREATMENT FOR ENHANCING PINEAPPLE WASTE SACCHARIFICATION

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Microwave-Assisted Alkali Pretreatment for Enhancing Pineapple Waste Saccharification

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ABSTRACT: The effectiveness of microwave-assisted sodium hydroxide pretreatments to enhance the saccharification performance of pineapple waste was evaluated. Microwave alkali pretreatments for short exposure times (up to 60 s) significantly improved the yield of the enzymatic hydrolysis compared with non-pretreated waste. The greatest increase of fermentable (35.7%) and total sugars (33.5%) was obtained at 6.375 W/g for 5 s. However, longer exposure times resulted in sugar degradation and released fermentation inhibitors, such as phenols or hydroxymethylfurfural (HMF) as a consequence of thermal degradation. Nevertheless, the obtained phenols values were not sufficient to inhibit subsequent fermentation. Scanning electron microscope (SEM) images confirmed that applying microwaves for short exposure times promoted structural changes that improved enzymatic hydrolysis. By contrast, an increase in the severity of the treatment led to a compacted structure, which hindered access to enzymes and consequently reduced the release of sugars into the medium.

KEYWORDS: Microwave-assisted alkali pretreatment; Enzymatic saccharification; Pineapple waste

INTRODUCTION

Pineapple (*Ananas comosus*) is one of the most important and appreciated tropical and subtropical fruit crops for the processing industry; it is used for juice, canned, and minimally processed fruit (Bartholomew *et al.* 2002; Reinhardt and Rodriguez 2009). Up to 75% of the whole processed fruit is industrial waste, consisting of peeled skin, core, and crown (Buckle 1989; Abdullah 2007). Pineapple industrial waste has been investigated as an interesting source for bromelain enzyme extraction, phenolic antioxidants, organic acids, fiber, vinegar, and biogas (Larrauri *et al.* 1997; Roda *et al.* 2016). Moreover, this waste is a potential raw material for bioethanol production, as it contains a high amount of fermentable sugars and potentially hydrolyzable cellulose and hemicellulose (Nigam 1999; Abdullah and Hanafi 2008; Ruangviriyachai *et al.* 2010).

The use of lignocellulosic biomass for biorefining continues to be a challenge due to the cellulose crystallinity and the complexity of the structure. Cellulose and hemicellulose are densely covered by layers of lignin, which protects them against enzymatic saccharification (Dalgaard *et al.* 2006). For these reasons, pretreatment of lignocellulose is necessary to disrupt their recalcitrant structure and successfully hydrolyze the biomass. Pretreatments are one of the least technologically mature and most expensive steps in the bioethanol production process (Laser *et al.* 2002). Of the different pretreatment methods, alkali pretreatment is known for its ability to alter the lignin composition and, therefore, increase the digestibility of the biomass (Durot *et al.* 2003; Kristensen *et al.* 2008; Pedersen and Meyer 2010). However, this chemical pretreatment usually requires a high temperature, generally reached by conduction or convection heating (Pedersen and Meyer 2010). The high energy cost reduces the efficiency of the process. In addition, sugars and lignin may be degradated to furfural, hydroxymethylfurfural, and phenolic compounds, which strongly inhibit microbial fermentation (Taherzadeh and Karimi 2007).

Microwave irradiation is a clear alternative to conventional heating in many areas because of its high energy-efficiency, rapid heating, and easy operation (Binod *et al.* 2012). Xiong *et al.* (2000) found that microwaves change the ultrastructure of cellulose and break down the hemicellulose and lignin layers. Thus, microwave heating enhances enzymatic hydrolysis (Azuma *et al.* 1984). Moreover, microwaves can be easily combined with chemical pretreatments to increase efficiency and reaction rate (Zhu *et al.* 2006; Hu and Wen 2008; Binod *et al.* 2012).

The present study examined the efficiency of microwave-assisted sodium hydroxide pretreatment in pineapple waste. The effect of microwave power and exposure time on enzymatic saccharification and the release of fermentation inhibitors was evaluated. Low temperature scanning electron microscopy (cryo-SEM) and infrared thermography were applied to assess microstructural changes and microwave heating during saccharification, respectively.

EXPERIMENTAL

Raw Material and Sample Preparation

Pineapples (*Ananas comosus* [L.] Merr., "MD-2" cv.) were washed in 0.1% sodium hypochlorite for 5 min. The crown and the pulp were removed, and the peel and core waste were pressed in a laboratory screw press at 2.5 bar (CP-4, Vincent Corporation, Tampa, FL, USA) to separate the liquid phase from the original pineapple waste, as it already contains fermentable sugars. Finally, the resulting press cake (solid phase) was ground in a blender (Avance Collection Blender HR2097/00 800 W, Philips, Amsterdam, The Netherlands) and stored at -22 °C.

Microwave-assisted Alkali Pretreatment (MAP)

A combined alkali and microwave pretreatment was carried out by mixing 40 g of thawed, ground solid pineapple waste and 40 mL of NaOH 0.5 N (Panreac Química S.L.U., Barcelona, Spain) at room temperature (20 °C) for 1 h in the line of previous experiences by Zhu

et al. (2006), Hu and Wen (2008), and Binod *et al.* (2012). The samples were vacuum filtered in a filter flask attached to a Büchner funnel and a vacuum pump (N86KN.18 model Laboport®, KNF Neuberger GmbH, Freiburg, Germany). The retentate (solid phase) was treated in a microwave oven with a turntable plate (LG MH63340F / MH6340FS), with a frequency of 2.45 GHz at 170 W, 340 W, or 510 W. The applied powers were 2.125 W/g, 4.25 W/g, and 6.375 W/g, with exposure times of 5 s, 10 s, 20 s, 40 s, 60 s, 120 s, and 180 s. The appearance of calcination phenomena defined the time exposure limits. Samples were reconstituted by mixing the solid waste and the liquid phase (permeate). Water loss during microwave processing was determined by the difference in weight and restored. Finally, the samples were adjusted to pH 5 with 37% HCl (Panreac Química, S.L.U., Barcelona, Spain) for subsequent enzymatic hydrolysis. Experiments were carried out in triplicate.

Study of Microwave Heating by Infrared Thermography

A Testo 870-1 thermal imaging camera (Testo AG, Lenzkirch, Germany) with a spectral wavelength range from 7.5 µm to 14 µm, 9 Hz image refresh rate, and an infrared resolution of 160 x 120 pixels was used to estimate the temperatures reached during MAP. An image of the bottom surface of the container was taken at the end of each microwave pretreatment. The infrared images were analyzed by Testo AG IRSoft software.

Enzymatic Hydrolysis

Enzymatic saccharification was conducted by mixing 0.4% (w/w) of cellulase (1.13 U/mg solid, Sigma-Aldrich Química SL, Madrid, Spain) and 0.1% (w/w) of hemicellulase (1.5 U/mg solid, Sigma-Aldrich Química SL) from *Aspergillus niger* (L.) with the pretreated wastes in a 100-mL glass beaker, which was placed in an incubation oven (Incudigit, JP Selecta S.A., Barcelona, Spain) at 50 °C for 24 h.

Non-pretreated samples were used as a reference for assessing the effectiveness of MAP. To do this, 40 g of thawed, ground solid

pineapple waste was diluted in distilled water in 1:1 (w/w) as described above for MAP before saccharification.

Sugars Determination

Sugars in the liquid phase for both the pretreated and non-pretreated wastes were measured by high-performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) on a Metrohm chromatograph system (Herisau, Switzerland) consisting of a 716 IC Compact module and Metrohm ICnet 2.0 software for data analysis. For separation, a Metrosep Carb 1 250/4.6 column (250 mm x 4.6 mm I.D.) was coupled to a guard column at 32 °C, 8.8 MPa, injection volume of 20 µL, and 0.1 M sodium hydroxide as the mobile phase (1 mL/min). The samples were diluted (1:2000 v/v in bidistilled water) and then filtered (0.45 µm nylon filter) before injection. High-purity standards (Sigma-Aldrich Química SL; purity ≥ 99%) of glucose, fructose, sucrose, arabinose, and xylose were used to prepare standard calibration curves (2.5 ppm, 5 ppm, 10 ppm, 15 ppm, 25 ppm, and 50 ppm). All measurements were conducted in triplicate. The applied potentials and time periods were as follows: t_1, 400 ms / E_1 = +0.05 V (detection); t_2, 200 ms / E_2 = +0.75 V (cleaning); t_3, 400 ms / E_3 = -0.15 V (regeneration).

In this study, the term fermentable sugars means glucose, fructose, and sucrose, as these sugars are naturally fermented by *Saccharomyces cerevisiae* (Hahn-Hagerdal *et al.* 2007; Matsushika *et al.* 2009). Likewise, the term total sugars refers to fermentable sugars plus pentoses (arabinose and xylose) released from saccharified pineapple wastes.

Determination of Fermentation Inhibitors

The following inhibitory compounds were determined in triplicate in the liquid phase of the non-pretreated and MAP samples followed by enzymatic saccharification.

Determination of total phenolic content

The total phenolic content was measured using the method developed by Waterhouse *et al.* (2001), with Folin-Ciocalteu reagent (Sigma-Aldrich Química SL) and monohydrate gallic acid (Sigma-Aldrich Química SL) as the standard. Results were expressed as mg of gallic acid equivalents per mL of pineapple waste (mg GAE/mL).

Determination of furfural and hydroxymethylfurfural

The furfural (F) and hydroxymethylfurfural (HMF) contents were determined by high-performance liquid chromatography (HPLC) as described by Blanco-Gomis *et al.* (1991). An Alliance® HPLC system (Waters, Milford, MA, USA) equipped with a degasser, a 2695 separation module, and coupled to a diode array detector (DAD 2996, Waters). The chromatographic separation was performed on a Kromasil® 100 C-18 column (3 µm × 250 mm × 4.6 mm inside diameter) (Sigma-Aldrich). Analyses were done isocratically at 25 °C, an injection volume of 10 µL, and using acetonitrile/water (8:92 v/v) as the mobile phase (1 mL/min). Final ultraviolet (UV) detection was conducted at 280 nm. Standard solutions of F (0 to 5 µg/mL) and HMF (1 to 100 µg/mL) were prepared by dissolving analytical grade reagents (Sigma-Aldrich; purity ≥ 98%) in water with 10% (v/v) methanol.

Low Temperature Scanning Electron Microscopy (Cryo-SEM)

Microstructural changes in pretreated and unpretreated wastes were analyzed by cryo-SEM on a Cryostage CT-1500C unit (Oxford Instruments, Witney, UK), coupled to a Jeol JSM-5410 scanning electron microscope (Jeol, Tokyo, Japan). Samples were sublimated in the microscope stage for 20 min at -90 °C and -5 kV and then moved to another stage and coated with gold for 3 min at vacuum conditions (0.2 kPa). Samples were observed at 15 kV, 15 mm working distance, and a temperature ≤ -130 °C.

Statistical Analysis

Statgraphics Centurion XVI® (Manugistics Inc., Rockville, MD, USA) was used for statistical analyses, including one-way and multifactor analyses of variance (ANOVA) across the different results. Multiple regression analysis using the Pearson product moment correlations between each pair of variables were conducted. A 95% confidence level was used in all cases; a p-value lower than 0.05 indicated a statistically significant difference.

RESULTS AND DISCUSSION

Effect of Microwave-alkali Pretreatment on Sugar Yield

To assess the suitability of the microwave pretreatment, the total and fermentable sugars in each pretreated sample were determined and compared with the non-pretreated controls. All reported values correspond to sugar content after enzymatic hydrolysis (Figure 3.3.1.). Fermentable and total sugars followed similar patterns for all treatments because fermentable sugars (glucose, fructose, and sucrose) comprise most of the total sugars.

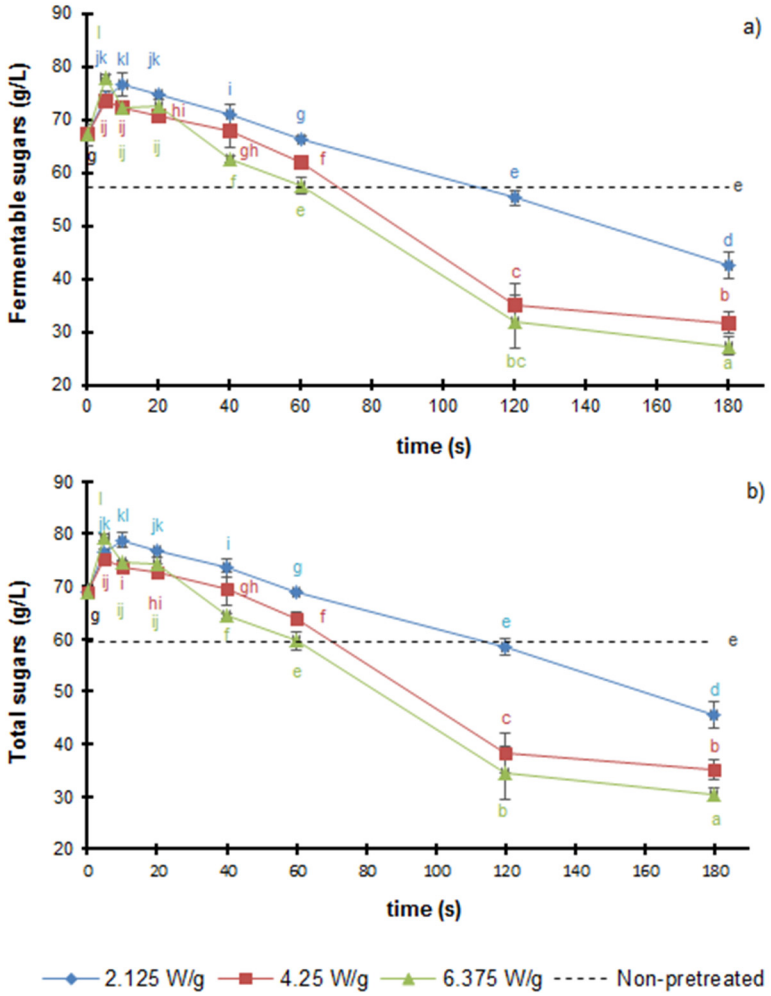

Figure 3.3.1. a) Fermentable sugars (g/L) and b) total sugars (g/L) of MAP samples at different power and exposure times followed by enzymatic saccharification. Data followed by different lowercase letters are statistically different according to the multiple range test (95% confidence level).

However, the applied microwave power, the exposure time, and the combination of both factors caused a statistically significant increase in sugar content. For all applied powers, microwave irradiation at short exposure times (up to 60 s) generated an increase in total and fermentable sugars yields. At a low microwave power (2.125 W/g), the

highest increases in fermentable (32.6%) and total sugars (33.6%) were obtained at t = 10 s, but there were no statistically significant differences with the results at t = 5 s and t = 15 s. At an intermediate power (4.25 W/g), the largest increases in fermentable (27.9%) and total sugars (26.7%) were achieved at t = 5 s, although no statistically significant differences were observed with the results at t = 10 s and 20 s. The highest increases of fermentable (35.7%) and total sugars (33.5%) were obtained at t = 5 s when high microwave power was applied. However, these values had no statistically significant differences with any pretreatment up to 20 s. Therefore, as suggested by Binod *et al.* (2012), for every microwave irradiation power, there was an optimal treatment time. Longer exposure times resulted in decreased sugar content. Higher applied microwave power accelerated decreases in sugar content. Binod *et al.* (2012) observed the same phenomenon in MAP for sugar cane bagasse, and charring was found for any applied microwave power at different exposure times. However, Zhu *et al.* (2005) and Singh and Trivedi (2011) did not report sugar reduction in wheat and rice straw at long exposure times. Notably, there was no further reduction of sugar yield with high microwave power (6.375 W/g) and 120 s exposure time, suggesting that sugars may have been entirely degraded at this point. Some useful components and sugars might be decomposed by pretreatments at high temperatures with high microwave power and long exposure times (Zhu *et al.,* 2005).

Analysis of Microwave Heating of Pineapple Waste by Thermography

Some of the effects produced by the application of microwaves may be due to the temperatures reached during pretreatment. Consequently, the temperatures of the samples during microwave pretreatment in an alkaline medium were evaluated *via* thermal images of the base of the waste containers. Figure 3.3.2 shows the thermography data and the maximum temperature or hot spot (HS) in each image. With short exposures ($t < 60$ s), the appearance of the HS was followed by inhomogeneous heating of the sample by the microwaves, leading to conductive heating from these points. This

evolution is characteristic for microwave heating (Lidström *et al.* 2001; Hu and Wen 2008) and is due to polar regions of the lignocellulosic material being selectively heated. The recalcitrant lignocellulosic structure may be disrupted by an "explosion effect" at the hot spots (Hu and Wen 2008).

Despite the initially heterogeneous heating, homogeneity of the entire sample was reached within a few minutes, although this effect took longer at lower power levels. In particular, more than 180 s was needed at the lowest power (2.125 W/g), more than 120 s was needed at intermediate power (4.25 W/g), and over 60 s was needed at the highest power (6.375 W/g). The results were similar to another report where longer exposure times were required at lower power levels; an applied power of 100 W required 150 s to reach a uniform temperature in the waste (Kumar *et al.,* 2014).

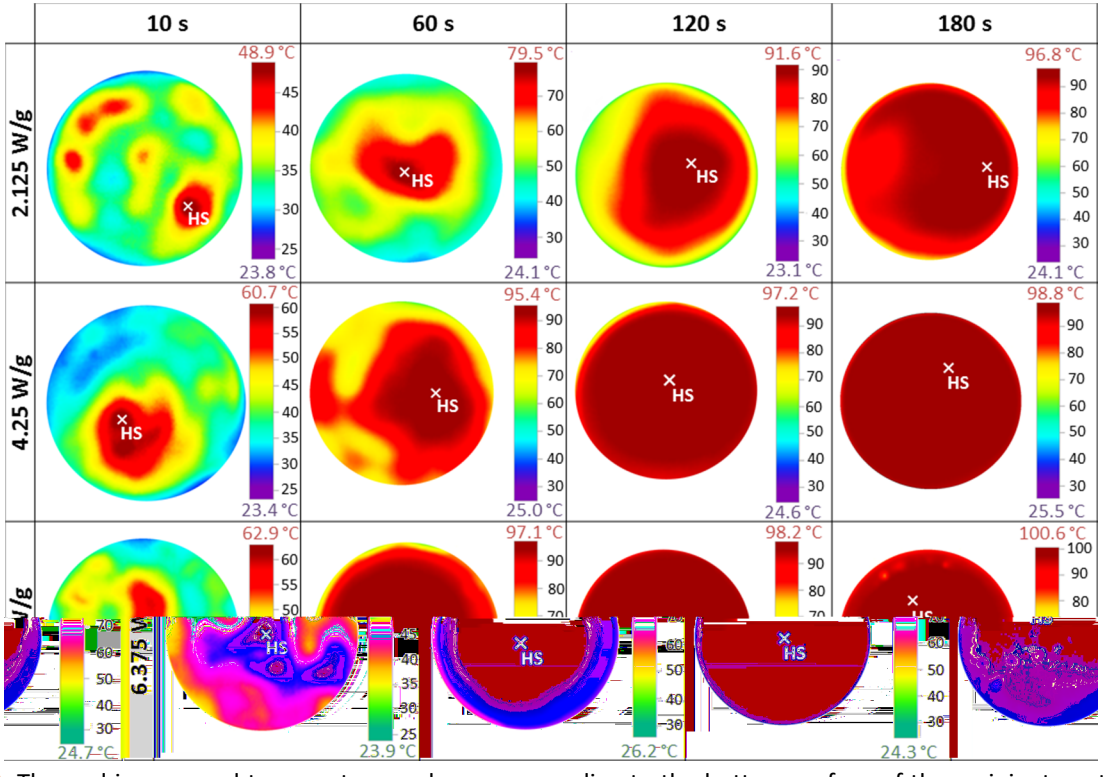

Figure 3.3.2. Thermal images and temperature scales corresponding to the bottom surface of the recipient containing MAP pineapple waste at different powers: 2.125 W/g, 4.25 W/g, and 6.375 W/g, and exposure times: 10 s, 60 s, 120 s, and 180 s; white cross, hot spot (HS).

Figure 3.3.3. shows the maximum and average temperatures throughout the treatment for all power levels. Exposure time, applied power, and the interaction between these two factors each had a statistically significant effect on the maximum and average temperatures. The evolution of the average and maximum temperature was fairly similar for all power levels. When 4.25 W/g and 6.375 W/g were applied, there was a more pronounced increase in temperatures up to 40 s of treatment; from then on, the temperature changed more gradually until the end of treatment. With the lowest applied power (2.125 W/g), the greatest temperature increase was between 20 and 40 s, and its evolution was considerably slower than in the previous cases. This result suggested that microwave power was less efficient at long times rather than at short times, where the greatest temperature changes were recorded (Kumar *et al.,* 2014). This initially faster warming was due to the emergence of the HS, while the subsequent slow evolution overlapped with conductive heating. The maximum temperature did not change greatly after 20 s for the highest power, after 20 s to 40 s for the intermediate power, or after 40 s for the lowest power. Although the final temperatures were significantly different depending on the power applied, the greatest differences between the various power levels occurred between 10 s and 120 s of treatment. Finally, heating was not the only effect that microwaves exerted on the residue structure. The electromagnetic field might have created non-thermal effects that also accelerate the destruction of the crystalline structures (De la Hoz *et al.,* 2005).

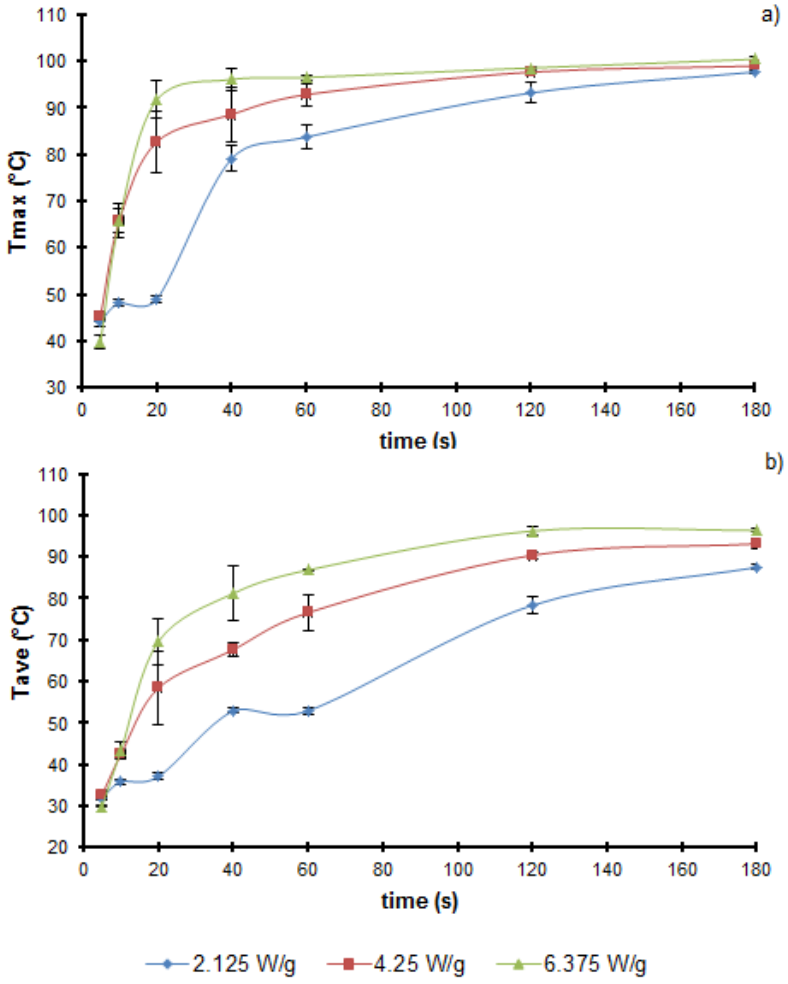

Figure 3.3.3. a) Maximum temperature (T_{max}) and b) average temperature (T_{ave}) in pretreated pineapple waste at different powers (2.125 W/g, 4.25 W/g, and 6.375 W/g) and exposures (5 s to 180 s)

Effect of the Pretreatments on the Release of Fermentation Inhibitors

Figure 3.3.4. shows the total phenol content in the liquid phase of the MAP and subsequently saccharified waste. Microwave pretreatment in an alkaline medium produced a significant increase in the total phenol content compared to non-pretreated waste (0.145 mg GAE/mL

± 0.004). In general, the phenol content was gradually raised as the time of exposure and the power applied were increased. Statistical analysis indicated that the time of exposure, the power applied, and the interaction between these two factors had a significant effect on the total phenols.

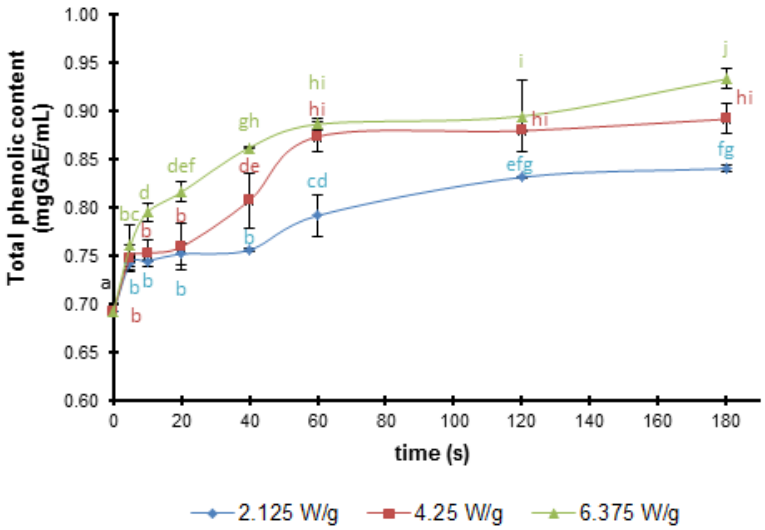

Figure 3.3.4. Total phenolic content (mg GAE/mL) in microwave-assisted alkali pretreated samples followed by enzymatic saccharification. Different lowercase letters indicate a statistical difference according to the multiple range test (95% confidence level).

Hu and Wen (2008) suggested that the increase in temperature *via* microwave pretreatment promotes lignin degradation and, thus, a rise in the phenol content. Vázquez-Gutiérrez *et al.* (2013) applied high pressures to onion waste and showed that increasing the severity of the treatment enhances the total phenol content.

Ando *et al.* (1986) showed a 30% reduction in the yield of phenolic compounds from *S. cerevisiae* yeast when 1 g/L of 4-hydroxybenzoic acid was added, while Palmqvist and Hahn-Hägerdal (2000) reported no significant effects when adding 2 g/L of this phenolic compound. In

this case, the obtained values were not sufficient to inhibit subsequent fermentation.

Figure 3.3.5. shows the HMF content of MAP and subsequently saccharified waste. Furfural was not detected in the liquid phase of the analyzed waste, which could be due to the low pentose content in the initial waste that can be considered negligible (Conesa *et al.,* 2016). In addition, the microwave pretreatment in an alkaline medium caused a marked increase in HMF content, such that both nominal power applied and exposure time were significant. The interaction between these two factors was also significant because the differences due to applying different power levels declined as exposure time increased. All values were significantly higher than those obtained when microwaves were not applied to the waste. These values were consistent with those reported in other studies. Specifically, Gabhane *et al.* (2013) found that increasing the application time and power of the microwave pretreatment of banana waste increases the temperature, resulting in greater degradation of hexoses to HMF. In this case, all of the values obtained ranged from 1.282 ± 0.015 g/L to 2.148 ± 0.018 g/L, with the lowest produced at a power of 2.125 W/g and a time of 5 s. The negative effects on the growth and fermentation rate of *S. cerevisiae* were reported for HMF concentrations above 1.0 g/L (Banerjee *et al.* 1981; Taherzadeh *et al.* 2000). Sanchez and Bautista (1988) hypothesized that the main effect of HMF (2 g/L) was the prolongation of the lag phase on fermentation.

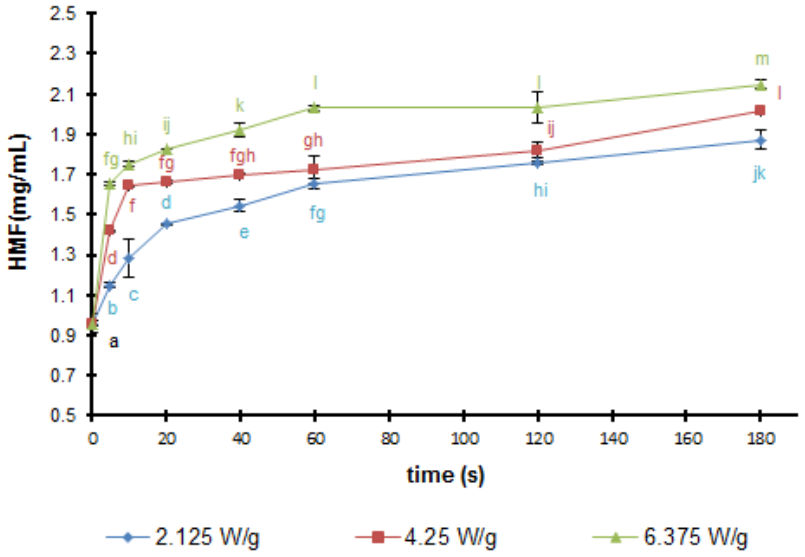

Figure 3.3.5. HMF content (mg /mL) of microwave-assisted alkali pretreated samples followed by enzymatic saccharification; data followed by different lowercase letters are statistically different according to the multiple range test (95% confidence level).

Consequently, the use of MAP at short exposure times (t < 60 s) significantly improved the yield of the enzymatic hydrolysis and kept the concentration of the generated inhibitors below the level to inhibit the subsequent fermentation. As the purpose of the study is to ferment the pretreated and saccharified waste without any extraction, these inhibitors will not be concentrated in the samples avoiding problems in the fermentation step.

Analysis of the Relationships between the Study Variables

Table 3.3.1. shows the Pearson correlation coefficients among each pair of analyzed variables. Each pair of variables had a significant linear relationship ($p < 0.05$). Fermentable and total sugars were strongly and negatively correlated with all the studied variables, meaning that these variables tended to decrease as the HMF, total phenolic content, and average and maximum temperatures increased. These results

were in line with those previously obtained because HMF is generated from hexose degradation as temperature increases.

The remaining variables were strongly and positively correlated with the maximum and average temperatures. The total phenolic content showed a positive correlation with the HMF content and the measured temperatures. This effect was due to further degradation of the lignin structure and the subsequent production of HMF as the temperatures rose. All temperatures were interrelated.

Table 3.3.1. Pearson Product Moment Correlations between Each Pair of Analyzed Variables.

	FS	TS	TPC	HMF	T_{max}	T_{ave}
FS	1.000	0.9997*	-0.8251*	-0.6717*	-0.6747*	-0.8460*
TS	0.9997*	1.000	-0.8287*	-0.6761*	-0.6759*	-0.8483*
TPC	-0.8251*	-0.8287*	1.000	0.8740*	0.8473*	0.9233*
HMF	-0.6717*	-0.6761	0.8740*	1.000	0.8749*	0.8661*
T_{max}	-0.6747*	-0.6759*	0.8473*	0.8749*	1.000	0.9443*
T_{ave}	-0.8460	-0.8483*	0.9233*	0.8661*	0.9443*	1.000

* Indicates statistically significant correlations ($p < 0.05$)

FS, fermentable sugars; TS, total sugars.; TPC, total phenolic content; HMF, hydroxymethylfurfural; T_{max}, maximum temperature; T_{ave}, average temperature.

Analysis of Physical Structure Changes by Cryo-SEM

Cryo-SEM observations of non-pretreated and MAP pineapple waste at 6.25 W/g and 10 s or 180 s showed that pretreatments induced structural changes in the lignocellulosic biomass (Figure 3.3.6.). The non-pretreated sample (Figure 3.3.6a.) possessed rounded packed cells with continuous surface areas. Moreover, protoplasts and cell walls were clearly identified. In contrast, microwave pretreated samples at a short exposure time (10 s) had a rougher surface area, and the separation of the plasmatic membrane from the cell wall was clearly identified. As suggested by Binod *et al.* (2012), microwave-assisted alkali pretreatments remove external fibers and increase the surface area, which makes cellulose more accessible to enzymes. These effects were directly related to the sugar increase for MAP at

short exposure times (< 60 s). Similar structural changes have been reported by Binod *et al.* (2012) for sugarcane bagasse microwave-alkali pretreated at short exposure times. With increasing microwave exposure time (Figure 3.3.6c.), cell wall roughness was greater, which suggested tissue dehydration. Laivins and Scallans (1996) indicated that dehydratation had a negative effect on enzyme accessibility to the substrate due to small pore sizes and narrowed pore size distributions in cellulose fibers. These results were in line with those obtained for high exposure times, in which MAP reduced the saccharification performance compared with non-pretreated samples.

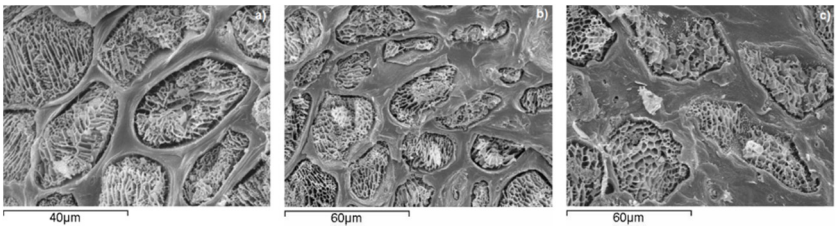

Figure 3.3.6. Cryo-SEM images of a) unpretreated sample, b) MAP waste treated at 6.375 W/g for 10 s, and c) MAP pretreated at 6.375 W/g for 180 s.

CONCLUSIONS

The use of microwave alkaline pretreatments for short exposure times (up to 60 s) improved the yield of enzymatic hydrolysis compared with non-pretreated waste. The highest increase in fermentable (35.7%) and total sugars (33.5%) was obtained at t = 5 s when high microwave power was applied (6.375 W/g). However, longer exposure times resulted in sugar degradation.

The content of fermentable and total sugars showed a statistically significant negative correlation with the maximum and average temperatures in the samples pretreated by microwave in an alkaline medium.

The content of fermentation inhibitor compounds, such as total phenols or HMF, increased as microwave power and exposure time rose. This increase was correlated with the rise in temperatures. However, the total amount of phenol values was not sufficient to

inhibit subsequent fermentation. Nevertheless, the effect of HMF content on pineapple waste fermentation should be studied.

Applying microwaves during short exposure times promoted structural changes that improved enzymatic hydrolysis. In contrast, an increase in the severity of the treatment compacted the structure and thus hindered access by the enzymes, which reduced the sugars released into the medium.

ACKNOWLEDGMENTS

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EVALUATION OF "ROJO BRILLANTE" PERSIMMON INDUSTRIAL RESIDUES AS A SOURCE FOR ANTIOXIDANT COMPOUNDS AND SUBSTRATE FOR BIOETHANOL PRODUCTION

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ABSTRACT: The industrial waste of "Rojo Brillante" persimmon was evaluated as a source of bioethanol and antioxidant compounds. Antioxidant properties were evaluated by analyzing total phenol content, soluble tanins, flavonoids and antioxidant capacity of the waste, consisting of the peel and calyx. On the other hand, fermentation and saccharification processes were assayed in order to valorize the waste as a substrate for bioethanol production: direct fermentation (DF) of the diluted waste was carried out and compared with simultaneous saccharification and fermentation (SSF) of diluted and non-diluted waste. The amount of phenols (59.2 ± 0.4 mg AGE/100 g FW), flavonoids (7.5 ± 0.4 mg QE/100 g FW) and tannins (11.43 ± 0.08 g AGE/100 g FW), as well as the antioxidant capacity (16.67 mg TE/100 g FW) of persimmon industrial waste were in the range of the pulp values, whereas β-carotene (400 ± 7 µg/100 g FW) and lycopene (194.3 ± 0.7 µg/100 g FW) content were found to be higher in the residues than in the pulp. Alcohol yield was significantly higher when SSF process was applied (0.3563 ± 0.0103 $g_{ethanol}/g_{sugar}$), as compared to DF (0.298 ± 0.002 $g_{ethanol}/g_{sugar}$). Results suggest that "Rojo Brillante" persimmon waste is a good candidate for obtaining value-added products.

Keywords: Persimmon, antioxidant properties, food waste, waste valorization, bioethanol.

INTRODUCTION

Persimmon (*Diospyros kaki* Thunb.) is an important commercial fruit in Asia that has expanded rapidly in the Mediterranean basin (Del Bubba *et al.*, 2009). In particular, the cultivation of the

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3.4. ARTÍCULO 4

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INTRODUCTION

Persimmon (*Diospyros kaki* Thunb.) is an important commercial fruit in Asia that has expanded rapidly in the Mediterranean basin (Del Bubba *et al.,* 2009). In particular, the cultivation of the “Rojo Brillante" variety has increased considerably in Spain, having grown from 2,437 hectares in 2004 to 11,510 hectares in 2014, its production reaching 220,000 tons in 2015 (Agricultura y Cooperación, 2015). The fast increase in the production and devoted cultivated surface is a result of the successful application of postharvest techniques in order to eliminate the astringency of the fruit, while fruit firmness and sweet flavour are preserved. This technology, which consists of 48 h of exposure to a CO_2 rich atmosphere, makes "Rojo Brillante" persimmon fruit much appreciated and commercially viable for the European market (Ben-Arie and Sonego, 1993; Taira et al., 1997; Arnal and Del Río, 2003).

One of the main drawbacks of persimmon consists of its seasonality. In the case of the "Rojo Brillante", harvesting takes place between October and December and cold storage can only preserve it for a month or two. Thus, the sector’s main priority is to extend the product’s sales calendar by improving harvesting techniques and storage methods whilst also maximizing the commercialization of discarded produce, which accounts for 20% of total production and making use of market surpluses. Therefore, a series of fruit derived products are being developed including dried persimmons (Kim *et al*., 2003), smoothies (Hernández-Carrión *et al.,* 2015), sauces (Han *et al.,* 2012), juices and purées for the production of jams, cakes, pastries and bread products (Brun, 2015). In addition, recent advances in postharvest technology have meant that "Rojo Brillante" persimmon can now be marketed as a fresh-cut commodity (Ghidelli *et al.,* 2013; Sanchís *et al.,* 2015). The main consequence of the increasing cultivation and industrialization of this fruit is the generation of a

significant amount of residue, mainly the peel and calyx. Some research has focused on the use of persimmon waste to produce vinegar (Kim *et al.,* 2011), or flours for the preparation and preservation of food products (Kim and Kim, 2005).

As for the fruit properties, persimmon has been claimed to have beneficial effects in fighting diabetes as well as a number of degenerative and cardiovascular diseases (George and Redpath, 2008; Park *et al.,* 2008; Piretti, 1991; Uchida *et al.,* 1990), due to the antioxidant compounds present the fruit. Takayuki (2005) has also suggested that the fruit has chemo-preventative effects against several types of cancer cells. According to Garcia-Alonso *et al.* (2004), antioxidant activity in "Rojo Brillante" persimmon can be two fold higher than other excellent sources of antioxidant compounds such as blueberries or blackberries. Due to its antioxidant properties, persimmon has recently been processed to produce functional milkshakes (Hernández-Carrión *et al.,* 2015) and juices (Endrizzi *et al.,* 2009). However, phenolic content in the persimmon fruits significantly depends on the astringency of the fruit, for which deastringency practices may result in a decreased antioxidant content (Del Bubba *et al.,* 2009). On the other hand, several studies state that the antioxidant capacity and total phenolic content of some fruits is higher in the peel than in the pulp (Ajila *et al.,* 2007; Okonogi *et al.,* 2007; Kunradi-Vieria *et al.,* 2009). Therefore, persimmon waste presents as a potential source of bioactive compounds to be used in the food, pharmaceutical o cosmetics industry (Deng *et al.,* 2012).

A different approach on the valorization of industrial persimmon waste consists of the evaluation of this residue as a substrate for microorganism growth and the production of interesting metabolites. In particular, there is great interest in the development of technologies for the use of agro-industrial biomass as a raw material for energy production (EC, 2009). Bioethanol production has traditionally used food crops as a carbon source, but their limited supply can lead to competition with food provision. This, together with price volatility of

food markets, has increased interest in developing processes for obtaining bioethanol from renewable resources at reasonable costs (Balat, 2011). Therefore, the use of residual lignocellulosic biomass for second-generation bioethanol is currently being encouraged.

Bioethanol is the most important biofuel used worldwide, and can be used on its own (in modified engines), mixed with conventional gasoline, or either be employed as an additive to replace methyl tert-butyl ether (MTBE), potentially toxic to humans (Song *et al.,* 2006). It has the advantage or reducing CO_2 and pollutants emissions into the atmosphere (Sánchez and Cardona, 2008; González-García *et al.,* 2009; Chen and Qiu, 2010; Balat, 2011). Bioethanol production from lignocellulosic biomass usually involves two different stages that can be carried out consecutively or simultaneously: the hydrolysis of cellulose and hemicellulose into mono and di-saccharides and the fermentation of the resulting sugars to obtain bioethanol. Hydrolytic enzymes used in the saccharification stage are usually obtained from fungi and consist of cellulases, which hydrolyze cellulose firstly into small oligosaccharides and then into glucose; and hemicellulases, which hydrolyse hemicellulose into monomeric sugars (Sehnem *et al.,* 2006). According to the literature, simultaneous saccharification and fermentation (SSF) provides higher ethanol yield and lower energy consumption but usually requires higher concentration of enzymes (Öhgren *et al.,* 2007), since control parameters are maintained closer to the optimum for fermentation rather than to the optimum for enzymes performance.

Hence, the main objective of this study was to evaluate the potential of the industrial waste of “Rojo Brillante” persimmon as a source for antioxidant compounds and as a substrate for the production of second-generation bioethanol. Both value-added products would contribute to the valorisation of persimmon fruit residues, which are expected to increase to a higher extent in subsequent years.

MATERIALS AND METHODS

Plant material and sample preparation

Persimmon waste was received from Anecoop S. Coop fresh-cut industry, Valencia, Spain. The cultivar used was "Rojo Brillante" Protected Designation of Origin "Kaki Ribera del Xúquer". Persimmons used in the processing line correspond to the discarded fruits of the fresh packing industry and the produced waste is mainly composed of the peel and calix of the fruit. In order to conduct the laboratory assays, persimmon waste was mashed in a blender (Avance Collection Blender HR2097/00 800 W, Philips, Amsterdam, the Netherlands) and stored at –20 °C until use.

Reagents and solvents

The following standards were used for the determination of sugar profile, total phenolic content, soluble tannins determination, flavonoids and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay: glucose, fructose and sucrose (purity ≥ 99%); gallic acid (purity = 99.7%); quercetin (purity ≥ 95%); DPPH (purity = 100%) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) (purity ≥ 97%) from Sigma-Aldrich. Co, San Luis, MO, USA. Methanol (PanReac® AppliChem, Barcelona, Spain purity = 100%) and acetonitrile (Sigma-Aldrich, Co., purity = 99.8%) were used as eluents in some determinations. Folin-Ciocalteu reagent and sodium carbonate (purity = 99.8%) were purchased from Sigma-Aldrich, Co. for determining total phenol content. Aluminum chloride ($AlCl_3$) (purity ≥ 98%) and hexane (purity = 95%) from Sigma-Aldrich, Co. were used for carotenoids extraction.

Reagents used in the saccharification and fermentation assays were: yeast extract peptone dextrose broth (YEPD) and yeast malt agar (YM Agar) culture media supplied by Scharlab S.L., Barcelona, Spain. Enzymes cellulase (1.13 U/mg solid) and hemicellulase (1.5 U/mg solid) from *Aspergillus niger* (Sigma-Aldrich., Spain). Other chemicals used for pH adjustment and physiological saline solution preparation: HCl

(37% w/v) (PanReac® AppliChem) and NaCl (Scharlab S.L.). Finally, ethanol content was determined with an ethanol assay kit (ethanol-UV method) obtained from Roche Diagnostics S.L. (Barcelona, Spain).

Evaluation of antioxidant properties of persimmon waste

The following analytical determinations were performed in triplicate.

Total phenolic content

The extraction of total phenolics was carried out according to the protocol described by Veberic *et al.* (2010). Thus, 20 ml of methanol and 5 g of waste were blended and kept in an ultrasonic bath for 30 min at room temperature (USC300T model, VWR® Ultrasonic cleaner, Radnor, PA, USA). After extraction, samples were centrifuged (Medifriger BL-S, J.P. Selecta®, Barcelona, Spain) for 7 min at 1000 rpm and 10 °C and the supernatant was filtered through a 0.45 µm of nylon filter (Filtros Anoia S.A., Barcelona, Spain). Then, total phenolic content was analyzed spectrophotometrically with the Folin-Ciocalteau reagent method (Singleton and Rossi, 1965). 6 ml of distilled water, 100 µl of the sample extract and 500 µl of Folin–Ciocalteu reagent were mixed and kept for 6 min at room temperature. Then, 1.5 ml of a 20% of sodium carbonate solution was added. After 90 min, the absorbance was measured at 760 nm using a spectrophotometer (632OD, Jenway, Stone, UK). The measurement was compared to a standard curve of gallic acid solutions in the range of 0.1 to 3 mg/ml and expressed as gallic acid equivalents (GAE) in mg per 100 g of FW. A mixture of water and reagents was prepared as a blank.

Soluble tannins

Soluble tannins content was analyzed using the Folin-Denis method described by Taira (1996). Thus, 5 g of the crushed persimmon waste were added to a 25 ml of methanol 80% solution and kept in an ultrasonic bath for 30 min at room temperature. Samples were centrifuged at 10.000 rpm for 20 min at 4 °C and vacuum filtered and the supernatant was stored at 4 °C. More supernatant was extracted from the precipitate by repeating homogenization, centrifugation and

filtration operations. The total supernatant was brought to 100 ml with distilled water. Next, 1 ml of this sample solution, 6 ml of distilled water and 0.5 ml of Folin-Ciocalteau reagent were added and stirred. After 3 min, 1 ml of 20% Na2CO3 and 1.5 ml of distilled added water were added and stirred. The absorbance was measured after 90 min with a spectrophotometer at 725 nm. The measurement was compared to a standard curve of gallic acid solutions and expressed as GAE in g per 100 g of FW. Blank was prepared in the same way without adding sample.

Carotenoid content

Lycopene and β-carotene contents were determined by UV-vis spectrophotometry (Porter and Anderson, 1967). Then, 20 ml of ethanol and 15 ml of hexane were added to 5 g of sample. The mixture was horizontally shooked (UNITRONIC-OR, J.P. Selecta®) for 1 hour in order to enhance the components extraction. Next, 1 ml of distilled water was added to separate the soluble and liposoluble phases. Finally, the spectrophotometric measurement was performed by collecting 0.5 ml of the apolar phase at 503 nm and 478 nm, for lycopene and β-carotene measurements respectively. Blank was prepared with hexane. Lycopene and β-carotene contents were calculated using equation 1 and 2:

$$\text{Lycopene (mg/100g)} = \frac{A_{503} * Pm_{lyc} * V}{m * E_{lyc}} * 100 \qquad \textbf{Equation 1}$$

Where A_{503} = Absorbance at 503 nm; Pm_{lyc} = molecular weight of the lycopene (537 g/mol); V = hexane phase volume (ml); m = sample weight (g); E_{lyc} = lycopene Extinction Coefficient in hexane (172,000 m/m) (Sadler *et al*. 1990).

$$\beta\text{–carotene (mg/100 g)} = \frac{[A_{478} - (A_{503} * 0{,}9285)] * Pm_{\beta-car} * V}{m * E_{\beta-car}} \qquad \textbf{Equation 2}$$

Where A_{503} = Absorbance at 503 nm; A_{478} = Absorbance at 478 nm; $Pm_{\beta\text{-car}}$ = molecular weight (533.85 g/mol); V = hexane phase volume

(ml); m = sample weight (g); $E_{\beta\text{-car}}$ = β-carotene Extinction Coefficient in hexane (139,000 m/m) (Sadler *et al.* 1990).

Total flavonoids content

Total flavonoids were determined according to Luximon-Ramma *et al.* (2014) using the aluminum chloride colorimetric method. Sample extraction was conducted as described for total phenolic content. 1.5 ml of a solution of 2% (m/v) $AlCl_3$ in methanol was added to 1.5 ml of the extract sample. The mixture was vigorously shaken and the absorbance was read at 337 nm after 10-15 min of incubation. The measurement was compared to a standard curve of quercetin solutions and expressed as quercetin equivalents (QE) in mg per 100 g of FW. A mixture of water and reagents was used as a blank.

Antioxidant capacity by the DPPH Free Radical Scavenging Assay

The antioxidant activity of persimmon waste was assessed by determining the radical scavenging activity through the DPPH method as described by Shahidi *et al.* (2006) with some modifications. Extraction of antioxidant compounds was conducted by adding 10 mL of 80% (v/v) methanol-water dissolution to 5 g of crushed persimmon waste and stirring for 2 min. Then the sample was centrifuged at 4000 rpm and 10 °C for 5 min. Then the supernatant was stored at 0-4 °C before analysis. 3.9 ml of a DPPH solution (80:20; methanol:water) was added to 0.1 ml of the obtained supernatant. The mixture was kept in a dark place at room temperature for 30 min and the absorbance of the sample was measured at 515 nm. Methanol was used as a blank. The percentage of DPPH inhibition was determined using the equation 3:

$$\%\ \text{DPPH inhibited} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \qquad \textbf{Equation 3}$$

Where A_{blank} = Absorbance of the blank; A_{sample} = Absorbance of the sample.

Results were compared with a standard Trolox curve and expressed as mg of Trolox equivalents (TE) in 100 g of FW.

Saccharification and fermentation for bioethanol production

Preparation of inoculum

Ethanol-producing yeast strain *Saccharomyces cerevisiae* Meyen ex E.C. Hansen 1883 provided by the Colección Española de Cultivos Tipo (CECT) of the Universitat de València was used (CECT 1319). The lyophilized yeast was reconstituted and kept on YM Agar at 4 °C. The inoculum was prepared by growing the microorganisms in tubes containing 3 mL of sterile YEPD medium in an incubation oven (Incudigit, J.P. Selecta®) during 48 h at 28 °C, in order to obtain an inoculum medium containing $2 \cdot 10^6$ Colony Forming Units per mL (CFU/mL).

Saccharification and fermentation experiments

Fermentations were performed either directly in the solid waste (DF: Direct Fermentation), or by simultaneously hydrolyzing polymeric sugars in a Simultaneous Saccharification and Fermentation (SSF) process.

Persimmon waste was diluted before the DF process since, according to Sassner *et al.* (2006), high solid loadings of biomass increase viscosity preventing homogeneity and effective distribution of microorganisms. Therefore, persimmon waste was diluted by adding distilled water (25 % v/v) before undergoing fermentation. Next, about $2 \cdot 10^6$ CFU/mL of *S. cerevisiae* inoculum were inoculated to 50 g of blended waste, in 100 mL Erlenmeyer flasks. The need for a previous thermal treatment in order to prevent microbial spoilage was evaluated by introducing the diluted waste in an autoclave (Systec GmbH, VB-40 model, Linden, Germany) at 121 °C for 5 min. Fermentations were underwent at pH 5 (adjusted with HCl 37% v/v) and 28 °C in an incubation oven. Experiments were conducted in triplicate.

Saccharification of the waste material was also assayed in order to maximize the amount of sugars available for fermentation. Since SSF is known to increase ethanol yield as compared to a consecutive saccharification and fermentation process, 1% (w/w) cellulase and

hemicellulose enzymes from *Aspergillus niger* were added to the blended waste (medium) together with the *S. cerevisiae* inoculum. Since the action of the enzymes could reduce the medium viscosity and facilitate enzyme and microorganism distribution, SFF was assayed in diluted and non-diluted waste. As for SSF conditions, these were similar to the DF process.

Analytical determinations

Colony Forming Units (CFU), sugar profile (glucose, fructose, sucrose) and ethanol content were determined at different time intervals (0 h, 24 h, 48 h and 78 h) during the fermentation experiments.

Sugar extraction was conducted as suggested by Veberic *et al.* (2010). Thus, 10 g of crushed fruit waste were blended with 50 ml of bidistilled water and shaken for 30 min at room temperature with a magnetic stirrer (BASIC ACS-160 model, SBS® Instruments S.A., Barcelona, Spain). Then, the sample was centrifuged at 10.000 rpm for 7 min at 10 °C. Finally, the supernatant was filtered through a 0.45 µm nylon filter and diluted (1:2000 v/v with bidistilled water). Identification and quantification of sugars were performed by High-Performance Anion-Exchange Chromatography with a Pulsed Amperometric Detector (HPAEC-PAD), using a Metrohm IC chromatograph system (Herisau, Switzerland) equipped with a 716 Compact module and an ICnet 2.0 software program for data analysis. A three-step PAD setting was used with the following time periods (ms) and potentials (V): t_1, 400 ms / E_1 = +0.05 V (detection); t_2, 200 ms / E_2 = +0.75 V (cleaning); t_3, 400 ms /E_3 = -0.15 V (regeneration). For separation, a Metrosep Carb 1 250/4.6 column (250 mm x 4.6 mm I.D.) was coupled to a guard column at 32 °C, 8.8 MPa, injection volume of 20 µL and using sodium hydroxide 0.1 M as the mobile phase (1 ml/min). Standards of glucose, fructose and sucrose were used to prepare standard calibration curves (2.5 ppm, 5 ppm, 10 ppm, 15 ppm, 25 ppm and 50 ppm).

CFU were determined by the serial dilution method, by serially diluting in 5 tubes containing 9 mL of a 0.9% NaCl solution. 0.1 mL of the less concentrated tubes were plated in YPD-agar medium in order to

obtain the 10^{-4}, 10^{-5} and 10^{-6} dilutions. Counting was performed on plates containing 30 to 300 CFU after 48 h of incubation at 28 °C. Ethanol concentration (% ethanol) was determined using the ethanol kit specified in the reagents and solvents section. It is based in various spectrophotometric measurements performed on a dilution of the sample (1:1000 v/v in bidistilled water), after reacting with the enzymes provided. Measurements were performed at 340 nm and the ethanol percentage calculated by the relationship given in the kit specifications. Results are shown as the average and standard deviations from triplicates.

Statistical analysis

Statgraphics Centurion XVI® (Manugistics Inc.; Rockville, MD, USA) was used for statistical analyses. Statistically significant differences were determined by using one-way analyses of variance (ANOVA) at 95% confidence level (p-value < 0.05).

RESULTS AND DISCUSSION

Antioxidant properties of persimmon waste

Antioxidant properties of persimmon waste are summarized in Table 3.4.1. Total phenolic content, soluble tannins, total flavonoids, carotenoids (β-carotene and lycopene) and antioxidant capacity values are given.

Table 3.4.1. Antioxidant properties of industrial persimmon waste (mean ± standard deviation of three replicates).

Antioxidant property	Value
Total phenolic content (mg GAE/100 g FW)	59.2 ± 0.4
Total flavonoid content (mg QE/100 g FW)	7.5 ± 0.4
Soluble tannins (mg GAE/100 g FW)	11.43 ± 0.08
B-carotene (µg/100 g FW)	400 ± 7
Lycopene (µg/100 g FW)	194.3 ± 0.7
Antioxidant capacity (mg TE/ 100 g FW)	18.15

GAE: Gallic Acid Equivalents; QE: Quercetin Equivalents; TE: Trolox Equivalent; FW: Fresh Weight).

Results indicate that all antioxidant compounds evaluated are present in the persimmon residues. Although there is still incomplete knowledge of the chemical structure of persimmon polyphenols, it is known that simple polyphenols together with highly polymerized flavanols are present in the fruit and, among the phenolic compounds in fully ripe persimmon fruit, catechin and gallic acid are the predominant (Denev and Yordanov, 2013). Phenolic content of the fruit can vary significantly depending on the ripening stage or the astringency of the cultivar studied (Giordani *et al.,* 2011; Denev and Yordanov, 2013). In astringent cultivars, reported polyphenol content is between 1.3 mg GAE/100 g (Gorinstein *et al.,* 2001; Park *et al.,* 2006) and 1480 mg GAE/100 g of product (Katsube *et al.,* 2004). In addition, the wide variability reported in the literature may not only be due to the type of astringent cultivar analyzed, but also to the environment and the extraction method used in the analysis (Giordani *et al.,* 2011). In addition, treatment with CO_2 to eliminate astringency decreases the total phenol content in the fruit (Del Bubba *et al.,* 2009; Giordani *et al.,* 2011). In particular, Del Bubba *et al.* (2009) reported a total phenolic content of 220 mg GAE/100 g for persimmons treated with ethanol vs. 31 mg GAE/100 g for those treated with CO_2, performing a methanol-water extraction on the "Rojo Brillante" variety. Results of the present study reveal that the phenolic content in deastringent "Rojo Brillante" persimmon waste (59.2 ± 0.4 mg GAE/100 g), is above the values reported in the literature for the whole fruit (Del Bubba *et al*., 2009), and in the lower range of the astringent cultivars. As compared to other residues (Deng *et al*., 2012), total phenols in persimmon peel are similar to the values reported for pear or red plum peels (≈ 0.6 mg GAE/g), higher than peach or water melon peels (0.2-0.4 mg GAE/g), but lower than apple or orange peels (1.0-1.6 mg GAE/g).

As for total flavonoids, although they have been said to represent a very small part of the phenols present in the fruit (between 0.8-2.7%), results suggest that they are more present in the residue by representing 12.7% of total phenolic constituents of persimmon waste. Flavonoid content of peel and calix appear to be significantly

higher than the reported by other authors (Denev and Yordanov, 2013) for different cultivars and ripening stages (0.9-4.2 mg QE/100 g).

Regarding soluble tannins, results of the analysis reveal a remarkable low value. This is in line with the observed by other authors (Salvador *et al.,* 2007; Del Bubba *et al.,* 2009), and is the result of the deastringency treatment with CO_2. Soluble tannins are the polyphenol fraction responsible for persimmon unripe typical astringency (Itoo, 1986; Giordani *et al.,* 2011), for which applying a CO_2 postharvest treatment necessarily implies a reduction in the soluble tannins content. Exposure to CO_2 activates anaerobic respiration in the fruit, which gives rise to an accumulation of acetaldehyde, which reacts with soluble tannins and become insoluble.

Carotenoids are responsible for the colour of persimmon fruit (Zhou *et al.,* 2009), β-carotene and lycopene being among the different carotenoids identified in persimmon and especially in persimmon peel (Daood *et al.,* 1992). β-carotene presence in the persimmon residues analysed in the present work (Table 3.4.1.) was in the range of the reported in the literature for persimmon peel. Veberic *et al.* (2010) obtained values ranging from 239 ± 28 µg/100 g in the peel of the "Thiene" cv. to 875 ± 50 µg/100 g in the peel of “Tenjin O'Gosho” cv. In turn, Giordani *et al.* (2011) suggested that β-carotene content varies between 253 ± 13 and 420 ± 100 µg/100 g FW depending on the cultivar. Results also confirm that β-carotene content in persimmon peel is higher than in the whole fruit, 124 mg/100 g and 12-115 µg/100 g, as reported by De Ancos *et al.* (2000) and Zhou *et al.,* (2009), respectively.

As said, persimmon is also a richer source of lycopene than most commonly consumed fruits, with an average content of 159 µg/100 g (Giordani *et al.,* 2011). Different investigations reveal considerable variability in the lycopene content of persimmon. Wright and Kader (1997) found figures of 242 µg/100 g in the non-astringent “Fuyu” variety; on the other hand, De Ancos *et al.* (2000) obtained a content of 534 µg/100g in the pulp of “Rojo Brillante” persimmon, which is

higher than the obtained in the present study. Accordingly, Zhou *et al.* (2009) reported lycopene contents between 2.5 and 112 µg/100 g when analyzing more than forty persimmon cultivars. Results varied significantly, regardless of the level of astringency. Giordani *et al.* (2011) attributed the wide degree of variability associated with the lycopene content to the genotype effect or environmental factors such as exposure to light. In addition, other authors rely on factors such as the specific phase of ripening of the fruit (Von Elbe and Schwartz, 1996).

Finally, the antioxidant capacity of persimmon residue was calculated as Trolox Equivalents by the DPPH method, obtaining results in line with the reported by Martinez-Las Heras *et al.* (2016), who studied the influence of pre-harvest treatments on the antioxidant properties of "Rojo Brillante" persimmon during storage, and after CO_2 postharvest deastringency treatment. According to Del Bubba *et al.* (2009), postharvest treatments with ethylene or CO_2 cause a significant decrease in the antioxidant capacity of the fruit, up to 20-30%. For the specific case of persimmon, antioxidant capacity in the peel was not observed to be higher than the antioxidant capacity of the pulp. This result is consistent with Peng-Min (2011), who reported higher antioxidant capacity in the pulp than in the peel of astringent cultivars. As compared to other fruit residues (Deng *et al.,* 2012), the antioxidant capacity of "Rojo Brillante" persimmon waste is in the lower range of the values reported for the water-soluble fraction of different fruit wastes. CO_2 deastringency treatment, as well as the high dependence on the extraction and quantification methods would explain the low antioxidant capacity value observed.

Study on obtaining bioethanol from persimmon waste

As a lignocellulosic residual biomass, persimmon waste was analyzed as a source for the production of second-generation bioethanol. As previously mentioned, persimmon waste was first diluted by adding 25% water in order to undergo fermentation, either thermally treated

or not; and it was also simultaneously saccharified and fermented, either diluted or not.

Figure 3.4.1. shows the evolution of CFU content during fermentation (72 h) for the different processes assayed. CFU count was registered every 24 h from the onset of fermentation so as to determine the adaptation of yeasts to the medium and their evolution during the process. Initially, a sufficient amount of inoculum was introduced in the medium so as to supersede the growth of other microorganisms that might contaminate the medium. The significant growth of yeast within 24 hours, deduced from viable counting, indicates a good adaptation of the yeasts to the culture medium (Figure 3.4.1). These early hours of culture coincide with the exponential growth phase and are followed by a stationary phase in which microorganisms grow at a constant rate, yielding the maximum CFU count. The stationary phase lasted 48 h and was followed by a significant decrease in CFU in all cases, indicating the decline or death phase, which points towards the end of the fermentation process (Owen, 1988).

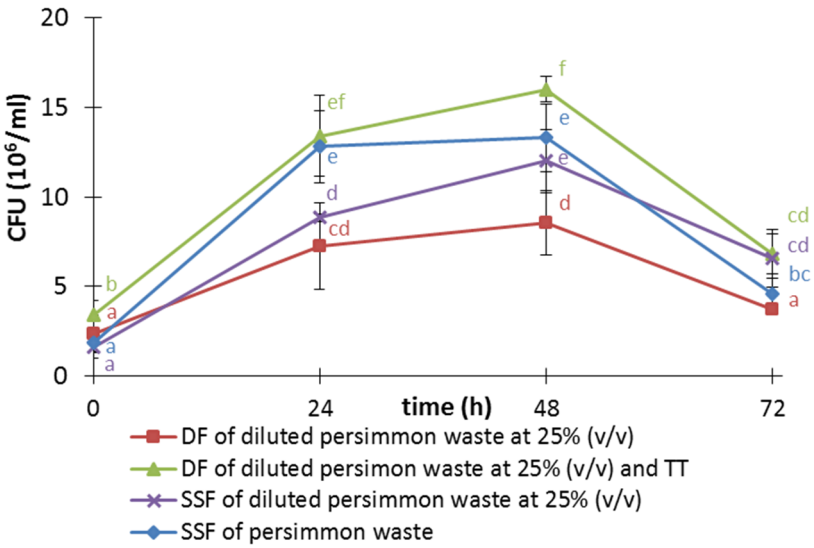

Figure 3.4.1. Cell growth (Colony Forming Units, CFU 10^6.ml^{-1}) during 72 hours of fermentation (DF) or simultaneous saccharification and fermentation (SSF).

Evolution of sugars and ethanol production during DF and SSF processes is shown in figure 3.4.2. Regarding initial sugar content in the liquid phase of persimmon waste, glucose and fructose were found in similar amounts, being fructose slightly higher (≈ 54% of the total sugar content). However, literature suggests that fructose, glucose and sucrose are the main sugars present in the flesh of mature fruits (Zheng and Sugiura, 1990; Senter *et al.,* 1991; Daood *et al.,* 1992; Ittah, 1993; Del Bubba *et al.,* 2009). In fact, very different glucose/fructose ratios and sucrose concentrations are found depending on the extraction method adopted and the cultivar analyzed (Zheng and Suguira, 1990; Ittah, 1993). It should be pointed out that persimmon has a much higher invertase activity than other fruits (e.g. about two fold higher than apples) (Hirai and Kondo, 2002). This fact might explain the absence of sucrose in the analyzed samples. Total sugar content values found in the non-diluted persimmon waste samples (Figure 3.4.2.d.) were lower than values reported for the mature flesh by Del Bubba *et al.* (2009) (14.9 g/100 g).

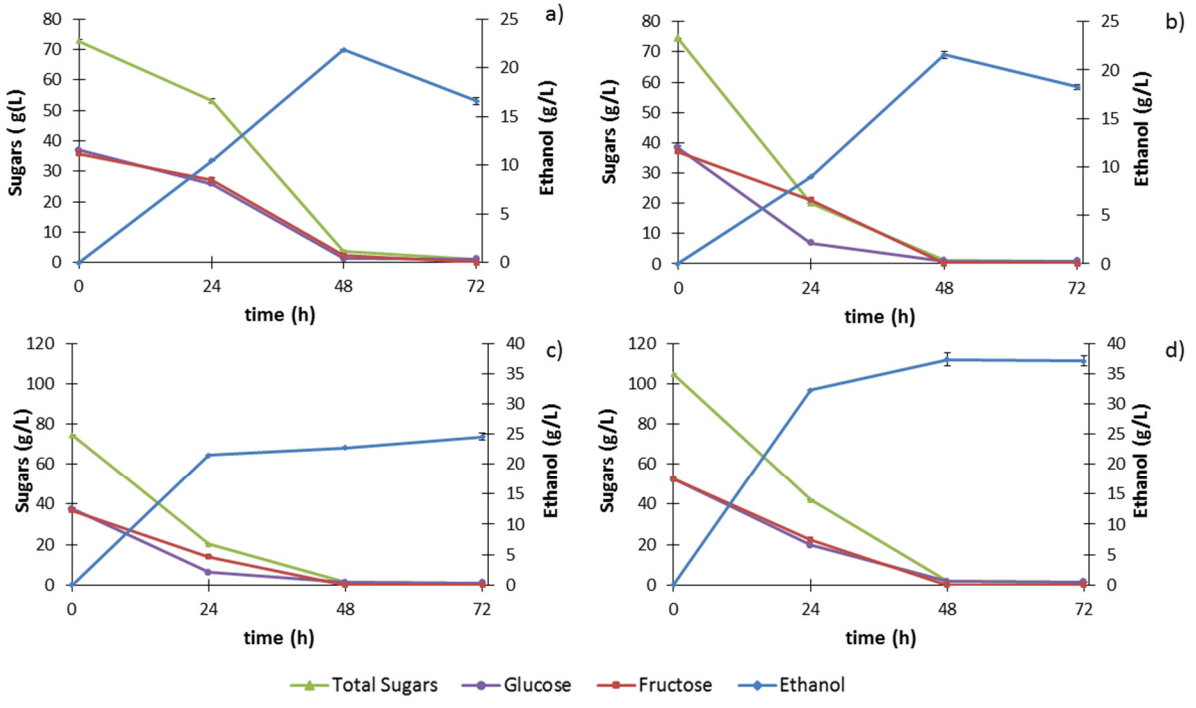

Figure 3.4.2. Glucose (g/l), fructose (g/l), total sugars (g/l) and ethanol (g/l) contents during the DF of persimmon waste diluted at 25% (v/v): a) with previous TT or b) without TT or duting SSF of both: c) diluted at 25% (v/v) and d) non-diluted persimmon waste.

As for the progress during fermentation, fermentable sugars decreased in line with an increase in the alcohol content. When comparing the results obtained for the diluted waste, as for the appropriateness of performing a thermal treatment (Figures 3.4.2.a. and 3.4.2.b.), the total sugar content was found to decrease and virtually disappear at 48 h, with the decline being even more marked when thermal treatment was applied. On the other hand, the ethanol content increased gradually, reaching its peak value at 48 h. Results reveal no statistically significant differences (p-value <0.05) as for ethanol production, which means that thermal treatment has no advantages over carrying out fermentation on unsterilized residue after 48 h of fermentation. Fermentation was considered to have finished 48 h after yeasts inoculation. Lengthening the fermentation stage resulted in a decrease in the alcoholic content, which was more significant in the non-sterilized waste, probably as a result of the growth of other microorganisms such as acetic bacteria.

SSF was evaluated in the diluted (3.4.2.c.) and non-diluted waste (3.4.2.d.). Results indicated that SSF implied higher ethanol production than DF. Apart from the sugars initially present in the solid waste, SSF allows for the gradual generation of fermentable sugars as a result of complex carbohydrates hydrolysis, and further fermentation to produce bioethanol. This results in a significantly increased bioethanol production during the first 24 h of treatment, which continues up during the 72 hours registered, albeit at a much slower pace than previously. Saccharification of the solid waste produced fluidification of the medium, which could have facilitated microorganism distribution and subsequent access to the substrate, this implying a faster fermentation rate. In addition, SSF process reduced contamination by unwanted microorganisms due to the presence of ethanol in the medium (Elumalai and Thangavelu, 2010). Therefore, SSF was also tested in the non-diluted waste. The analysis of the results obtained for SSF treatment when no water is added to the residue (Figure 3.4.2.d) implied higher levels of ethanol production compared to the diluted medium (p-value <0.05). The amount of total sugars

initially available for fermentation were also higher in this case, for which the alcohol increase was expected (Figure 3.4.2.c.). Again, fermentation could be considered as having finished after 24 h, which is technically advantageous since processing time is of paramount importance at industrial level.

Yeast sugar conversion into ethanol, i.e. alcohol yield (g of alcohol produced/g of sugar consumed) was calculated for each of the treatments assayed (Table 3.4.2). First, it is evidenced that sterilizing the medium does not imply better yeast performance. In contrast, results reveal that SSF has advantages versus DF of the waste, since ethanol yields were significantly higher for both SSF treatments (p-value <0.05). In fact, SSF have been found to be a more effective treatment (Viikari *et al.,* 2007) due to the simultaneous generation of fermentable sugars and fermentation, thus reducing yeast inhibition due to high sugar levels, which enhances yeast fermentation performance. Advantages of SSF were more evidenced when increasing the solid loading, since the best conversion yields were obtained for the SSF of the non-diluted waste.

Table 3.4.2. Ethanol yield (gethanol/gsugar) obtained in the different saccharification and fermentation processes assayed.

	DF of the waste diluted at 25% (v/v)	DF of the waste diluted at 25% with TT (v/v)	SSF of the diluted waste at 25% (v/v)	SSF of the waste
Ethanol yield ($g_{ethanol}$/ g_{sugar})	0,298 (0,002)[a]	0,285 (0,005)[a]	0,330 (0,009)[b]	0,3563 (0,0103)[c]

DF: Direct Fermentation. TT: Thermal treatment; SSF: Simultaneous Saccharification and Fermentation.

[a,b,c,…]Different lowercase letters indicate statistically differences according to the multiple range test (95% confidence level).

CONCLUSIONS

Nowadays, agri-food waste recovery is of capital importance because of its environmental impact and potential economic benefits. Specifically, one of the main problems of “Rojo Brillante” persimmon

is the large amount of waste generated as a consequence of its seasonality and the dramatic rise in its production.

Industrial persimmon residue offers considerable potential for the production of high-added-value compounds, such as antioxidants. Specifically, the level of phenols, tannins, flavonoids and antioxidant capacity was similar to that found in the whole fruit by other authors, although their content were not particularly remarkable when compared to other fruit residues, probably due to antioxidant content reduction as a consequence of deastringency treatments. Nevertheless, concentrations of β-carotene and lycopene were found to be higher than in the fruit, and persimmon fruit is already a considerable good source of carotenoids. Therefore, persimmon waste can be considered an inexpensive and readily available resource of bioactive compounds, mainly carotenoids. Potential applications of these bioactive compounds in the food, pharmaceutical o cosmetics industry are not only possible after their extraction, which would raise the price of the resulting product, but the use of a crude flour or powder obtained from persimmon residue after drying and grinding could also have several applications as a natural preservative, flavoring or coloring agent.

On the other hand, persimmon waste has also shown potential as a substrate for bioethanol production. Among the different processes assayed, the simultaneous saccharification and fermentation process implied higher ethanol yields. The use of lignocellulose residual biomass to produce second generation bioethanol is being encouraged nowadays. In the present work, persimmon waste has successfully undergone fermentation and has shown potential to be used for bioethanol production, either alone or mixed with other food residual biomass.

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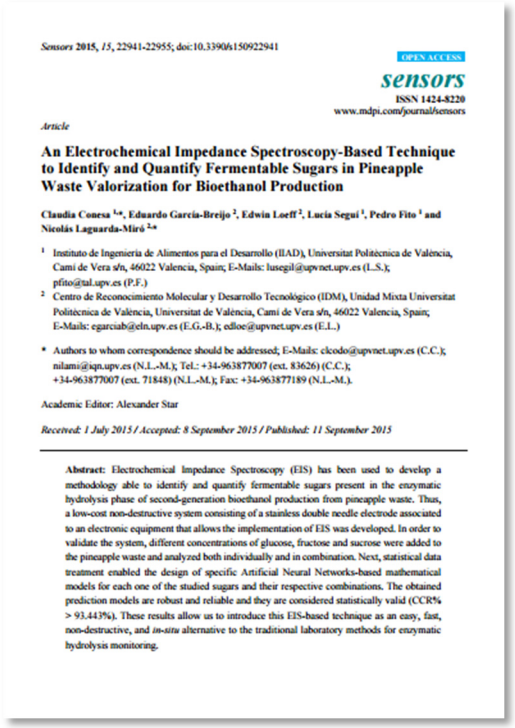

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Article

An Electrochemical Impedance Spectroscopy-Based Technique to Identify and Quantify Fermentable Sugars in Pineapple Waste Valorization for Bioethanol Production

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Abstract: Electrochemical Impedance Spectroscopy (EIS) has been used to develop a methodology able to identify and quantify fermentable sugars present in the enzymatic hydrolysis phase of second-generation bioethanol production from pineapple waste. Thus, a low-cost non-destructive system consisting of a stainless double needle electrode associated to an electronic equipment that allows the implementation of EIS was developed. In order to validate the system, different concentrations of glucose, fructose and sucrose were added to the pineapple waste and analyzed both individually and in combination. Next, statistical data treatment enabled the design of specific Artificial Neural Networks-based mathematical models for each one of the studied sugars and their respective combinations. The obtained prediction models are robust and reliable and they are considered statistically valid (CCR% > 93.443%). These results allow us to introduce this EIS-based technique as an easy, fast, non-destructive, and *in-situ* alternative to the traditional laboratory methods for enzymatic hydrolysis monitoring.

3.5. ARTÍCULO 5

AN ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY-BASED TECHNIQUE TO IDENTIFY AND QUANTIFY FERMENTABLE SUGARS IN PINEAPPLE WASTE VALORIZATION FOR BIOETHANOL PRODUCTION

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ABSTRACT: Electrochemical Impedance Spectroscopy (EIS) has been used to develop a methodology able to identify and quantify fermentable sugars present in the enzymatic hydrolysis phase of second-generation bioethanol production from pineapple waste. Thus, a low-cost non-destructive system consisting of a stainless double needle electrode associated to an electronic equipment that allows the implementation of EIS was developed. In order to validate the system, different concentrations of glucose, fructose and sucrose were added to the pineapple waste and analyzed both individually and in combination. Next, statistical data treatment enabled the design of specific Artificial Neural Networks-based mathematical models for each one of the studied sugars and their respective combinations. The obtained prediction models are robust and reliable and they are considered statistically valid (CCR% > 93.443%). These results allow us to introduce this EIS-based technique as an easy, fast, non-destructive, and in-situ alternative to the traditional laboratory methods for enzymatic hydrolysis monitoring.

KEYWORDS: bioethanol; saccharification; electrochemical impedance spectroscopy; fermentable sugars; pineapple waste

INTRODUCTION

The search for sustainable and environmentally friendly energy sources alternative to fossil fuels is raising the investigation of agro-industrial wastes as potential inputs for second-generation bioethanol production. In this sense, pineapple is generating a growing interest as its world production is steadily increasing and has reached 24 million tons in 2014 (FAOSTAT, 2015). Nowadays, around 33% of its production is being processed, mainly by canning and juice industry (Reinhardt and Rodríguez, 2009) and its industrial waste (crown, pulp and peel), representing about 50% (W/W) of the total processed fruit (Ketnaea *et al.*, 2012), cannot be neglected. In addition, its bio-chemical composition reinforces the interest in this waste as a potential source for bioethanol production because of its high content of cellulose and hemicellulose (Tanaka *et al.,* 1999; Nigam, 2009; Ruangviriyachai *et al.,* 2010).

In order to produce bioethanol from lignocellulosic biomass, it is necessary to hydrolyze cellulose (polymer of d-glucose units linked by β-1,4-glycosidic bonds) and hemicellulose (polymer of pentoses, hexoses and uronic acids) into fermentable sugars (Scheller and Ulvskov, 2010). This is the most complex phase in the bioethanol production process and can be performed by chemical or enzymatic hydrolysis. The enzyme-based saccharification is more efficient than the chemical hydrolysis, showing higher selectivity and lower energy costs. On the contrary, it is particularly complex due to the mechanism of the enzymatic hydrolysis and the relationship between the enzyme and the substrate structure (Yang *et al.,* 2011).

Nowadays, there are several complex laboratory techniques for the identification and quantification of sugars generated during enzymatic hydrolysis processes, such as gas chromatography, high performance liquid chromatography, and enzymatic methods, even though the latter are generally applied for the quantification of a single type of sugar (Sánchez-Mata *et al.,* 2002; Karkacier *et al.,* 2003). These techniques are very precise and considered as a reference but they are

slow, expensive, destructive, and require skilled labor to be conducted.

Over the last few years, several electrochemical-based techniques have been raising and nowadays they are showing promising results for the identification of chemical compounds in an easy, rapid, non-destructive, and online way. In this regard, EIS is one of the most remarkable ones. This technique allows the analysis of the properties of the materials by a successive application of alternate electric signals at different frequencies (sinusoidal voltage or current) in the test sample, the subsequent registration of the current or voltage responses within an electrochemical cell and the calculation of the impedance value for each signal (Bard and Faulkner, 2001; Barsoukov and Macdonald, 2005). EIS has been successfully applied in several fields such as medicine (MacRae and Esrick, 1992; Piccoli *et al.,* 2002; Nescolarde *et al.,* 2004), materials science and engineering (Pan *et al.,* 2003; Prabakar and Mallikarjum, 2007; Cen *et al.,* 2015), water (Houssin *et al.,* 2010) and environmental engineering (Rosborg and Pan, 2008). EIS has also been widely applied in food engineering: study of salt levels in food products (García-Breijo *et al.,* 2008; Masot *et al.,* 2010; Karásková *et al.,* 2011; Alcañiz *et al.,* 2012) quality control of fish (Fernández-Segovia *et al.,* 2012; Fuentes *et al.,* 2013; Pérez-Esteve *et al.,* 2014) and meat products (Labrador *et al.,* 2010; De Jesús *et al.,* 2014), and novel food processes (Wu *et al.,* 2008; Rizo *et al.,* 2013).

In these electrochemical techniques, an appropriate statistical treatment of the obtained data becomes fundamental because of its large size. In this sense, Principal Components Analysis (PCA) and Partial Leasts Squares (PLS) are quite usual and efficient but nowadays Artificial Neural Networks (ANNs) have been raised as very promising and alternative methods to conduct sample classifications and pattern recognition (Bishop, 1995). These methods are called neural networks because of their similarity to the way the human brain processes information (Grossberg, 1973). ANNs, as a biological brain, have a set of neurons linked together in a complex way and are able to treat

information in a multifunctional process. In addition, ANNs are able to learn in their training process in order to improve themselves and find the optimal conditions to work showing high flexibility and adaptive capacity. In addition, ANNs are being used in a wide range of applications such as electronic noses (Llobet *et al.,* 1999; Brezmes *et al.,* 2005) and tongues (Moreno-Barón *et al.,* 2006; Gil *et al.,* 2011) and they are showing very interesting prediction models in several fields such as water (Ibáñez-Civera *et al.,* 2011), food (García-Breijo *et al.,* 2013) and the environment (Laguarda-Miró *et al.,* 2012; Martínez-Gil *et al.,* 2013).

According to this, the aim of the present work is to study the suitability of EIS-based techniques to identify and quantify fermentable sugars present in the enzymatic saccharification process of pineapple wastes for bioethanol production by an optimized prediction system.

EXPERIMENTAL SECTION

Raw Material and Sample Preparation

Pineapple fruits selection (MD-2 cultivar, Extra Sweet or Golden Sweet) was based on external factors such as the absence of injuries, ripeness and weight. In order to prepare the samples, pineapples were first washed in a sodium hypochlorite solution (0.1%) for 5 min. Next, the crown was removed, and the pulp was separated from the rest of the fruit by using a pineapple cutter. Peel and core (waste) were cut into smaller pieces and grinded in a blender (Solac Inox Professional 1000 W Mixer). The resulting product was then frozen and kept at −22 °C until the experiments were conducted.

Electrochemical Impedance Spectroscopy Equipment

EIS measurements were carried out using a system developed by the Group of Electronic Development and Printed Sensors (GED + PS) belonging to the Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) at the Universitat Politècnica de València (UPV). This system consists of a device called AVISPA (Advanced Voltammetry, Impedance Spectroscopy & Potentiometry Analyzer)

(Figure 3.5.1.) associated with a specific software application that is able to apply different sinusoidal voltage signals with amplitudes up to 1 Vpp and frequency sweep from 0.01 Hz to 10 MHz using up to 32 current scales. The hardware consists of an Altera Cyclone II EP2C5T144C8N Field Programmable Gate Array (FPGA), clocked at 100 MHz, a 12-bit THS5661A Digital-to-Analog Converter (DAC), two identical ADS6125 12-bit Analog-to-Digital Converters (ADC), and various analog blocks to adapt signals to the required levels. It also contains hardware to be able to select 32 current scales, by means of various shunt resistors, to increase the sensitivity of the current measurement.

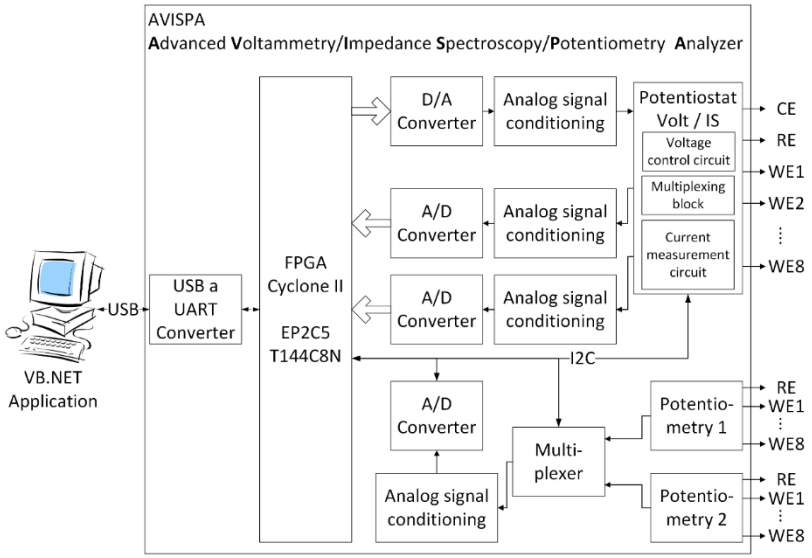

Figure 3.5.1. Block diagram of the AVISPA device.

The user can configure, by means of the software, the start and end frequency, the number of frequencies of the sweep and the amplitude of the sine wave to be generated. The user also has the option to fix the currents scale, or let the software choose the appropriate current scale at each measurement dynamically: if the values are below 20% or above 80% of the full range of the ADC, the software selects a higher or lower shunt resistor, respectively.

Once the measurement is started, the software calculates the digital values to be sent to the DAC, using a previously generated calibration file, and sends the data to a memory block inside the FPGA. Once the FPGA receives the last byte, it starts to generate the signal and acquire the data of the two ADCs simultaneously, which are written to two separate memory blocks. Once the signal generation and acquisition stage has been finished, the FPGA sends the obtained data to the PC software, where the digital data is converted to analog values by the use of a calibration file. The software calculates modulus and phase values and plots these values into a graph. This is repeated for each of the frequencies in the sequence obtaining a frequency response plot of the sample.

The sine wave generated is formed by 1000 points per cycle wherever allowed, taking into account the generated frequency and the working frequency of the FPGA. The worst case scenario is the generation of a 10 MHz signal that can only contain 10 points per cycle due to the clock speed of the FPGA at 100 MHz.

Electrochemical Impedance Spectroscopy Sensor

Previous studies suggested using non-oxidizable materials instead of oxidizable ones (e.g., Cu, Co, Ni, Ag) due to their rust resistance and easy handling, avoiding complex cleaning treatments of the electrodes (Sierra *et al.,* 2008; Martínez-Gil *et al.,* 2013). Moreover stainless steel was selected among other non-oxidizable materials (e.g., Pt, Au, Ir, Rh) because of a clear economic motivation and its successful and extended use in food industry applications (García-Breijo *et al.,* 2008; Labrador *et al.,* 2010; Rizo *et al.,* 2013). A double needle electrode (working and counter electrodes) composed of two parallel stainless steel needles 1.5 cm long and 1 mm in diameter, separated by a distance of 1 cm was used (Figure 3.5.2.). The plastic frame containing the needles was designed with a 3D printer (EKOCYCLE™ Cube®, Cubify 3DSYSTEMS®) and fixed with an epoxy kit (RS 199-1468). This design keeps both a constant the separation between the needles and also a constant electrode surface in contact with the samples during the

measurements as protects the electrical connections to transmit information to the device. The specific design of the sensor assures that the distance between electrodes is enough to consider a stable electric field preventing polarization effects. In addition, the use of parallel electrodes compared to other kind of designs (e.g., coaxial electrodes) generates a homogenous electric field distribution (Masot *et al.,* 2010); thus, an easier interpretation of the obtained measures is possible, so that the design is particularly appropriate for liquid samples.

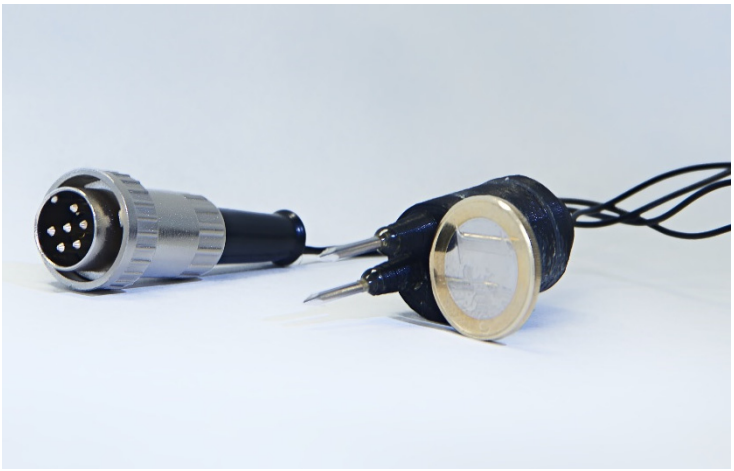

Figure 3.5.2. A view of the designed double needle electrode.

Electrochemical Impedance Spectroscopy Measurements

EIS measures were conducted in thawed pineapple waste samples with pH adjusted to 5 by adding a few drops of NaOH 1N (Panreac Química, S.L.U.). The penetration depth of the electrodes into the samples was 1 cm and it was a constant for all the assays. Analyses were made in triplicate at 25 °C by using a thermostatic bath (PolyScience®) and samples were selected randomly in order to avoid any memory effect in the measurements.

First of all, individual identification and quantification of sugars was carried out taking into account that the absence of added sugars in some specific samples did not mean the absence of endogenous

sugars in the raw material, which was considered as a baseline for these determinations. In addition, the presence or effect of any potential interfering compound in the samples was negligible due to the use of the same homogenous pineapple waste for all the analyses. Previous works in this research line determined the behavior of the existing sugars along the enzymatic hydrolysis in pineapple samples (Conesa *et al.,* 2014). Thus, the concentration range for each sugar was selected attending to these results. Accordingly, seven different concentrations were added to the pineapple waste samples and then analyzed for each studied sugar: 0 g/L, 5 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, and 50 g/L for glucose, and 0 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, and 30 g/L for sucrose and fructose. Analyses were conducted in triplicate for a total of 63 samples (189 analyses).

Next, identification and quantification of combined sugars was conducted. In order to assess the ability of EIS to identify and quantify combinations of three sugars, a total of 81 pineapple waste samples (241 analyses) were prepared by mixing the three studied sugars (glucose, fructose and sucrose) at three different added concentrations (0, 25 and 50 g/L).

Once the samples were thermostated and the AVISPA device was ready, EIS measurements started by placing the double needle electrode into the assayed sample. Then, the system carried out the procedure described in Electrochemical Impedance Spectroscopy Equipment Section in order to instantly show the modulus and phase of the signal on the PC screen and compile the data into the corresponding file for further analyses.

Statistical Analysis

PCAs were carried out with data obtained from the samples in order to assess the feasibility of the EIS technique to discriminate among different sugar concentrations both individually and in combination. PCAs were performed using just the specific impedance modulus and phase data obtained in the frequency range in which the sensor

showed the highest sensitivity. In addition, PLS analyses were also carried out to create predictive models for each studied sugar from their respective EIS measurements. According to the literature and previous studies in this research line, PLS prediction models were created using two series of the experimental data (66% of the data for the calibration set). The model was then validated with the remaining series of experimental data (34% for the validation set) (Martínez-Gil *et al.,* 2013; Hastie *et al.,* 2009). The accuracy was given by the root mean square error of prediction (RMSEP) and the coefficient of determination (R^2). All multivariate analyses were performed using SOLO© (Eigenvector Research, Inc., Manson, WA, USA).

A commercial ANN software (Alyuda Neurointelligence 2.2©, Alyuda Research Inc., Los Altos, CA, USA) was used throughout this study in order to create alternative, flexible and more adaptive predictive models to PLS (Brezmes *et al.,* 2005; Laguarda-Miró *et al.,* 2012; García-Breijo *et al.,* 2013). Multi-layer feed forward neural networks and a single hidden layer ANN structure were selected and on-line back propagation training algorithms were used for fitting the network.

The optimal network topology was selected by developing several artificial neural network structures in order to determine the number of neurons of the hidden layer. Similarly, several trials suggested the selection of logistic-type transfer functions for the output layer neurons and hyperbolic tangent-type functions for the hidden nodes. Random data division was used by Alyuda Neurointelligence 2.2© in order to select the samples for training (70%), validation (15%) and test (15%) data (Ibáñez-Civera *et al.,* 2011; Laguarda-Miró *et al.,* 2012; García-Breijo *et al.,* 2013). In addition, overfitting was avoided by using proportional number of nodes in the network architecture (Del Brío and Molina, 2001), cross validation and early-stopping in the training phase, so that the difference between training and validation mean square errors was minimal. As described before, the accuracy of the model was given by the root mean square error of prediction (RMSEP)

and the coefficient of determination (R^2) in the case of numerical prediction models. On the other hand, when classification models were developed, the accuracy of the model was given by the correct classification rate (CCR%) and the confusion matrix.

RESULTS AND DISCUSSION

Individual Identification and Quantification of Sugars

The AVISPA device generated 200 data per analyzed sample corresponding to the modulus and phase of the 100 analyzed frequencies in each test. Analyses were carried out independently for each fermentable sugar at the above mentioned different concentrations. As shown in Figure 3.5.3., the frequency range showing the highest sensitivity to sucrose concentration was the one between 5.96×10^5 Hz and 7.47×10^5 Hz. Glucose and fructose showed their respective highest sensitivity in similar ranges. Consequently, this was the selected frequency range for data treatment and mathematical modeling.

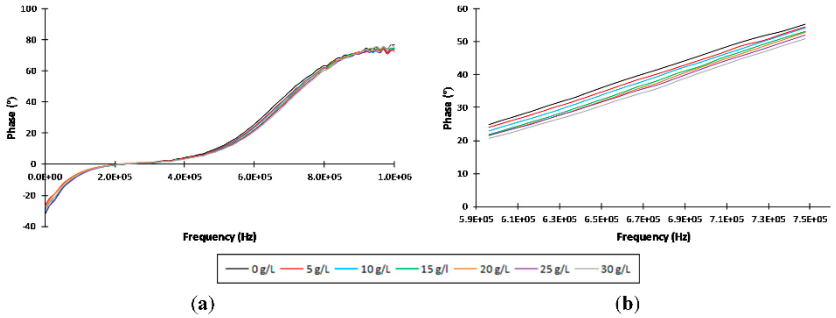

Figure 3.5.3. Averaged phase values of the impedance spectra of different sucrose concentration measurements for (a) the entire analyzed frequency range and (b) the selected range for data treatment (5.96×10^5 Hz–7.47×10^5 Hz).

PCA analyses showed a high percentage of the total variability (>99%) being explained just with the first two principal components for all the studied sugars. Specifically, variability for fructose data was explained up to 97.93% by the first component (PC1) and component 2 (PC2)

explained the remaining 1.86% of the total variability. For sucrose, PC1 and PC2 explained 96.60% and 2.82% respectively of total variability (Figure 3.5.4.). Finally, glucose variability was explained up to 97.57% and 2.19% by PC1 and PC2, respectively. Therefore, the results indicate that these concentrations can be discriminated with only one main component in the studied ranges.

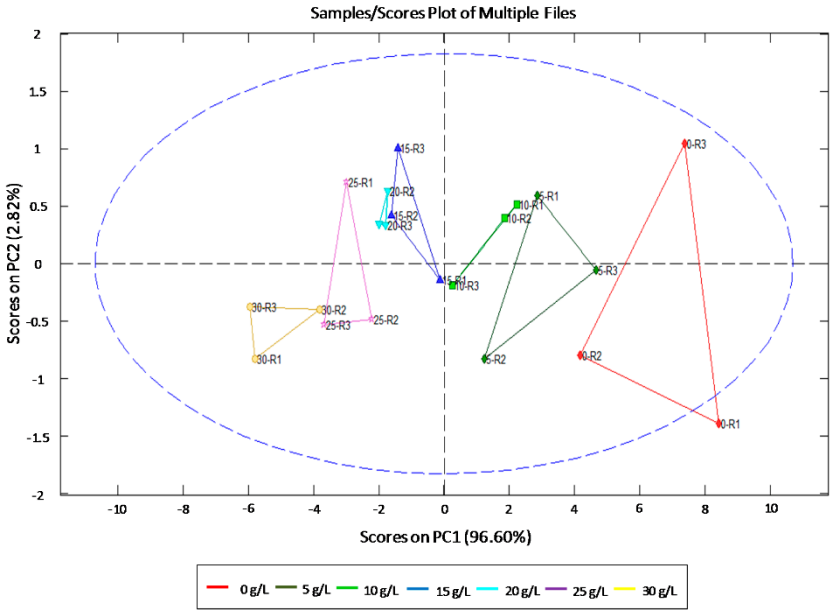

Figure 3.5.4. Principal component analysis (PCA) for the studied sucrose concentrations. R1–3: average of each replicate. The blue ellipsis indicates 95% confidence level.

Since PCA analysis showed that EIS analyses with the double needle sensor can discriminate different concentrations of glucose, fructose and sucrose, a PLS analysis was performed to predict these concentrations from EIS measures.

As shown in Table 3.5.1., good correlations were obtained for all the analyzed sugars with $R^2 = 0.958$ or above and RMSEP = 2.272 or below. These results demonstrated an accurate fitting between predicted and experimental values and, consequently, the obtained models can be considered statistically valid. The best correlation was obtained for

sucrose as shown in Figure 3.5.5. Moreover, the PLS analysis for sucrose showed that a reliable mathematical model can be obtained using just one latent variable. Thus, the phase data for just one frequency is enough to quantify sucrose in a sample. Consequently, the prediction model could be very simple and accurate.

Table 3.5.1. Statistic values of Partial Least Square (PLS) discriminant analysis for the quantification of the studied fermentable sugars. (R^2: coefficient of determination; RMSEP: Root Mean Square Error of Prediction; LV: Latent Variables.)

Sugars	Statistics		
	R^2	RMSEP	LV
Glucose	0.979	2.272	3
Fructose	0.958	2.103	2
Sucrose	0.983	1.576	1

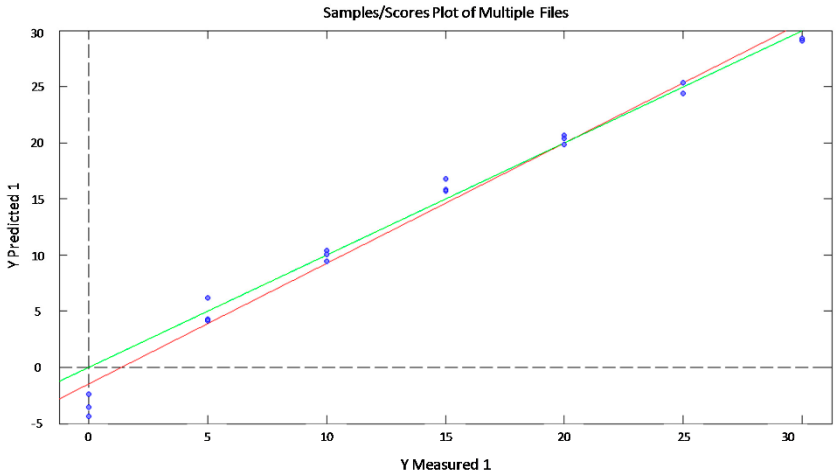

Figure 3.5.5. Correlation plot between experimental and predicted values of sucrose (g/L) by PLS statistical model (red line) and ideal behavior (green line).

Consequently, the obtained results demonstrate that EIS is a robust and reliable methodology to quantify the concentration of the three main fermentable sugars in the studied ranges.

However, as an alternative method to PLS analyses, Artificial Neural Networks-based models (ANN) were designed using the same data set. In order to do this, different net architectures were tested for each analyzed sugar to optimize the fitting between the EIS data and the expected response. Thus, a (16-8-1) architecture was designed for glucose that means 16 input nodes connected to an 8-node hidden layer and a final output layer. For fructose and sucrose, (16-21-1) and (16-2-1) architectures were selected. The training phase of these ANN generated mathematical models that are summarized in Table 3.5.2. The obtained models showed determination coefficients higher than 0.95 and RMSEP lower than 3.96. Figure 3.5.6. shows the regression line obtained by ANN for sucrose. These results demonstrate that the designed ANN model generates noticeable results with sufficient accuracy and reliability for modeling sugar concentration depending on the EIS response.

Table 3.5.2. Artificial neural network (ANN) results for the studied fermentable sugars (R^2: coefficient of determination; RMSE: Root Mean Square Error).

		R^2	RMSE
Glucose	**Training**	0,99	1,41
	Validation	0,88	3,39
	Test	0,95	3,96
Fructose	**Training**	0,96	1,39
	Validation	0,99	0,27
	Test	0,95	1,63
Sucrose	**Training**	0,99	0,09
	Validation	0,88	1,26
	Test	0,99	0,40

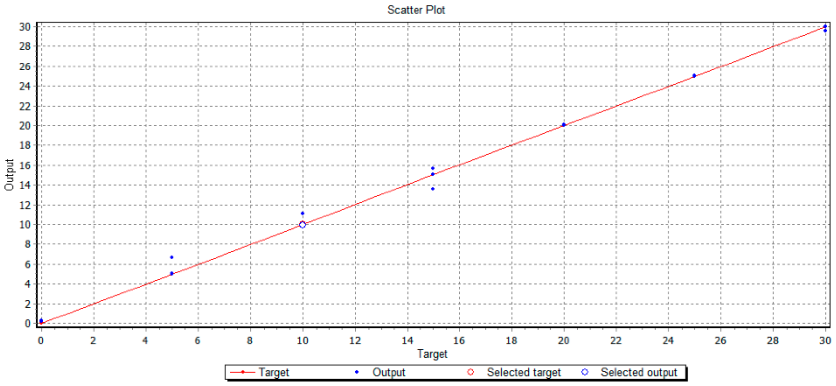

Figure 3.5.6. Regression line plot of the obtained ANN model for the studied sucrose concentrations (g/L).

Considering R^2 and RMSEP parameters for both PLS and ANN models, it follows that the fitting and accuracy of the models are quite similar (Table 3.5.1. and Table 3.5.2.). However, slight differences in RMSEP are observed, so that a better fit for fructose and sucrose is obtained by ANN as glucose is better fit by PLS.

Finally, a PCA analysis was performed to assess the ability of EIS to discriminate different fermentable sugars, comparing individual electrochemical responses in the studied frequency range (Figure 3.5.7). It is observed that the first two components explain a high percentage of the total variability (99.74%). Specifically, the first component (PC1) explains 92.66% and component 2 (PC2) explains the remaining 7.08% of the total variability. Therefore, the obtained results indicate that all three fermentable sugars can be easily identified with just one principal component using the electrochemical data in the studied ranges. It means that the phase data for just one frequency in the studied frequency range is enough to identify the kind of sugar in a sample. Consequently, the prediction model could be very simple and accurate.

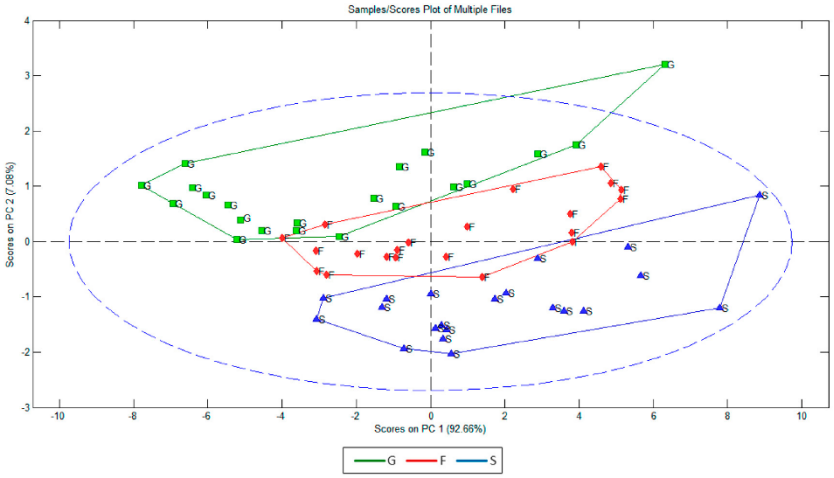

Figure 3.5.7. Principal component analysis (PCA) for the studied fermentable sugars: glucose (G), fructose (F) and sucrose (S).

Combined Identification and Quantification of Glucose, Sucrose and Fructose

Once the EIS technique and double needle electrode was demonstrated to be sensitive to the presence of fermentable sugars in pineapple waste samples, the next step was to assess its sensitivity to the combined presence of the three studied sugars. In order to do this, mixtures of these three sugars at three different concentrations (0 g/L, 25 g/L and 50 g/L) were analyzed in pineapple waste samples.

As in the previous cases, the AVISPA device generated 200 electrochemical data per assayed sample in the form of module and phase corresponding to 100 frequencies in the selected ranges. As happened before, phase data was the one showing the best sensitivity although in this specific case, the highest sensitivity was achieved in two different frequency ranges (1 Hz–1.41 × 10^5 Hz) and (5.76 × 10^5 Hz–8.48 × 10^5 Hz).

Then, a PCA analysis was carried out in order to determine whether different combinations of the three fermentable sugars could be discriminated by EIS in the two studied frequency ranges. The result of

this analysis showed that 97.29% of the total variability was explained with just two principal components as PC1 and PC2 explained 77.78% and 19.51% of the variability. Therefore, these results indicate that mixtures of the three studied sugars can be discriminated with just two principal components.

Next, PLS analyses were performed to generate a mathematical model able to predict concentrations of combined fermentable sugars. In order to do this, different PLS were conducted to check the capability of the system to detect and quantify each fermentable sugar from different mixtures of sugars in pineapple waste samples. The obtained results (R^2 > 0.841 RMSEP < 8.23) indicate that PLS modeling for the combination of fermentable sugars is slightly lower than the ones shown in the previous cases. However, these results are not far from those obtained in other scientific studies in similar fields [39,41].

Therefore, ANN models were studied as an alternative to improve accuracy of the ones obtained by PLS. In this specific case, (11-38-9) was the selected ANN architecture to predict the combined concentration of the three fermentable sugars in pineapple waste samples.

The obtained ANN-based mathematical models generated very promising results showing CCR% values higher than 93.443% and confusion matrices like the ones shown in Table 3.5.3. for the combined quantification of the studied sugars. These results demonstrate that ANN-based models are remarkable complements to PLS models for predicting the combined concentrations of fermentable sugars in pineapple waste samples via EIS determinations, generating significant results with sufficient accuracy and reliability. Future studies will focus on studying the suitability of this EIS-based technique to monitor industrial saccharification and fermentation processes.

Table 3.5.3. Confusion matrices for combined sugars quantification.

Glucose: *Mean CCR% = 93.443%*

Training				Validation				Test				Overall			
Target output:	25	50	0	Target output:	25	50	0	Target output:	25	50	0	Target output:	25	50	0
25	16	0	0	25	1	1	0	25	2	0	0	25	19	1	0
50	0	16	0	50	1	3	0	50	0	4	0	50	1	23	0
0	0	0	13	0	1	0	1	0	1	0	1	0	2	0	15

Fructose: *Mean CCR% = 96.721%*

Training				Validation				Test				Overall			
Target output:	25	50	0	Target output:	25	50	0	Target output:	25	50	0	Target output:	25	50	0
25	12	0	0	25	2	0	0	25	3	0	0	25	17	0	0
50	0	16	0	50	1	2	1	50	0	2	0	50	1	20	1
0	0	0	17	0	0	0	2	0	0	0	3	0	0	0	22

Sucrose: *Mean CCR% = 100%*

Training				Validation				Test				Overall			
Target output:	25	50	0	Target output:	25	50	0	Target output:	25	50	0	Target output:	25	50	0
25	15	0	0	25	4	0	0	25	4	0	0	25	23	0	0
50	0	13	0	50	0	2	0	50	0	2	0	50	0	17	0
0	0	0	17	0	0	0	2	0	0	0	2	0	0	0	21

CONCLUSIONS

In the current energy outlook, the search for alternatives to fossil fuels is of strategic importance. In this sense, second-generation bioethanol production from agricultural and industrial food waste is a strategy that must be taken into account. Within this option, pineapple has a remarkable potential use due to its extensive worldwide market, the generation of an important waste volume in its industrial processing, and the bio-chemical composition of these wastes.

This work introduces an EIS-based methodology for monitoring and managing the concentration of sugars in the most complex phase for second generation bioethanol production: the enzymatic hydrolysis. In order to do this, an AVISPA device has been used as it is able to generate and receive EIS signals from an especially designed double needle sensor made of stainless steel. Statistical treatment of the data allowed to build reliable and robust ANN-based mathematical models (mean CCR% > 93.443%) to identify and quantify the main fermentable

sugars (glucose, fructose and sucrose) in pineapple waste samples both individually and jointly. Furthermore, this methodology is easy, rapid, non-destructive, and in-situ. Thus, it can be considered as a promising alternative to the traditional laboratory techniques for enzymatic hydrolysis monitoring and management in second-generation bioethanol production not just from pineapple wastes but also from many other lignocellulosic sources.

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AUTHOR CONTRIBUTIONS

C.C. and L.S. carried out the electrochemical measurements, E.L. developed the electronic device and the sensor, E.G.-B. participated in the data analysis and interpretation, and P.F. participated in the coordination of the study. N.L.-M. conceived and also coordinated the study. Finally, C.C. and N.L.-M. wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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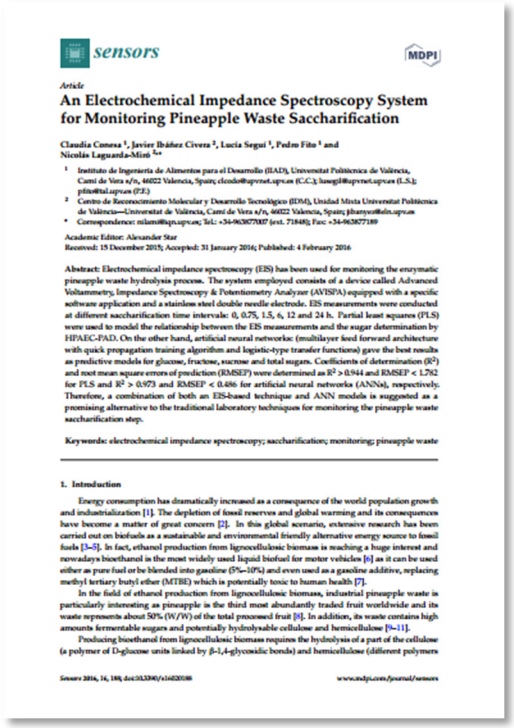

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An Electrochemical Impedance Spectroscopy System for Monitoring Pineapple Waste Saccharification

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Abstract: Electrochemical impedance spectroscopy (EIS) has been used for monitoring the enzymatic pineapple waste hydrolysis process. The system employed consists of a device called Advanced Voltammetry, Impedance Spectroscopy & Potentiometry Analyzer (AVISPA) equipped with a specific software application and a stainless steel double needle electrode. EIS measurements were conducted at different saccharification time intervals: 0, 0.75, 1.5, 6, 12 and 24 h. Partial least squares (PLS) were used to model the relationship between the EIS measurements and the sugar determination by HPAEC-PAD. On the other hand, artificial neural networks (multilayer feed forward architecture with quick propagation training algorithm and logistic-type transfer functions) gave the best results as predictive models for glucose, fructose, sucrose and total sugars. Coefficients of determination (R^2) and root mean square errors of prediction (RMSEP) were determined as $R^2 > 0.944$ and RMSEP < 1.782 for PLS and $R^2 > 0.973$ and RMSEP < 0.486 for artificial neural networks (ANNs), respectively. Therefore, a combination of both an EIS-based technique and ANN models is suggested as a promising alternative to the traditional laboratory techniques for monitoring the pineapple waste saccharification step.

Keywords: electrochemical impedance spectroscopy; saccharification; monitoring; pineapple waste

1. Introduction

Energy consumption has dramatically increased as a consequence of the world population growth and industrialization [1]. The depletion of fossil reserves and global warming and its consequences have become a matter of great concern [2]. In this global scenario, extensive research has been carried out on biofuels as a sustainable and environmental friendly alternative energy source to fossil fuels [3–5]. In fact, ethanol production from lignocellulosic biomass is reaching a huge interest and nowadays bioethanol is the most widely used liquid biofuel for motor vehicles [6] as it can be used either as pure fuel or be blended into gasoline (5%–10%) and even used as a gasoline additive, replacing methyl tertiary butyl ether (MTBE) which is potentially toxic to human health [7].

In the field of ethanol production from lignocellulosic biomass, industrial pineapple waste is particularly interesting as pineapple is the third most abundantly traded fruit worldwide and its waste represents about 50% (W/W) of the total processed fruit [8]. In addition, its waste contains high amounts fermentable sugars and potentially hydrolysable cellulose and hemicellulose [9–11].

Producing bioethanol from lignocellulosic biomass requires the hydrolysis of a part of the cellulose (a polymer of D-glucose units linked by β-1,4-glycosidic bonds) and hemicellulose (different polymers

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An Electrochemical Impedance Spectroscopy System for Monitoring Pineapple Waste Saccharification

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ABSTRACT: Electrochemical impedance spectroscopy (EIS) has been used for monitoring the enzymatic pineapple waste hydrolysis process. The system employed consists of a device called Advanced Voltammetry, Impedance Spectroscopy & Potentiometry Analyzer (AVISPA) equipped with a specific softwabre application and a stainless steel double needle electrode. EIS measurements were conducted at different saccharification time intervals: 0, 0.75, 1.5, 6, 12 and 24 h. Partial least squares (PLS) were used to model the relationship between the EIS measurements and the sugar determination by HPAEC-PAD. On the other hand, artificial neural networks: (multilayer feed forward architecture with quick propagation training algorithm and logistic-type transfer functions) gave the best results as predictive models for glucose, fructose, sucrose and total sugars. Coefficients of determination (R^2) and root mean square errors of prediction (RMSEP) were determined as $R^2 > 0.944$ and RMSEP < 1.782 for PLS and $R^2 > 0.973$ and RMSEP < 0.486 for artificial neural networks (ANNs), respectively. Therefore, a combination of both an EIS-based technique and ANN models is suggested as a promising alternative to the traditional laboratory techniques for monitoring the pineapple waste saccharification step.

KEYWORDS: electrochemical impedance spectroscopy; saccharification; monitoring; pineapple waste

INTRODUCTION

Energy consumption has dramatically increased as a consequence of the world population growth and industrialization (Ma *et al.,* 2008). The depletion of fossil reserves and global warming and its consequences have become a matter of great concern (Betiku and Taiwo, 2015). In this global scenario, extensive research has been carried out on biofuels as a sustainable and environmental friendly alternative energy source to fossil fuels (Cadoche and López, 1989; Duff and Murray, 1996; Binod *et al.,* 2010). In fact, ethanol production from lignocellulosic biomass is reaching a huge interest and nowadays bioethanol is the most widely used liquid biofuel for motor vehicles (Sarkar *et al.,* 2012) as it can be used either as pure fuel or be blended into gasoline (5%–10%) and even used as a gasoline additive, replacing methyl tertiary butyl ether (MTBE) which is potentially toxic to human health (Song *et al.,* 2006).

In the field of ethanol production from lignocellulosic biomass, industrial pineapple waste is particularly interesting as pineapple is the third most abundantly traded fruit worldwide and its waste represents about 50% (W/W) of the total processed fruit (Ketnawa *et al.,* 2012). In addition, its waste contains high amounts fermentable sugars and potentially hydrolysable cellulose and hemicellulose (Nigam, 1999; Tanaka *et al.,* 1999; Ruangviriyachai *et al.,* 2010).

Producing bioethanol from lignocellulosic biomass requires the hydrolysis of a part of the cellulose (a polymer of D-glucose units linked by β-1,4-glycosidic bonds) and hemicellulose (different polymers of pentoses, hexoses and uronic acids) into fermentable sugars by fungal enzymatic complexes: cellulases and hemicellulases, respectively (Scheller and Ulvskov, 2010). However, saccharification is the most complex step in the bioethanol production process (Taherzadeh and Karimi, 2007). In fact, the mechanism of the enzymatic hydrolysis and

the relationships between the enzyme and the substrate structure are particularly complex (Yang *et al.,* 2011). Moreover, several authors have suggested that some sugars released during saccharification could inhibit the hydrolysis reaction, specifically cellobiose and to a lesser extent glucose (Eklund and Zacchi, 1995; Hari Krishna and Chowdary, 2000; Kádár *et al.,* 2004; Linde *et al.,* 2007). Therefore, monitoring enzymatic saccharification is of capital importance for maximizing sugar yields and ethanol production.

Nowadays, different chromatographic techniques such as high pressure liquid chromatography (HPLC) using various columns and detectors (Stefanssom and Westerlund, 1996; Raessler, 2011) or gas chromatography-mass spectrometry (GC-MS) (Terrab *et al.,* 2001; Sanz *et al.,* 2004) have been successfully used for carbohydrate analyses. Specifically, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is considered as the most highly sensitive and reliable method for all sugars (LaCourse *et al.,* 2002). However, these conventional techniques are destructive, time consuming, require specialized sample preparation with hazardous chemicals and are labor intensive (Jie *et al.,* 2014). In contrast, electrochemical impedance spectroscopy (EIS) is a non-destructive, rapid, simple, real-time analysis methid, with no use of toxic reagents and with relatively reduced operational costs. EIS allows the analysis of the properties of materials and systems by applying alternate electric signals (voltage or current) of different frequencies to them and measuring the corresponding electric output signal (current or voltage) (Bard and Faulkner, 2001; Barsoukov and Ross, 2005). EIS has been shown to be a powerful technique for monitoring the effects of industrial processing methods such as heat treatments (Fuentes *et al.,* 2014), freezing (Ohnishi *et al.,* 2004) or cold injuries (Wu *et al.,* 2008) on agricultural products (fruits and vegetables).

For further data processing, EIS requires powerful mathematical and statistical tools in order to get robust and reliable responses from the

huge amount of data generated. This is the specific case of principal component analysis (PCA) and PLS that have been shown as excellent mathematical tools for this kind of data (Ulrich *et al.,* 2007; Olivati *et al.,* 2009), but also the case of ANNs that are widely used to perform sample classification (Kumar and Buchheit, 2008; Eddahech *et al.,* 2012). In fact, ANNs outperform these methods due to their enormous flexibility and adaptive capacity, accurate fit to nonlinear systems and ability to learn from their mistakes. In addition, ANN-based classification/modeling systems are clear, easy to use, have a low computational burden and their algorithms are easily implementable on a PC or microprocessor. Potential applications of ANNs in microprocessors are of particular interest as they allow the design of portable devices that can in situ analyses, with great flexibility, a wide range of applications, easy operation and low power consumption. For such applications, (Kasuba, 1993) other authors (Rajasekaran and Vijayalakshmi Pai, 2004; García-Breijo *et al.,* 2011) have developed simplified ANN versions by simplifying the architecture and learning equations and further reducing the computational costs. Thus, the system has less computational requirements, being faster to program and easier to operate, while offering almost the same reliability (García-Breijo *et al.,* 2013).

In previous studies, a system consisting of a stainless steel double needle electrode associated with specific electronic equipment was designed. This device was called Amperometry, Voltammetry, Impedance Spectroscopy and Potentiometry Analyzer (AVISPA) and it allowed the implementation of EIS analyses on previously prepared samples (Conesa *et al.,* 2015). This previous step in this research showed that a combination of both EIS-based technique and the designed ANN could be considered a promising alternative to traditional laboratory techniques for identifying and quantifying glucose, fructose and sucrose added to pineapple wastes. Therefore, this study aims to verify that natural enzymatic saccharification of pineapple waste can be accurately monitored by using an EIS system.

EXPERIMENTAL

Sample Preparation

Pineapple (*Ananas comosus* L. cv. "MD-2") fruit selection was based on the visual absence of external defects. Next, a NaClO (0.1%) solution was used to wash the pineapples. Then, the crown was removed and the pulp was separated from the rest of the fruit using a pineapple cutter. Next, peel and core (waste) were cut into smaller pieces and crushed in a blender (Avance Collection Blender HR2097/00 800 W, Philips, Amsterdam, The Netherlands). Finally, the mashed product was frozen and stored at −22 °C. Enzymatic hydrolysis was carried out by adding 0.4% (w/v) of cellulase (1.13 U/mg solid, Sigma-Aldrich Química SL, Madrid, Spain) and 0.4% (w/v) of hemicellulase (1.5 U/mg solid, Sigma-Aldrich Química SL, Madrid, Spain) from *Aspergillus niger* to 40 g of thawed pineapple waste in a 100 mL glass beaker. Samples were placed in a thermostatic bath (Precisdig, JP SELECTA S.A., Barcelona, Spain) at 40 °C for 24 h.

Electrochemical Impedance Spectroscopy Equipment

EIS measurements were carried out using a measurement system developed by the Group of Electronic Development and Printed Sensors (GED+PS) belonging to the Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) at the Universitat Politècnica de València (UPV). This system consists of a device called Advanced Voltammetry, Impedance Spectroscopy & Potentiometry Analyzer (AVISPA) associated to a specific software application that is able to apply different sinusoidal voltage signals with amplitudes up to 1 Vpp and frequency sweep from 0.01 Hz to 10 MHz using up to 32 current scales (Conesa *et al.,* 2015). It means that the frequency range, the number of the sweep frequencies, the current scale and the amplitude of the sine wave applied to the sensor can be completely configured by means of the software.

The applied sensor was the same as the one previously used in the identification and quantification of sugars added to pineapple waste

(Conesa *et al.,* 2015), consisting of a 1.5 cm in length and 1 mm in diameter double needle electrode (working and counter electrodes) made of stainless steel and encapsulated in epoxy resin to assure a constant separation of 1 cm between the needles.

Electrochemical Impedance Spectroscopy Measurements

EIS measures were conducted in saccharified pineapple waste samples at different time intervals: 0, 0.75, 1.5, 6, 12 and 24 h. The sensor was completely introduced into the samples (1.5 cm) in order to assure a constant contact surface between the electrode and the samples. A thermostatic bath (PolyScience®, Niles, IL, USA) was used to conduct assays in triplicate at 25 °C for an total of 54 EIS measurements (Figure 3.6.1.).

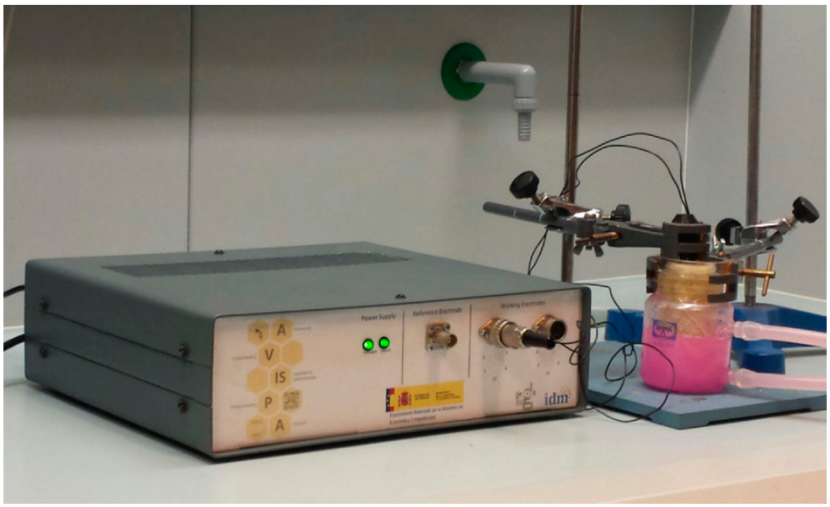

Figure 3.6.1. A view of the experimental set-up.

Memory effects were minimized by a random selection of the analyzed samples. The use of the same homogenous pineapple waste for all the analyses avoided any effect of potential interfering compounds in the samples. The AVISPA device started measuring the response plot of the applied EIS signal and sending it to a PC to calculate modulus and phase values and generate the corresponding plot. This step was

repeated for all the frequencies in the sequence in order to have a complete graph of the sample response.

High Performance Anion-Exchange Chromatography-Pulsed Amperometric Detection (HPAEC-PAD) Analyses

Sugars present in the liquid phase of the same pineapple waste samples were measured at identical time intervals as stated before by using an IC chromatograph system (Metrohm, Herisau, Switzerland) equipped with a 716 Compact module and an ICnet 2.0 software program for interpreting the results. A three-step PAD setting was applied with the following path intervals (ms) and potentials (V): t_1: $400/E_1$ = +0.05 (detection); t_2: $200/E_2$ = +0.75 (cleaning); t_3: $400/E_3$ = −0.15 (regeneration). The column used was a Metrosep Carb 1 250/4.6 column (250 mmL × 4.6 mmID) coupled to a guard column. Analyses were done at 32 °C, 8.8 MPa, injection volume: 20 µL and using sodium hydroxide 0.1 M as the mobile phase (1 mL/min). Chromatographic measurements required filtration of the liquid (0.45 µm nylon filter) and dilution of the resulting filtered sample (1:2000 v/v in bidistilled water). High-purity (≥99%) standards of glucose, fructose and sucrose (Sigma-Aldrich Química SL) were used to prepare standard calibration curves (2.5, 5, 10, 15, 25 and 50 ppm). All the determinations were carried out in triplicate.

Statistical Analysis and Modeling

Statgraphics Centurion XVI® (Manugistics Inc.; Rockville, MD, USA) was used for analyzing the HPAEC-PAD results. Statistically significant differences between sugar yields during saccharification were determined using one-way analyses of variance (ANOVA) with Multiple Range Test (95% confidence level).

The electrochemical data were analyzed using multivariate techniques, applying the software SOLO© (Eigenvector Research, Inc., Manson, WA, USA). Principal Component Analyses (PCA) were carried out with data obtained from the specific impedance modulus and phase data obtained in the frequency range in which the sensor

showed the highest sensitivity. Partial Least Squares (PLS) were used to model the relationship between the array of dependent variables Y (EIS measurements) and the array of independent variables X (sugar determination by HPAEC-PAD). According to (Ulrich *et al.,* 2007; Hastie *et al.,* 2009), PLS prediction models were created using the 66% of the experimental data (calibration set) and the model was then validated with the remaining 34% (validation set). The accuracy was given by the root mean square error of prediction (RMSEP) and the coefficient of determination (R^2).

Alyuda Neurointelligence 2.2© (Alyuda Research Inc., Los Altos, CA, USA) was used to design and implement the ANN. This software enables users to select the main network parameters (e.g., the training algorithm type, the number of neurons in hidden layer and transference functions). It also randomly divides the experimental data into the following three sets: training (70%), validation (15%) and test (15%) data (Civera *et al.,* 2011; García-Breijo *et al.,* 2011; Laguarda-Miró *et al.,* 2012). The training data was used to compute the network parameters and the testing data, to ensure robustness of the network parameters. A proportional number of nodes in the network architecture (Laguarda-Miró *et al.,* 2012), cross validation and early-stopping in the training phase were used in order to avoid the "overfitting" phenomenon, so that the difference between training and validation mean square errors was minimal. Therefore, multi-layer feedforward networks with a single hidden layer were used to predict glucose, fructose, sucrose and total sugars evolution during saccharification process. Quick propagation training (a modified version of the back propagation algorithm), was selected for fitting the network (Priddy and Keller, 2005). The optimal network topology (architecture and number neurons in each hidden layer) was selected by testing several artificial neural network structures. Similarly, several trials suggested the selection of logistic-type transfer functions for both output layer neurons and hidden nodes. As described before, the performance of the designed ANN models was assessed on the basis

of RMSEP and R^2 between the predicted values of the network and the experimental data.

RESULTS AND DISCUSSION

Sugar Determination by HPAEC-PAD

Table 3.6.1. summarizes the evolution of the sugar profile of pineapple waste throughout the enzymatic treatment. To that end, sugars present in the hydrolyzed samples (0 h–24 h) were identified and quantified through the chromatograms obtained from the HPAEC-PAD analysis of the liquid phase (Figure 3.6.2.).

Table 3.6.1. Sugar profile of pineapple waste during saccharification at different time intervals (0 h to 24 h). Values correspond to the average of three replicates (Standard deviation).

Saccharification time (h)	Glucose (g/L)	Fructose (g/L)	Sucrose (g/L)	Total Sugars[1] (g/L)
0	33,5 (1,2)[a]	33,18 (1,1)[a]	28,1 (0,3)[e]	94,8 (0,3)[a]
0,75	36,4 (1,6)[b]	36,69 (0,15)[b]	20,6 (1,5)[d]	95,7 (0,2)[b]
1,5	36,1 (0,9)[b]	41,3 (0,5)[c]	19,0 (0,7)[c]	96,5 (1,4)[b]
6	44,3 (0,5)[c]	51,3 (1,1)[d]	9,4 (0,3)[b]	105,0 (0,9)[c]
12	47,63 (1,07)[d]	58,9 (1,2)[e]	0,0 (0,0)[a]	107,0 (0,7)[d]
24	48,8 (0,8)[d]	60 (2)[e]	0,0 (0,0)[a]	108 (3)[d]

[a,b,c,d,e] Similar lowercase letters indicate statistically homogeneous groups with a confidence level of 95%; [1] Total Sugars obtained by adding glucose, fructose and sucrose contents

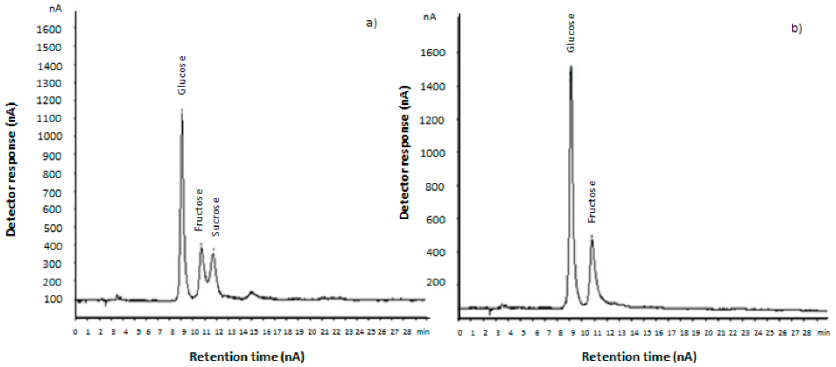

Figure 3.6.2. HPAEC-PAD chromatograms (dilution 1:2000 v/v) for (a) pineapple waste before saccharification (0 h); and (b) saccharified pineapple waste (24 h).

As shown in Table 3.6.1., glucose, fructose and sucrose were identified in the liquid phase of the pineapple waste before saccharification (0 h). Glucose and fructose were present in similar amounts, while sucrose concentration was slightly lower. These results are similar to those reported in (Krueger *et al.,* 1992; Abdullah and Hanafi, 2008).

The enzymatic treatment produced a statistically significant increase in the glucose (45%) and fructose (81%) contents, whereas the sucrose content decreased and eventually disappeared as the saccharification process proceeded. Specifically, glucose release is probably due to the action of the fungal cellulase complex that consists of three groups of enzymes: (1) the endoglucanases (EC 3.2.1.4.) act by randomly hydrolyzing the internal glycosidic linkages of the cellulose chain; (2) the cellobiohydrolases, also known as exoglucanases (EC 3.2.1.74), act on the ends of the chains, releasing glucose monomers, cellobiose and low molecular weight oligosaccharides; and (3) the β-glucosidases (EC 3.2.1.21) convert cellobiose to glucose (Goyal *et al.,* 1991; Ting *et al.,* 2009). On the contrary, the sucrose decrease and fructose increase would not be the result of the enzymatic action but rather of sucrose inversion (Ban-Koffi and Han, 1990), considering the acid pH of the medium and the fact that selected enzymes are not potentially capable of reversing sucrose. As a consequence, total sugars in the

hydrolyzed samples (24 h) increased by 24% compared to the original one (0 h).

Electrochemical Impedance Spectroscopy Measurements

For each analyzed sample, the AVISPA device generated 200 datasets corresponding to the modulus and phase of the 100 applied frequencies (between 1 and 10^6 Hz) at 0, 0.75, 1.5, 6, 12 h and 24 h since the saccharification process started. The graph of the modulus showed no relevant information, whereas the representation of the phase revealed that frequencies between 6.5×10^5 Hz and 7.0×10^5 Hz (6 frequency data) were the ones that showed the highest sensitivity to total sugars along the saccharification process (Figure 3.6.3.). Consequently, this frequency range was selected for further data treatment and mathematical modeling.

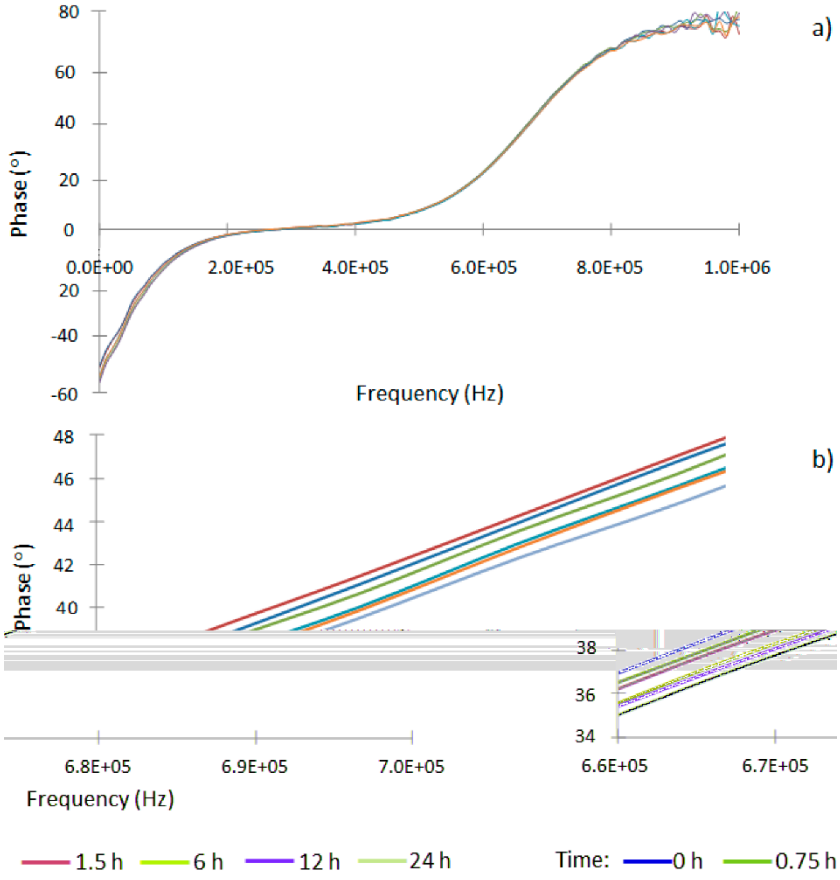

Figure 3.6.3. Phase data for pineapple waste saccharification at different time intervals (0 to 24 h) for (a) the complete analyzed frequency range; and (b) the studied frequency range (6.6×10^5 Hz–7.0×10^5 Hz).

PCA bi-dimensional graphic analyses showed a high percentage of the total variability (98.22%) being explained just with the first two components (Figure 3.6.4). The first component (PC1) and second component (PC2) explained 97.31% and 0.91% of the graphic variability, respectively. Therefore, the results indicate that variation of total sugars can be discriminated in the studied ranges with only one principal component. Obviously, this PC1 is directly correlated to the time variable in the saccharification process.

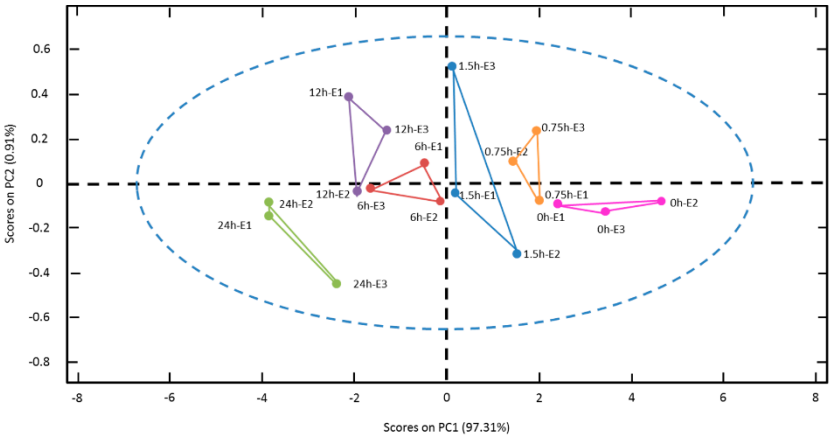

Figure 3.6.4. Principal component analyses (PCA) biplot for EIS phase data (6.6×10^5 Hz–7.0×10^5 Hz) during pineapple waste saccharification (0 to 24 h). R1-3: average of each replicate. The blue ellipsis indicates 95% confidence level.

Since the PCA analysis demonstrated the ability of the designed EIS system to discriminate total sugar concentrations, PLS analyses were performed to predict glucose, fructose and sucrose concentrations from EIS measurements. Based to the results, models for glucose, fructose, sucrose and total sugars were built in the range of 0 to 24 h. Meanwhile the time range used for sucrose was just from 0 to 12 h due to its exhaustion.

The obtained correlations for the studied sugars are shown in Table 3.6.2. These results demonstrated accurate fitting between experimental and predicted data. Consequently, the designed models can be considered statistically valid. Moreover the PLS analyses showed that just one latent variable is enough to generate reliable mathematical models for the three analyzed sugars. Consequently, the prediction models could be simple and accurate as just one frequency data is enough to quantify these sugars along the saccharification process.

Table 3.6.2. Statistical values of Partial Least Square (PLS) discriminant analysis for the quantification of the studied fermentable sugars for EIS phase data from 6.6×10^5 Hz–7.0×10^5 Hz. (R^2: coefficient of determination; RMSEP: Root Mean Square Error of Prediction; LV: Latent Variables).

Sugars	Statistics		
	R^2	RMSEP	LV
Glucose	0.955	1.306	1
Fructose	0.970	1.782	1
Sucrose	0.975	1.645	1
Total Sugars	0.944	1.353	2

In the present work, artificial neural networks (ANNs) were studied as an alternative modeling method to PLS analyses. Thus, different multilayer feed forward net architectures with quick propagation training algorithms and logistic-type transfer functions were tested. Specifically, a (6-5-1) architecture was designed for fructose that means six input nodes (corresponding to the six analyzed frequencies) connected to a 5-node hidden layer and a final output layer. For glucose, sucrose and total sugars, (6-4-1), (6-9-1) and (6-1-1) architectures were selected, respectively.

Table 3.6.3. shows the mathematical models obtained for the corresponding ANN training, validation and test phase and Figure 3.6.5 shows the regression line obtained for fructose. As indicated, at least $R^2 \geq 0.991$ and RMSEP ≤ 0.901 were obtained for all the test phases. Thus, accurate and reliable models for determining sugar concentrations depending on EIS measurements were designed. Considering R^2 and RMSEP parameters for both PLS and ANN models, a better fit was obtained by ANN than by PLS for all the studied sugars.

Table 3.6.3. Artificial neural network (ANN) results for the studied fermentable sugars for EIS phase data from 6.6 × 10^5 Hz–7.0 × 10^5 Hz (R^2: coefficient of determination; RMSE: Root Mean Square Error).

		R^2	RMSEP
Glucose			
	Training	0,970	0,686
	Validation	0,995	0,251
	Test	0,998	0,233
Fructose			
	Training	0,986	0,414
	Validation	0,998	0,309
	Test	0,996	0,486
Sucrose			
	Training	0,999	0,161
	Validation	0,982	0,489
	Test	0,973	0,393
Total Sugars			
	Training	0,989	0,420
	Validation	0,991	0,298
	Test	0,991	0,333

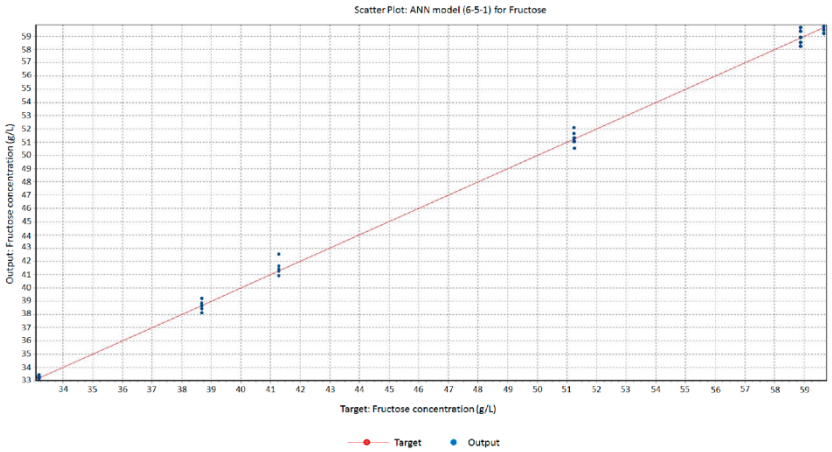

Figure 3.6.5. Scatter plot showing the relationship between analyzed (HPAEC-PAD) and predicted (ANN model) fructose concentrations in saccharified pineapple waste (g/L) for the studied EIS phase data (6.6 × 10^5 Hz–7.0 × 10^5 Hz).

CONCLUSIONS

Pineapple industrial waste is generating increasing worldwide interest as an alternative energy source because of its large volume and useful biochemical properties that allow for high efficiency in bioethanol production. However, this raw material must be previously hydrolyzed and an accurate monitoring of this process is of interest as enzymatic hydrolysis is a particularly complex process. In this study, the validation of an EIS-based method to monitor the saccharification process of industrial pineapple waste is introduced as an innovative analytical procedure. The use of the AVISPA device associated to a stainless steel double needle sensor allowed the development of electrochemical measurements and further comparison between EIS and HPAEC-PAD results. Statistical tools such as PLS and ANN allowed the design of robust and reliable mathematical prediction models for glucose, fructose, glucose and total sugars (R^2 > 0.970 and RMSEP < 1.206). Therefore, the introduced EIS-based technique combined with ANN models is validated and it is suggested as an easy, non-destructive and economic alternative to the traditional laboratory techniques for sugar monitoring in saccharification processes.

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AUTHOR CONTRIBUTIONS

Claudia Conesa and Lucía Seguí carried out the electrochemical measurements, Javier Ibáñez Civera contributed to data analysis and interpretation, and Pedro Fito participated in the coordination of the study. Nicolás Laguarda-Miró conceived and also coordinated the study. Finally, Claudia Conesa and Nicolás Laguarda-Miró wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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3.7. ARTÍCULO 7

ETHANOL QUANTIFICATION IN PINEAPPLE WASTE BY AN ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY-BASED SYSTEM AND ARTIFICIAL NEURAL NETWORKS

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Ethanol quantification in pineapple waste by an electrochemical impedance spectroscopy-based system and artificial neural networks

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ABSTRACT: Electrochemical impedance spectroscopy (EIS) technique has been applied to determine the ethanol concentration in pineapple waste samples. To do this, six different concentrations of ethanol were added to the pineapple samples and were analyzed using the system designed by our research group and consisting of the Advanced Voltammetry, Impedance Spectroscopy & Potentiometry Analyzer (AVISPA) device associated to a stainless steel double needle electrode. Results indicated that phase data in frequencies between 6.0×10^5 Hz and 8.0×10^5 Hz showed the highest sensitivity to ethanol concentrations. A principal component analysis (PCA) confirmed the potential discrimination and partial least squares (PLS) regression showed mathematical models able to quantify ethanol in samples accurately. In order to implement flexible and precise models in programmable equipment, different types of artificial neural networks (ANNs) have been studied: Fuzzy ARTMAP and multi-layer feed-forward (MLFF) algorithms. As a result, a coefficient of determination (R^2) = 0.996 and a root mean square error of prediction (RMSEP) = 0.408 have been obtained. Therefore, it allows us to introduce this technique as an alternative method for ethanol quantification along

the fermentation of pineapple waste in an easy, low-cost, rapid and portable way.

KEYWORDS: Electrochemical impedance spectroscopy; ethanol; pineapple waste; artificial neural networks.

INTRODUCTION

Global energy demand is significantly increasing due to global population growth and the industrialization of the emerging countries (International Energy Agency, 2015). The depletion of fossil energy resources and the emission of pollutants into the atmosphere (Nigam and Singh, 2011) have led to the rise of new energy policies promoting renewable energies, such as bioethanol (Directive 2009/28/EC).

In this scenario, second-generation bioethanol, which is produced from the fermentation of lignocellulosic biomass such as agricultural, forestry or industrial wastes, deserves special attention. Unlike first generation bioethanol (obtained from sugar or starch-rich crops) second-generation of this biofuel helps to diversify energy supplies without competing in the global food market (Rutz and Janssen, 2008; Bacovsky, 2010). Furthermore, the use of waste as a source for bioethanol production would also add up value to the whole manufacturing process. This biofuel can be used singly, as an additive replacing methyl tert-butyl ether (MTBE), or can be used in mixtures with conventional gasoline in a variable percentage from 10% to 85% (Yue *et al.,* 2014; Manzettu and Andersen, 2015; Morales *et al.,* 2015). As a result, bioethanol is the most widely used biofuel in the transport sector (Balat, 2011). Nowadays, pineapple industrial waste is considered as a potential bioethanol source because of its high content of fermentable sugars and highly hydrolysable cellulose and hemicellulose (Nigam, 1999; Tanaka *et al.,* 1999; Ruangviriyachai *et al.,* 2010). This waste represents up to 50% (w/w) of the total processed fruit whose world production has reached 24 millions of tons in 2014 (FAO, 2016).

Hydrolysis, fermentation, distillation and dehydration are the necessary steps for bioethanol production from lignocellulosic biomass (Scott *et al.,* 2013). In the specific case of the fermentation step, performance depends on the operating conditions (pH, oxygen content and temperature), the raw material properties (initial fermentable sugars content) and the used microorganisms (yeast strain and the viability of cell populations) (Cesaro and Belgiorno, 2015). Therefore, a proper monitoring of the process is of capital importance. To date, chromatographic and enzymatic methods are highlighted among the current techniques to determine ethanol content. Chromatographic methods such as gas-chromatography (G-C) and high-performance liquid chromatography (HPLC) are accurate and considered as a reference but they are time-consuming and complex pretreatments are needed (distillation, pervaporation) (Mohammed Al-Mhanna and Huebner, 2011). On the other hand, enzymatic methods are easier although spectrophotometric techniques are generally required to follow the enzyme-catalyzed reactions. As a consequence, they are undesirable for complex media due to the presence of interfering substances (Azevedo *et al.,* 2005). In contrast, electrochemical methodologies are emerging as an alternative to the traditional ones in order to identify chemical compounds in an easy, fast, non-destructive and on-line way. Specifically, electrochemical impedance spectroscopy (EIS) is one of the most remarkable ones. It consists in analyzing properties of the samples by means of electric alternating signals (current or voltage) at different frequencies and measuring the corresponding electric response (voltage or current) within an electrochemical cell (Bard and Faulkner, 2001; Barsoukov and Ross Macdonald, 2005).

In previous studies, identifying and quantifying fermentable sugars in pineapple waste as well as monitoring its enzymatic saccharification has been possible by using a device called Advanced Voltammetry, Impedance Spectroscopy & Potentiometry Analyzer (AVISPA) that combines EIS with a double needle stainless steel sensor (Conesa *et al.,* 2015; Conesa *et al.,* 2016). Furthermore, it has been necessary to

use artificial neural networks (ANNs) for a proper statistical treatment of the EIS data. ANNs are mapping structures simulating the neural system of the human brain. These structures are able to solve problems involving complex and non-linear data even in case of imprecise and noisy data sets. Among the different ANNs, Fuzzy ARTMAP and multi-layer feed-forward (MLFF) algorithms are able to be implemented in portable equipment (Laguarda-Miró *et al.,* 2012). These algorithms are characterized by a high accuracy, fast calculation, ease to use and low memory requirements that facilitate their application in programmable components such as microcontrollers, Digital Signal Processors (DSP) or Field Programmable Gate Arrays (FPGA).

Consequently, the aim of the present study was to validate the designed EIS-based system to quantify added ethanol in real pineapple waste samples and create reliable ANNs-based models able to be implemented into programmable devices.

MATERIAL AND METHODS

Electrochemical impedance spectroscopy equipment

The EIS measuring system has been designed by the Group of Electronic Development and Printed Sensors (GED+PS) belonging to the Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) at the Universitat Politècnica de València (UPV). It consists of three parts: the AVISPA device, the sensor and the specific software. The AVISPA device is able to run tests for potentiometry, pulse and cyclic voltammetry and impedance spectroscopy. It includes a Field Programmable Gate Array (FPGA), a 12-bit Digital-to-Analog Converter (DAC), two 12-bit Analog-to-Digital Converters (ADC) and different analog blocks (Conesa *et al.,* 2015). The software application was developed using Visual Basic® 6.0 (Microsoft, Redmond, WA, USA) to be run in a PC. It includes a section for EIS that allows the selection of the frequency sweep (from 0.01 Hz to 10 MHz), the current scale (up to 32 current scales) and the amplitude of the variable sinusoidal voltage signals (amplitude up to 1 Vpp). Finally, a double needle

electrode (working and counter electrodes) made of stainless steel was designed. The needles dimensions were 1.5 cm long and 1 mm in diameter and they were separated by 1 cm in order to create a stable electric field.

Laboratory analyses

Pineapple fruits (*Ananas comosus* L. cv. "MD-2") were selected avoiding external defects. Once the fruits were washed in a NaClO (0.1%) solution and the pulp and the crown were removed, peel and core (waste) were mashed in a blender (Avance Collection Blender HR2097/00 800W, Philips, Amsterdam, The Netherlands). Finally, the pH was adjusted to 5 by adding NaOH 1N (Panreac Química, S.L.U., Barcelona, Spain). The use of the same raw material along the laboratory analyses prevented any effect of potential interfering compounds in the laboratory analyses.

Preliminary assays in this research line established the expected ethanol concentrations after alcoholic fermentation of pineapple waste (De Prados *et al.,* 2010). Attending to these results, EIS measures were conducted to ethanol (purity=96%, Panreac-Química, S.L.U.) dissolved in pineapple waste at six different concentrations: 0% v/v (0 g/l), 2.5% v/v (19.725 g/l), 5% v/v (39.45 g/l), 7.5% v/v (59.175 g/l), 10% v/v (78.9 g/l) and 20% v/v (157.8 g/l). Analyses were conducted in triplicate at 25 °C in a thermostatic bath (PolyScience®, Niles, IL, USA). Consequently, a total of 54 EIS measurements (6×3x3) were performed.

Once the sensor was completely introduced into each sample, the AVISPA device applied sinusoidal signals at 100 different frequencies (between 1 and 10^6 Hz). Then, the electronic device sent the data to the PC and the software program calculated the modulus and phase values for each frequency. Finally, the corresponding plots were generated.

Multivariate analyses of data

Specific multivariate analyses, such as principal component analysis (PCA) and partial least squares analysis (PLS), have been successfully applied to process complex data sets obtained using EIS techniques (Martínez-Gil *et al.,* 2013). The SOLO© software program (Eigenvector Research, Inc., Manson, WA. USA) was used for these analyses. On the one hand, PCA was conducted to reduce dimensionality of data variables and to detect structures in the relationships between variables (Fuentes *et al.,* 2014). On the other hand, PLS analysis was performed in order to find a robust linear relationship between two matrices, X (the obtained EIS measurements) and Y (the added ethanol concentrations) and check its statistical validity (Wold *et al.,* 2001; Hastie *et al.,* 2009). Finally, the coefficient of determination (R^2) and the root mean square error of prediction (RMSEP) are used to test the predictive significance of the obtained PLS-models as they are reference parameters for this purpose (Wold *et al.,* 2001).

Training the Fuzzy ARTMAP neural network

Fuzzy ARTMAP networks use the so-called adaptative resonance method and it is based on the use of prior actions to predict subsequent steps (Carpenter *et al.,* 1992; Martínez-Mañez *et al.,* 2005). The method of operation is based on finding similarity between the input data and those previously classified. If similarity is found, the network associates the input data to the corresponding previously established category. If not, a new output category is created for this data. The element determining similarity between input data and those previously existing is a vigilance parameter called ρ. On the other hand, another parameter called learning parameter (β) determines the learning rate of the network when new data are introduced as well as the robustness of the model. Fuzzy ARTMAP can be used to create supervised classification systems by using 66% of the data for the training phase and the remaining 33% of the data for the test or validation phase. The relationship between output nodes from both networks is performed by a specific module called mapfield

connection. The module is a memory register whose data vary and increase as new data are incorporated.

Fuzzy ARTMAP networks have been used in many applications such as electronic nose systems (Llobet *et al.,* 1999; Brezmes *et al.,* 2005). and electronic tongues (Gil *et al.,* 2010; Gil *et al.,* 2011; Tan *et al.,* 2015), showing good and reliable results even with a limited number of samples (Tan *et al.,* 2015). Despite their wide range of successful uses, algorithms can be complex and may present difficulties on computer applications particularly if there is a memory restriction. In most cases, the algorithm is implemented on a PC and the memory is usually large enough for the algorithm to work properly. The problem may arise when the Fuzzy ARTMAP is implemented on portable devices using low-cost microcontrollers with a limited memory. In these cases, low memory demand algorithms are needed.

In order to satisfy this requirement, Simplified Fuzzy ARTMAP (SFAM) was developed achieving lower computational requirements and easier network arquitectures (Kasuba, 1993). This simplified network was used by Garrett (2003) to develop a MATLAB (MathWorks, Natick, MA, USA) toolbox. Using this toolbox functions, our research group has developed a specific MATLAB® graphical user interface. This application is focused on optimizing the algorithm by both getting the best classification rate and the minimum mapfield size (García-Breijo *et al.,* 2013). Thus, we can achieve an accurate classification with low memory requirements. The designed application scans ρ and β parameters in a value range and steps that can be set by the user. For each combination of ρ and β, the application determines the mapfield size and the classification rate so that the optimal values for ρ and β can be selected. In this way, the memory requirements can be controlled as they are directly related to the amount of data for the training phase. This application has been successfully used in several food classifications (Gil *et al.,* 2015).

Training the MLFF neural network

MLFF algorithm is one of the most popular ANN and it has been applied to a wide range of chemistry-related problems (Zupan and Gasteiger, 1993). MLFF consists of neurons that are arranged into layers. The first layer is called the input layer, the last layer is called output layer, and the layers between are hidden layers. Moreover, each neuron in a particular layer is connected with all neurons in the next one. MLFF algorithms always require a training stage, where the weights of each neuron are set and reflect the degree of importance of the given connection, followed by a validation step (Svozil *et al.,* 1997).

In the present work, the commercial ANN software program Alyuda Neurointelligence 2.2© (Alyuda Research Inc., Los Altos, CA, USA) was used to design the MLFF. First, the experimental data was randomly divided into three sets: training (70%), validation (15%) and test (15%) by the software program (Laguarda-Miró *et al.,* 2012; Ibáñez Civera *et al.,* 2011).

On the one hand, on-line back propagation training algorithms, hyperbolic tangent-type function for the hidden nodes and logistic-type transfer functions for the output layer neurons were chosen to fit the network for ethanol classification. The optimal network topologies (architecture and number of neurons in the hidden layer) were selected by testing several MLFF network structures and functions. Finally, the accuracy of the model was given by the correct classification rate (CCR%) and the confusion matrix.

On the other hand, a single hidden layer and quick propagation training algorithms (a modified version of the back propagation algorithms) showed the best results to predict ethanol concentrations in pineapple waste (García-Breijo *et al.,* 2011). Logistic-type transfer functions for both output layer neurons and hidden nodes were selected by testing several artificial neural network structures and functions. Finally, the accuracy of the obtained model was also given by the RMSEP and R^2.

RESULTS AND DISCUSSION

Multivariate analysis of the obtained EIS response

For each analyzed sample, the AVISPA device generated 200 data corresponding to the modulus and phase of the 100 applied frequencies (between 1 and 10^6 Hz, in linear steps of approximately 10100 Hz). The modulus graph did not reveal important information whereas the representation of the average phase values demonstrated that frequencies between 6.0×10^5 Hz and 8.0×10^5 Hz (21 frequency data) showed the highest sensitivity to ethanol concentration (Figure 3.7.1). In fact, the phase of EIS in pineapple waste samples for this frequency range decreased as the added ethanol concentration increased. This decrease is observed in all the selected frequencies. Therefore, we obtain parallel plots, with a clear separation among them except for those corresponding to concentrations 2.5% and 5% (nearly overlapped). As a consequence, phase values between 6.0×10^5 Hz and 8.0×10^5 Hz were selected for further data treatment.

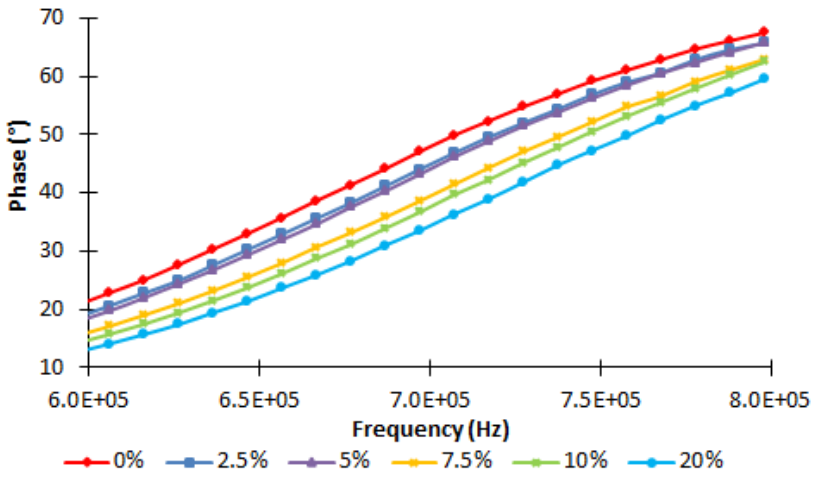

Figure 3.7.1. Average phase data for different ethanol concentrations in pineapple waste using EIS-based technique in the studied frequency range from 6.0×10^5 Hz to 8.0×10^5 Hz.

PCA analysis was conducted in order to check the ability of EIS-based technique to quantify the amount of added ethanol in pineapple waste. As shown in the biplot (Figure 3.7.2.), a clear discrimination of all the samples according to their corresponding ethanol concentration is obtained and they are allocated in ascending order from right to left in the graph. It is also noteworthy that the 99.65% of the total variability can be explained with only two principal components. The first (PC1) and the second component (PC2) explained 99.14% and 0.51% of the total variability, respectively. Consequently, ethanol content can be discriminated with only one principal component in the analyzed frequency range.

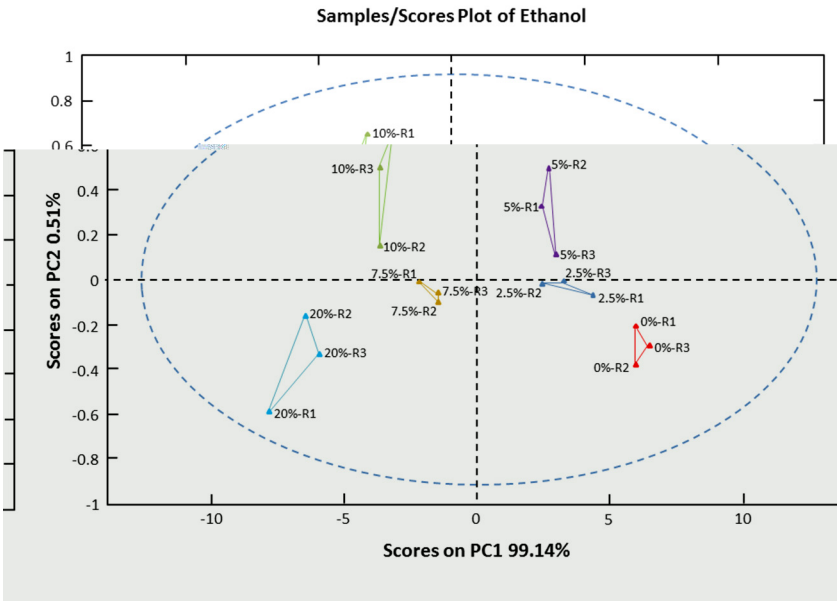

Figure 3.7.2. Principal component analyses (PCA) biplot for EIS phase data from 6.0 × 10^5 Hz to 8.0 × 10^5 Hz for the studied ethanol concentrations. R1-3: average of each replicate. The blue ellipsis indicates 95% confidence level.

Next, PLS analysis was conducted in order to create predictive models to quantify ethanol concentrations from the selected values of phase from EIS analysis in an easy and reliable way. Thus, Figure 3.7.3. shows the correlation between experimental values and those obtained by

the PLS prediction model using only one latent variable. In this specific case, the model is created by just one latent variable with R^2=0.974 and RMSEP=1.0588. Therefore, the designed model is considered statistically valid to predict ethanol content in pineapple waste from electrochemical signals.

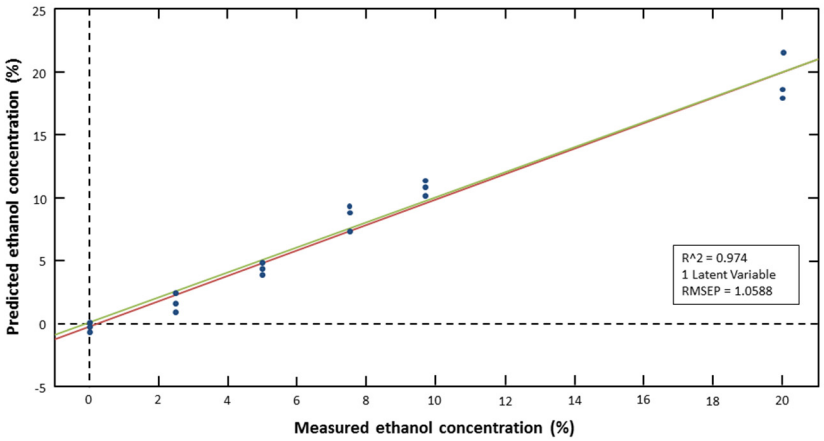

Figure 3.7.3. Scatter plot showing the relationship between added ethanol concentrations and predicted values of ethanol (%) by partial least square (PLS) statistical model (red line) and ideal pattern (green line) for the studied frequency range from 6.0 × 10^5 Hz to 8.0 × 10^5 Hz.

Modeling by Fuzzy ARTMAP neural networks

ANNs usually outperform multivariate methods in electrochemical applications (García-Breijo *et al.,* 2011) such as for fermentable sugar identification and quantification (Conesa et al., 2015) and saccharification monitoring (Conesa *et al.,* 2016). Moreover, ANNs can be implemented in programmable components to build portable devices as stated before. Therefore, data classification was performed by using Fuzzy ARTMAP neural networks. In order to reach a compromise between a high rate and a minimum mapfield, the ρ and β values have been changed in the range of [0.1–1.0]. For each combination, the mapfield size and the recognition rate were calculated (Table 3.7.1. and Table 3.7.2., respectively). Therefore, a

small sized mapfield has been obtained with ρ values in the range of [0.1–0.6] and β=0.8 with a maximum recognition rate of 94.4%, getting a mapfield of 10.

Table 3.7.1. Mapfield for EIS phase data from 6.0×10^5 Hz to 8.0×10^5 Hz.

	Mapfield						
ρ \ β	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
0.1	17	13	100	12	10	11	10
0.2	17	13	100	12	10	11	10
0.3	17	13	100	12	10	11	10
0.4	17	13	100	12	10	11	10
0.5	17	13	100	12	10	11	10
0.6	17	13	100	12	10	11	10
0.7	17	13	100	12	11	11	10
0.8	18	14	100	13	11	11	12
0.9	100	16	100	100	17	17	16

Table 3.7.2. Recognition rates for EIS phase data from 6.0×10^5 Hz to 8.0×10^5 Hz.

	Recognition rate						
P \ B	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
0.1	83.33	77.77	88.88	88.88	94.44	88.88	88.88
0.2	83.33	77.77	88.88	88.88	94.44	88.88	88.88
0.3	83.33	77.77	88.88	88.88	94.44	88.88	88.88
0.4	83.33	77.77	88.88	88.88	94.44	88.88	88.88
0.5	83.33	77.77	88.88	88.88	94.44	88.88	88.88
0.6	83.33	77.77	88.88	88.88	94.44	88.88	88.88
0.7	83.33	77.77	88.88	88.88	94.44	88.88	88.88
0.8	83.33	77.77	88.88	88.88	94.44	88.88	88.88
0.9	72.22	61.11	72.22	77.77	77.77	77.77	72.22

As a result, beta=0.8 and ρ=0.6 values were chosen and the obtained confusion matrix is shown in Table 3.7.3. The diagonal cells (in green) indicate the number of residue positions that were correctly classified for each of the 6 ethanol concentrations. The off-diagonal cells (in orange) represent the number of residue positions that were

misclassified. The grey cells show the total percentage of correctly predicted residues (top number in green color) and the total percentage of incorrectly predicted residues (bottom number in red color). As seen in Table 3.7.3., the designed Fuzzy ARTMAP neural network successfully matched 17 (94.4%) in 18 test measurements and failed in only one of them (5.6%). The prediction failure corresponds to a 2-type measurement (ethanol concentration of 2.5%) that has been classified as a 3-type (ethanol concentration of 5%). These results are consistent with those obtained in the PCA analysis (Figure 3.7.2.), where samples with 2.5% and 5% of ethanol content were clearly and only separated according to PC2 parameter. Finally, it is important to note that PC1 explained 99.14% of the total variability whereas PC2, 0.51%.

Table 3.7.3. Confusion matrix obtained with the Fuzzy Artmap ANN for EIS phase data from 6.0×10^5 Hz to 8.0×10^5 Hz. Target Class: 1: (0% v/v), 2: (2.5% v/v), 3: (5% v/v), 4: (7.5% v/v), 5: (10% v/v), 6: (20% v/v).

		Target Class						
		1	**2**	**3**	**4**	**5**	**6**	
Output Class	**1**	3 16.7%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	100% 0.0%
	2	0 0.0%	2 11.1%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	100% 0.0%
	3	0 0.0%	1 5.6%	3 16.7%	0 0.0%	0 0.0%	0 0.0%	75.0% 25.0%
	4	0 0.0%	0 0.0%	0 0.0%	3 16.7%	0 0.0%	0 0.0%	100% 0.0%
	5	0 0.0%	0 0.0%	0 0.0%	0 0.0%	3 16.7%	0 0.0%	100% 0.0%
	6	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	3 16.7%	100% 0.0%
		100% 0.0%	66.7% 33.3%	100% 0.0%	100% 0.0%	100% 0.0%	100% 0.0%	94.4% 5.6%

Modeling by MLFF neural networks

Different MLFF network architectures were tested in order to determine the number of neurons in the hidden-layer and optimize the fitting between the ethanol content and the EIS data set. As a consequence, a 21-6-6 architecture was designed that means 21 input nodes (corresponding to the 21 analyzed frequencies) connected to a 6-node hidden layer and a final 6-output layer (representing the 6 different concentrations of ethanol in to which the data has to be classified). The confusion matrices generated by the MLFF model for the corresponding training, validation and test phases are shown in Table 3.7.4. In the confusion matrices, diagonal cells (in green) show the number of data that were correctly classified for each ethanol concentration. The off-diagonal cells (in orange) indicate the number of data that were misclassified. Therefore, these results demonstrate that the designed MLFF is an accurate and reliable model for determining ethanol concentrations depending on EIS measurements (CCR%=96.15%). Similarly, errors in the range of 2.5% and 5% were observed with that method as a consequence of the overlapped data set for the above mentioned concentrations.

Table 3.7.4. Confusion matrices for the added ethanol concentrations (%) for EIS phase data from 6.0×10^5 Hz to 8.0×10^5 Hz (CCR%: correct classification rate).

Mean CCR% = 96.15%

Target (%) / Output (%)	Training						Validation						Test						Overall					
	0	2.5	5	7.5	10	20	0	2.5	5	7.5	10	20	0	2.5	5	7.5	10	20	0	2.5	5	7.5	10	20
0	7						1						1						9					
2.5		6						0	1					1	1					7	2			
5			7						1						1						9			
7.5				6						2						0						8		
10					6						2						1						9	
20						5						1						2						8

Finally, a 21-10-1 architecture was designed to quantify ethanol in pineapple waste samples that means 21 input nodes connected to a 10-node hidden layer and a final output layer. The obtained statistics for the corresponding training, validation and test phase and the scatter plot showing the correlation between predicted and added ethanol concentrations are shown in Table 3.7.5. and Figure 3.7.4 respectively. Consequently, accurate and reliable ANN-based models were designed to quantify ethanol in pineapple waste. Particularly, the best fit was obtained by MLFF as it can be seen by comparing R^2 and RMSEP parameters for both PLS and ANN models.

Table 3.7.5. Artificial neural network (ANN) results for the studied ethanol concentrations for EIS phase data from 6.0×10^5 Hz to 8.0×10^5 Hz (R^2: coefficient of determination; RMSE: Root Mean Square Error).

	R^2	RMSEP
Training	0.996	0.399
Validation	0.997	0.308
Test	0.996	0.408

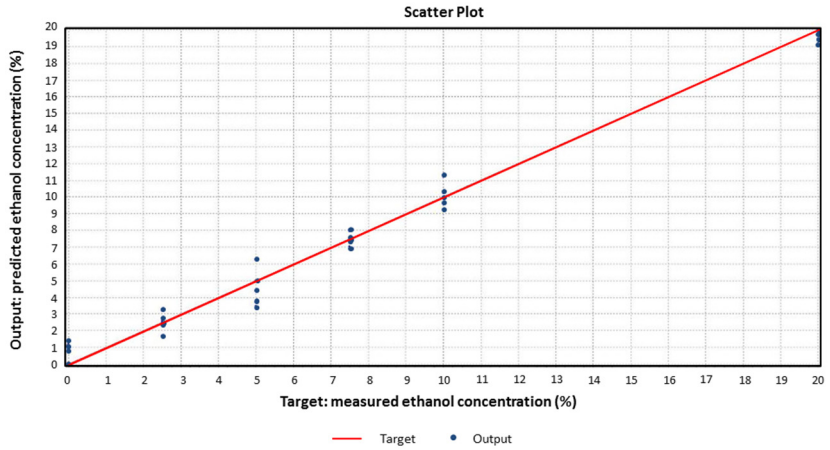

Figure 3.7.4. Scatter plot showing the relationship between the predicted and the added ethanol concentrations (%) in pineapple waste samples for the studied EIS phase data [6.0×10^5 Hz – 8.0×10^5 Hz].

CONCLUSIONS

The use of the AVISPA device associated to a stainless steel double needle electrode has allowed the application of a specific EIS frequency sweep [0.01 Hz–10 MHz] for ethanol quantification in real pineapple waste samples. The tests performed with this technique and PCA analyses have shown that is possible to discriminate different concentrations of ethanol using the phase data in frequencies from 6.0×10^5 Hz to 8.0×10^5 Hz. Further PLS analyses have accurately correlated EIS data to ethanol concentrations in the samples (R^2=0.974 and RMSEP=1.0588). Therefore, it is shown that ethanol concentration in pineapple waste samples can be quantified by EIS.

Moreover, different ANNs (Fuzzy ARTMAP and MLFF) have been studied in order to generate mathematical models able to be implemented into programmable systems. Results show that the appropriate combination of EIS assays and ANN (MLFF) analyses of the data is able to quantify ethanol content in pineapple waste samples in an accurate and reliable way (R^2=0.996 and RMSEP=0.408).

Finally, the obtained results are very promissory in the field of monitoring bioethanol production from lignocellulosic wastes due to a twofold reason: a) the obtained results allow us to suggest the implementation of ANN-based mathematical models on portable, easy and low cost EIS-based measurement systems to quantify ethanol production along the fermentation processes and b) this technique can be successfully applied not just in monitoring the pineapple waste fermentation process but in managing bioethanol production from many other similar lignocellulosic wastes.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests regarding the publication of this paper.

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4. DISCUSIÓN DE RESULTADOS

En la actualidad, es necesario seguir avanzando en la búsqueda de fuentes de energía alternativas a los combustibles fósiles, como es el caso del bioetanol 2G obtenido a partir de la biomasa lignocelulósica. Por este motivo, la presente Tesis Doctoral se ha centrado en los residuos industriales de frutas, tales como: la piña y el caqui.

En el caso de la piña (*Ananas comosus* (L.) Merr) de la variedad Golden-Sweet (MD-2), su volumen de residuos puede alcanzar más del 50% (p/p) de la fruta procesada (Ketnawa *et al.,* 2012). Además, la importancia comercial del cultivo y sus características fisicoquímicas lo convierten en una fuente potencial de obtención de bioetanol (Nigam, 1999; Tanaka *et al.,* Ruangvirichai *et al.,* 2010). No obstante, la tecnología desarrollada y los estudios realizados podrían ser aplicables a otros residuos agroindustriales.

Artículo 1: Hydrolytic performance of *Aspergillus niger* and *Trichoderma reesei* cellulases on lignocellulosic industrial pineapple waste for bioethanol production

Para obtener bioetanol a partir de biomasa lignocelulósica son necesarias dos etapas fundamentales: la hidrólisis de la celulosa y hemicelulosa a mono y disacáridos y su fermentación posterior (Galbe y Zacchi, 2002). Sun y Cheng (2002), al igual que otros autores, sugieren que la sacarificación enzimática presenta mayores ventajas que la aplicación de tratamientos químicos, puesto que las enzimas pueden trabajar a temperaturas más suaves y son mucho más específicas. No obstante, como afirma Verardi *et al.* (2012), su aplicación industrial sigue siendo limitada debido al elevado coste de los procesos de purificación y aislamiento de las enzimas y a su menor rendimiento con respecto a la sacarificación química. Entre las enzimas hidrolíticas, las celulasas producidas por las cepas mutantes de *Trichoderma (T. reesei; T. viride y T. longibrachitum)* son las más utilizadas para la sacarificación de la biomasa lignocelulósica debido a sus buenos rendimientos (Gusakov *et al*., 2007; Galbe y Zacchi, 2002; Choonut *et al.,* 2014). No obstante, existen varios estudios que utilizan las celulasas liberadas por *Aspergillus niger* para la sacarificación

enzimática de algunos residuos concretos (Sternberg *et al.,* 1997; Park *et al.,* 2002) y sugieren que podrían representar una alternativa más económica que las de *Trichoderma*. Es por ello que en este artículo, se comparó la acción hidrolítica de las celulasas comerciales producidas por *A. niger* y *T. reesei*, solas o combinadas con hemicelulasa procedente de *A. niger*, en la hidrólisis enzimática de los residuos industriales de piña.

Los resultados obtenidos demostraron que la velocidad inicial de sacarificación es mayor cuando se utilizan combinaciones enzimáticas con celulasa de *T. reesei* que de *A. niger*. No obstante, la baja actividad β-glucosidasa de la celulasa *T. reesei* (Sternberg *et al.*, 1997) produjo una acumulación de celobiosa y, por lo tanto, no se observaron diferencias significativas en el rendimiento final entre ambas celulasas tras 24 horas de hidrólisis, siendo 0,349±0,009 el incremento máximo de azúcares para las combinaciones con *T. reesei* y 0,34±0,05 para las de *A. niger*. Por otro lado, se observó que la celulasa de cualquier tipo y la hemicelulasa tienen un efecto sinérgico en la sacarificación, en la línea de lo observado por Medve *et al.* (1998) con *T. reesei*. Así, las celulasas producidas por *A. niger*, especialmente cuando se combinan con hemicelulasas, tienen un rendimiento similar a las liberadas por *T. reesei* y son más económicas.

Por lo tanto, se demostró que las celulasas comerciales de *A. niger* son una alternativa eficaz a las liberadas por *T. reesei* para la sacarificación de los residuos industriales de piña.

Artículo 2: Microwaves as a pretreatment for enhancing enzymatic hydrolysis of pineapple industrial waste for bioethanol production

Pretratar la matriz lignocelulósica es un paso previo e indispensable que complementa la hidrólisis enzimática ya que facilita el acceso de las enzimas dentro de la estructura y posibilita la obtención de altos rendimientos de azúcares (Sánchez *et al.,* 2010). En la actualidad, existe una gran variedad de pretratamientos físicos, fisicoquímicos, químicos y biológicos, así como combinaciones de ellos (Sun y Cheng,

2002). La mayoría de ellos requieren altas temperaturas que se alcanzan mediante calentamiento por conducción o convección (Liu y Wyman, 2005). Esto genera un elevado coste energético que condiciona la eficiencia del proceso y la posible aparición de compuestos inhibitorios de la fermentación. Como alternativa a estos pretratamientos convencionales, se estudió en este artículo la eficacia del calentamiento con microondas de los residuos industriales de piña.

Los resultados obtenidos demostraron que la aplicación de microondas a potencias intermedias (hasta 6.375 W/g) provocó la aparición de puntos calientes en el residuo que favorecieron la degradación de la estructura celular tal como describen Hu y Wen (2008). Estos cambios estructurales facilitaron la acción enzimática, aumentando el rendimiento de la sacarificación. Además, el contenido de compuestos inhibitorios de la fermentación medidos en el residuo pretratado, tales como furfural, hidroximetilfurfural y compuestos fenólicos no fue suficiente para generar una posible inhibición de la fermentación posterior. En concreto, pretratar con microondas a 6.375 W/g durante 4 min el residuo industrial de piña produjo un incremento del 27% en el contenido de azúcares totales con respecto al residuo sin pretratar. Por el contario, pretratar a potencias mayores, provocó la degradación térmica de los azúcares presentes en la piña y una compactación del tejido celular que disminuyó el rendimiento enzimático.

El detallado estudio comparativo mostrado en la Tabla 1.1., permite afirmar que las microondas tienen un menor requerimiento energético que los pretratamientos físicos convencionales (Cadoche y López, 1989), que este es un proceso notablemente más rápido que los biológicos tal como describe Saritha *et al.* (2012) y el coste es también sensiblemente menor, atendiendo a lo descrito por (Sun y Cheng, 2002) para los tratamientos químicos. Además, la aplicación de microondas a potencias intermedias genera un menor contenido en compuestos inhibitorios que la explosión de vapor, pese a ser el

método fisicoquímico más ampliamente utilizado como indica Duff y Murray (1996).

Por otro lado, el uso de la termografía infrarroja para el estudio de los perfiles de temperaturas permitió identificar diferentes periodos durante el calentamiento por microondas. El primer periodo se caracteriza por un calentamiento heterogéneo con temperaturas inferiores a 100 °C y la existencia de puntos calientes en la zona central de la muestra. En un segundo periodo, el calentamiento es homogéneo y la temperatura se mantiene cercana a los 100 °C. Finalmente, el calentamiento se vuelve de nuevo heterogéneo, aparecen puntos calientes y la temperatura asciende por encima de los 100 °C. Este estudio termográfico permite profundizar en el conocimiento del fenómeno de calentamiento por microondas, ayudando a identificar las variaciones térmicas en las muestras, la aparición de puntos calientes y por lo tanto el establecimiento de los rangos de tiempo y potencia en los que pueden ser utilizadas las microondas.

A tenor de los resultados obtenidos, el calentamiento por microondas a potencias intermedias de los residuos agroindustriales de piña puede ser considerado como un pretratamiento alternativo a los convencionales.

Artículo 3: Microwave-assisted alkali pretreatment for enhancing pineapple waste saccharification

Puesto que las microondas pueden combinarse con tratamientos químicos convencionales, en el siguiente artículo se evaluó la posibilidad de pretratar con microondas el residuo industrial de piña en medio alcalino con el fin de aprovechar las ventajas de ambos pretratamientos.

Los resultados demostraron que la aplicación de este pretratamiento a tiempos cortos de exposición (hasta 60 s), generó un aumento de temperatura en el medio alcalino y una degradación de la estructura celular similar a la descrita por Binod *et al.* (2012) en caña de azúcar.

De esta manera, se facilitó la acción enzimática y se aumentó el rendimiento enzimático. Por el contrario, tiempos altos de exposición compactaron los tejidos y degradaron térmicamente la lignina y las hexosas presentes en el medio a HMF y compuestos fenólicos. En concreto, el mayor incremento de azúcares totales (33,5%) se obtiene con tratamientos a 6.375 W/g durante 5 s, siendo estos valores significativamente mayores que los obtenidos con el pretratamiento con microondas del artículo anterior. Por lo tanto, el empleo de un medio alcalino permite disminuir el tiempo de tratamiento por microondas y aumentar la eficiencia del mismo. Tal y como sugiere Zhu *et al.* (2006) y Binod *et al.* (2012), las microondas actúan como catalizador de la reacción química. Además a diferencia de Durot *et al.* (2003) que aplica un tratamiento alcalino convencional a la paja de trigo, no se aplican altas temperaturas como las alcanzadas mediante conducción y convección que puedan producir compuestos inhibitorios de la fermentación.

Como líneas futuras de este artículo y del anterior, se sugiere fermentar las muestras pretratadas con microondas y comparar su rendimiento alcohólico con un residuo de piña sacarificado y fermentado pero sin pretratamiento previo. Asimismo, se podría plantear un estudio económico y técnico para comprobar si estas metodologías pueden implementarse a nivel industrial.

Artículo 4: Evaluation of “Rojo Brillante” persimmon industrial residues as a source for antioxidant compounds and substrate for bioethanol production

Según datos del Consejo Regulador de la DO Kaki Ribera del Xúquer la producción de caqui de la variedad “Rojo Brillante” ha crecido anualmente y alcanzó las 220.000 toneladas en 2015 (Agricultura y Cooperación, 2015). Este aumento de la producción y la elevada estacionalidad del cultivo, están promoviendo la búsqueda de alternativas para el aprovechamiento de sus posibles excedentes de producción, destríos y residuos industriales. Es por ello que en este artículo se evaluó el potencial de revalorización de dichos residuos.

Para ello, se recogieron muestras reales de una industria de IV gama de "Rojo Brillante" para la determinación de su potencial como fuente de obtención de compuestos antioxidantes y bioetanol.

Los resultados obtenidos sugieren que el contenido total de fenoles, flavonoides y taninos, así como la capacidad antioxidante de los residuos industriales de caqui fueron similares a los hallados en la pulpa del fruto por otros autores, tales como: Del Bubba *et al*. (2009), Denev y Jordanov (2013), Salvador *et al.* (2007) y Martínez-Las Heras *et al.* (2016), respectivamente. Si se compara el contenido de fenoles totales en caqui (59,2 ± 0,4 mg GAE/ 100g) con los hallados por Deng *et al.* (2012) en diferentes pieles de frutas, se observa un valor similar a los de la pera y ciruelo rojo (≈ 0,6 mg GAE/g), mayor a los del melocotón y sandía (0,2 – 0,4 mg GAE/g) y menor a los de las manzanas y naranjas (1,0 – 1,6 mg GAE/g). No obstante, la capacidad antioxidante medida fue muy baja en comparación con la encontrada por Deng *et al.* (2012) en los diferentes residuos analizados. Del Bubba *et al.* (2009) sugiere que los tratamientos poscosecha con CO_2 para eliminar la astringencia podrían disminuir dicho valor hasta un 30%. Por otro lado, el contenido hallado en β-caroteno y licopeno fue mayor al obtenido por De Ancos *et al.* (2000) y Giordani *et al.* (2011) en el fruto entero. Es por ello que se puede considerar el residuo de caqui como una fuente económica de obtención de compuestos bioactivos, principalmente carotenoides. De esta manera, se sugiere el uso del residuo de caqui para la obtención de harinas o polvos para su uso como conservante natural, aromatizante o colorante.

Por otro lado, la sacarificación y fermentación simultáneas (SFS) de los residuos permitió obtener una producción máxima de etanol a las 48 h del proceso de 37,39 g/L (0.3563 ± 0.0103 $g_{etanol}/g_{azúcar}$). Si se comparan estos resultados con los hallados en otros trabajos utilizando diferentes materias primas (Tabla 4.1.), los rendimientos de etanol son menores que en el caso de la cascarilla de arroz y el bagazo de caña de azúcar pero del mismo orden de magnitud que los residuos cítricos y la piña. Por lo tanto, a partir de los resultados obtenidos se

puede deducir que los residuos industriales de caqui son una fuente potencial de compuestos de alto valor añadido.

Tabla 4.1. Rendimientos en etanol en función del tipo de biomasa lignocelulósica.

Materia prima	Rendimiento en etanol
Cascarilla de arroz	0,25 L/Kg materia prima (Rojas y Cabanillas, 2008) 0,20 g/g (Saha y Cotta, 2007)
Bagazo de caña de azúcar	0.34 L/Kg materia prima (Forero, 2009)
Residuos cítricos	0.16 L/Kg materia seca (Coll, 2008)
Residuos industriales de piña	0,428 $g_{etanol}/g_{azúcar}$ (De Prados *et al.*, 2010)

Trabajos futuros podrían seguir avanzando en la mejora del rendimiento alcohólico mediante la aplicación de pretratamientos y optimización de las etapas de sacarificación y fermentación (microorganismos, temperatura, pH…). También, sería interesante estudiar la extracción y estabilización de los compuestos antioxidantes procedentes de residuos de caqui para su uso en la industria alimentaria. Finalmente, evaluar la posibilidad de utilizar diferentes residuos procedentes de otros cultivos para su revalorización conjunta en una planta de obtención de bioetanol.

Artículo 5: An electrochemical impedance spectroscopy-based technique to identify and quantify fermentable sugars in pineapple waste valorization for bioethanol production

Como se ha podido comprobar en los trabajos anteriores, la hidrólisis enzimática es una etapa compleja y su control es necesario para optimizar el proceso de obtención de bioetanol a partir de biomasa lignocelulósica.

Es por ello que en este artículo, se estudió la validez de la EIS para determinar el contenido de glucosa, fructosa y sacarosa en los residuos de piña. Para ello, se diseñó un sensor de doble aguja de

acero inoxidable para asociarlo al sistema de medida AVISPA. Éste equipo emite, recibe e interpreta la señal electroquímica mediante una aplicación software. La elección de este metal no oxidable para la fabricación del sensor permitió obtener un sistema de medida resistente, económico, de fácil manejo y autorizado para su uso en la industria agroalimentaria.

Los resultados obtenidos demostraron que esta metodología permite identificar y cuantificar los azúcares fermentables en el residuo de piña solos o combinados entre sí y es posible obtener modelos matemáticos robustos, flexibles y fiables (CCR% > 93,443%) mediante RNA. Éstos pueden ser implementados en sistemas portátiles de medida que además una vez entrenados se adaptan fácilmente a los cambios en el entorno y permiten obtener una respuesta rápida y en línea.

Por lo tanto, esta metodología basada en la EIS puede considerarse una alternativa eficaz a las técnicas de laboratorio tradicionales y consideradas de referencia para la determinación de azúcares, tales como la cromatografía (Cataldi *et al.*, 2000; Schütz *et al.*, 2006; Ronkart *et al.*, 2007; Matias *et al.*, 2011). A diferencia de éstas, la EIS es comparativamente fácil de utilizar, rápida, no destructiva, económica y no requiere de personal cualificado para su utilización (Jie *et al.*, 2014).

Por último, los resultados hallados están en línea con los obtenidos por otros autores utilizando otras técnicas electroquímicas. Rudnitskaya *et al.* (2006) y Beullens *et al.* (2006) han logrado con éxito determinar el contenido de glucosa, fructosa y sacarosa en manzana y tomate mediante lenguas electrónicas (e-tongues o ET) con sensores potenciométricos y electrodos de referencia de Ag/AgCl. La potenciometría requiere de un mantenimiento de los electrodos de referencia y los sensores necesitan un tiempo de estabilización largo. En comparación, la EIS es una técnica mucho más potente pues estudia la respuesta electroquímica en todo un rango de frecuencias pudiéndose realizar un análisis más complejo. Además, la técnica es

comparativamente más rápida pues no requiere de estabilización y más sencilla ya que no necesita de electrodo de referencia. Por lo tanto la aplicación de la EIS con este tipo de sensor supone un avance significativo en este campo.

Artículo 6: An electrochemical impedance spectroscopy system for monitoring pineapple waste saccharification

Trabajos previos como los presentados por Ohnishi *et al*. (2004), Wu *et al.* (2008) y Fuentes *et al.* (2014) demostraron que la EIS permite controlar en tiempo real diferentes procesos agroindustriales, tales como determinados tratamientos térmicos, la congelación y los daños por frío en diversas frutas y hortalizas. Estos estudios junto con los resultados obtenidos para la detección de azúcares en el artículo anterior, promovieron la decisión estudiar la validez de esta técnica para monitorizar en línea el proceso de sacarificación enzimática. Para ello, se compararon las medidas de EIS realizadas a diferentes tiempos de sacarificación con los datos de concentración de azúcares obtenidos mediante HPAEC-PAD.

Los resultados obtenidos demostraron que es posible diseñar modelos de predicción robustos y fiables (R^2 > 0.970 and RMSEP < 1.206) mediante PLS y RNA para la identificación y cuantificación de los azúcares fermentables durante el proceso de hidrólisis enzimática. Estos resultados junto con las cualidades de la EIS constatadas en el artículo anterior, postulan esta técnica como una alternativa real para el control en línea de la sacarificación, lo que supone una ventaja añadida a los métodos anteriormente descritos y abre un abanico de aplicaciones para la EIS.

Como líneas de investigación futuras se plantea la posibilidad de implementar los modelos desarrollados anteriormente en sistemas de medida portátiles. Finalmente, sería necesario comprobar si la metodología desarrollada en este capítulo y el anterior, puede ser utilizada a escala industrial.

Artículo 7: Ethanol quantification in pineapple waste by an electrochemical impedance spectroscopy-based system and artificial neural networks

En los procesos de obtención de bioetanol a partir de biomasa lignocelulósica, el rendimiento de la etapa de fermentación depende de numerosos factores. En un estudio realizado por Cesaro y Belgiorno (2015) se analizan los retos y oportunidades de la producción de bioetanol y se resalta la necesidad de controlar el proceso de fermentación.

Por lo tanto, en este artículo se planteó la posibilidad de extender el uso de la EIS, además de para las aplicaciones descritas en los artículos 5 y 6, para la cuantificación del contenido en etanol en muestras reales de residuos de piña y como paso previo a la monitorización de la fermentación. Para ello, se añadieron diferentes concentraciones de etanol en muestras de residuo de piña y se midió su respuesta electroquímica. Los resultados de un análisis PCA permitieron discriminar las muestras en función de su concentración de alcohol y los análisis PLS (R^2= 0.974 and RMSEP= 1.0588) permitieron correlacionar la señal electroquímica con la concentración de etanol en las muestras con exactitud. A continuación, la aplicación de RNA permitió la aplicación de modelos de predicción potentes (R^2 = 0.996 y RMSEP= 0.408) y fácilmente implementables en equipos programables de medida. Así, la metodología descrita se presenta como una alternativa sencilla, eficaz, económica y en línea, a las técnicas tradicionales de cuantificación de etanol, tales como la cromatografía de gases y el HPLC. Esta afirmación está en la línea de lo descrito por Mohammed Al-Mhanna y Huebner (2011) y Azevedo *et al.* (2005) que utilizan sensores de pH y biosensores para la cuantificación de etanol en muestras como métodos alternativos a la cromatografía.

Adicionalmente, los resultados obtenidos en este estudio son muy prometedores pues permiten postular la EIS como metodología eficaz en al menos una doble línea. Por una parte, sirve de base para el desarrollo de sistemas portátiles para la detección de etanol. Por otra,

abre la vía para la utilización de esta técnica para la monitorización del proceso de obtención de bioetanol a partir de otras materias lignocelulósicas.

Por último, sería interesante comprobar si es posible determinar el contenido en etanol y azúcares en los residuos durante la sacarificación y fermentación simultáneas. Asimismo, se plantea la necesidad de comprobar esta metodología a escala industrial y con otros residuos.

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5. CONCLUSIONES

The below described conclusions is a compilation of those obtained in each one of the published articles in the frame of this PhD Thesis:

Article 1: Hydrolytic performance of *Aspergillus niger* and *Trichoderma reesei* cellulases on lignocellulosic industrial pineapple waste intended for bioethanol production

Pineapple processing generates significant amounts of residues that need to be properly disposed in order to meet environmental requirements. The use of this waste material for the production of bioethanol is a good opportunity to give added value to this residue of the pineapple industry, in the context of the second generation of biofuels. In order to obtain fermentable sugars from this residual biomass, two different commercial cellulases (*Trichoderma reesei* and *Aspergillus niger*) have been assayed, either alone or combined with hemicellulase from *A. niger*. The increase in the total soluble solids present in the hydrolysate indicate that, in spite of exhibiting a significant slower initial hydrolytic rate than *T. reesei* cellulase, *A. niger* cellulase may lead to a similar amount of soluble solids present in the final hydrolysate. This trend has been also confirmed when analysing the specific sugars released to the medium, especially when combined with the enzyme hemicellulase. The fact that *T. reesei* cellulase tend to accumulate more cellobiose, could be responsible for a slowdown of the hydrolytic process, despite the faster initial rate. On the other hand, a synergistic effect of combining both cellulase and hemicellulase enzymes has been proven, since the addition of one enzyme conditioned the action of the other one.

In conclusion, this study shows that commercial *A. niger* cellulase represents an efficient alternative to *T. reesei* cellulase for the saccharification of industrial pineapple waste, especially when combined with a hemicellulase. Total sugars present in the final hydrolysates indicated that *A. niger* cellulase performed similarly at a lower cost, with no cellobiose accumulation. However, if processing time is a limiting factor, *T. reesei* cellulase could be the one preferred.

Article 2: Microwaves as a pretreatment for enhancing enzymatic hydrolysis of pineapple industrial waste for bioethanol production

In the context of biofuel production pretreatments of lignocellulosic biomass are being currently studied in order to improve the saccharification step; in particular, microwaves have been suggested as an alternative pretreatment of this residual biomass.

In the present work microwaves have been studied as a pretreatment for improving pineapple waste saccharification. Results of applying different powers and exposure times to the pineapple waste material indicate that microwave pretreatment may increase saccharification performance as long as mild treatments are used. However, low powers and short exposure times do not modify sugar content where as higher powers and/or exposure times may result in sugar decrease. Infrared thermography and Cryo-SEM microscopy observations indicated that both thermal sugar degradation and increased tissue compactness may be responsible for the lower yield when harsher microwave conditions are used. The presence of phenolic components as a result of lignin solubilization as well as sugar degradation to furfural and hydroxymethylfurfural have also been confirmed when lengthening the treatment, especially when higher powers are used. At milder conditions, however, explosion in hot spots and resulting tissue modifications facilitate enzyme action in the subsequent saccharification step.

The use of infrared thermography for the study of temperature profiles also allowed the identification of different periods during MW heating: a first period, characterized by a heterogeneous heating, the existence of a hot spot in the central region of the sample and temperatures below 100 °C; a second one, in which a homogeneous heating is reached and temperature remains around 100 °C; and a final period, when sample over-heating have been identified (temperatures > 100 °C) and new hot spots appear.

In conclusion, microwave pretreatments using the appropriate energy supply and exposure time allows to enhance the efficiency of lignocellulosics saccharification and, therefore, it may improve bioethanol yield in a subsequent step. This has been proved for

industrial pineapple waste, although it could be potentially applicable to other food industry residues.

Article 3: Microwave-assisted alkali pretreatment for enhancing pineapple waste saccharification

The use of microwave alkaline pretreatments for short exposure times (up to 60 s) improved the yield of enzymatic hydrolysis compared with non-pretreated waste. The highest increase in fermentable (35.7%) and total sugars (33.5%) was obtained at t = 5 s when high microwave power was applied (6.375 W/g). However, longer exposure times resulted in sugar degradation.

The content of fermentable and total sugars showed a statistically significant negative correlation with the maximum and average temperatures in the samples pretreated by microwave in an alkaline medium.

The content of fermentation inhibitor compounds, such as total phenols or HMF, increased as microwave power and exposure time rose. This increase was correlated with the rise in temperatures. However, the total amount of phenol values was not sufficient to inhibit subsequent fermentation. Nevertheless, the effect of HMF content on pineapple waste fermentation should be studied.

Applying microwaves during short exposure times promoted structural changes that improved enzymatic hydrolysis. In contrast, an increase in the severity of the treatment compacted the structure and thus hindered access by the enzymes, which reduced the sugars released into the medium.

Article 4: Evaluation of “Rojo Brillante” persimmon industrial residues as a source for antioxidant compounds and substrate for bioethanol production

One of the main problems of “Rojo Brillante” persimmon is the large amount of waste generated as a consequence of its seasonality and the dramatic rise in its production.

Industrial persimmon residue offers considerable potential for the production of high-added-value compounds, such as antioxidants. Specifically, the level of phenols, tannins, flavonoids and antioxidant capacity was similar to that found in the whole fruit by other authors, although their content were not particularly remarkable when compared to other fruit residues, probably due to antioxidant content reduction as a consequence of deastringency treatments. Nevertheless, concentrations of β-carotene and lycopene were found to be higher than in the fruit, and persimmon fruit is already a considerable good source of carotenoids. Therefore, persimmon waste can be considered an inexpensive and readily available resource of bioactive compounds, mainly carotenoids. Potential applications of these bioactive compounds in the food, pharmaceutical o cosmetics industry are not only possible after their extraction, which would raise the price of the resulting product, but the use of a crude flour or powder obtained from persimmon residue after drying and grinding could also have several applications as a natural preservative, flavoring or coloring agent.

On the other hand, persimmon waste has also shown potential as a substrate for bioethanol production. Among the different processes assayed, the simultaneous saccharification and fermentation process implied higher ethanol yields. The use of lignocellulose residual biomass to produce second generation bioethanol is being encouraged nowadays. In the present work, persimmon waste has successfully undergone fermentation and has shown potential to be used for bioethanol production, either alone or mixed with other food residual biomass.

Article 5: An electrochemical impedance spectroscopy-based technique to identify and quantify fermentable sugars in pineapple waste valorization for bioethanol production

This work introduces an EIS-based methodology for monitoring and managing the concentration of sugars in the most complex phase for second generation bioethanol production: the enzymatic hydrolysis. In order to do this, an AVISPA device has been used as it is able to generate and receive EIS signals from an especially designed double

needle sensor made of stainless steel. Statistical treatment of the data allowed to build reliable and robust ANN-based mathematical models (mean CCR% > 93.443%) to identify and quantify the main fermentable sugars (glucose, fructose and sucrose) in pineapple waste samples both individually and jointly. Furthermore, this methodology is easy, rapid, non-destructive, and in-situ. Thus, it can be considered as a promising alternative to the traditional laboratory techniques for enzymatic hydrolysis monitoring and management in second-generation bioethanol production not just from pineapple wastes but also from many other lignocellulosic sources.

Article 6: An electrochemical impedance spectroscopy system for monitoring pineapple waste saccharification

In this study, the validation of an EIS-based method to monitor the saccharification process of industrial pineapple waste is introduced as an innovative analytical procedure. The use of the AVISPA device associated to a stainless steel double needle sensor allowed the development of electrochemical measurements and further comparison between EIS and HPAEC-PAD results. Statistical tools such as PLS and ANN allowed the design of robust and reliable mathematical prediction models for glucose, fructose, glucose and total sugars (R^2 > 0.970 and RMSEP < 1.206). Therefore, the introduced EIS-based technique combined with ANN models is validated and it is suggested as an easy, non-destructive and economic alternative to the traditional laboratory techniques for sugar monitoring in saccharification processes.

Article 7: Ethanol quantification in pineapple waste by an electrochemical impedance spectroscopy-based system and artificial neural networks

The use of the AVISPA device associated to a stainless steel double needle electrode has allowed the application of a specific EIS frequency sweep [0.01 Hz–10 MHz] for ethanol quantification in real pineapple waste samples. The tests performed with this technique and PCA analyses have shown that is possible to discriminate different concentrations of ethanol using the phase data in frequencies from 6.0×10^5 Hz to 8.0×10^5 Hz. Further PLS analyses have accurately

correlated EIS data to ethanol concentrations in the samples (R^2=0.974 and RMSEP=1.0588). Therefore, it is shown that ethanol concentration in pineapple waste samples can be quantified by EIS.

Moreover, different ANNs (Fuzzy ARTMAP and MLFF) have been studied in order to generate mathematical models able to be implemented into programmable systems. Results show that the appropriate combination of EIS assays and ANN (MLFF) analyses of the data is able to quantify ethanol content in pineapple waste samples in an accurate and reliable way (R^2=0.996 and RMSEP=0.408).

Finally, the obtained results are very promissory in the field of monitoring bioethanol production from lignocellulosic wastes due to a twofold reason: a) the obtained results allow us to suggest the implementation of ANN-based mathematical models on portable, easy and low cost EIS-based measurement systems to quantify ethanol production along the fermentation processes and b) this technique can be successfully applied not just in monitoring the pineapple waste fermentation process but in managing bioethanol production from many other similar lignocellulosic wastes.

Final conclusions

It has been proved that commercial *A. niger* cellulase represents an efficient alternative to *T. reesei* cellulase for the saccharification of industrial pineapple waste, specially when combined with hemicellulase. Moreover, microwave pretreatments (alone or combined with alkali) with the appropiate power and exposure times enhance the saccharification performance of pineapple waste which would improve the subsequent fermentation step. Finally, persimmon waste can be considered as a potential low-cost source of bioethanol and antioxidant compounds, mainly carotenoids.

On the other hand, an EIS-based technique has been validated for fermentable sugars monitoring and ethanol quantification in pineapple waste. Thus, this methodology is suggested as an easy, fast, non-destructive and economic alternative to the traditional laboratory techniques.

These advances enable the development of interesting research lines for enhancing bioethanol production from lignocellulosic waste and the application of the latest technological developments for its control.

ANEXOS

CONTENIDOS

A.1. PRODUCTIVIDAD CIENTÍFICA

A.1.1. Artículos de investigación

Autores: Conesa, Claudia; Seguí, Lucía; Fito, Pedro
Título: Hydrolytic performance of *Aspergillus niger* and *Trichoderma reesei* cellulases on lignocellulosic industrial waste intended for bioethanol production
Editorial: Springer Netherlands
REF. Revista/ Libro (ISBN, ISSN, SUPV): Waste and Biomass Valorization (ISSN 1877-2641)
Volumen: 140 **Páginas:** 1 – 10 **Año:** 2017

Autores: Conesa, Claudia; Gil-Sánchez, Luis; Seguí, Lucía; Fito, Pedro; Laguarda-Miró, Nicolás
Título: Ethanol quantification in pineapple waste by an electrochemical impedance spectroscopy-based system and artificial neural networks
Editorial:
REF. Revista/ Libro (ISBN, ISSN, SUPV): Chemometrics and Intelligent Laboratory Systems (ISSN 0169-7439)
Volumen: 161 **Páginas:** 1 – 7 **Año:** 2017

Autores: Conesa, Claudia; Gil-Sánchez, Luis; Seguí, Lucía; Fito, Pedro; Laguarda-Miró, Nicolás
Título: An Electrochemical Impedance Spectroscopy System for Monitoring Pineapple Waste Saccharification
Editorial: Molecular Diversity Preservation International, Matthaeusstrasse 11, Basel, Switzerland, CH-4057
REF. Revista/ Libro (ISBN, ISSN, SUPV): Sensors (ISSN 1424-8220)
Autores: Conesa, Claudia; Seguí, Lucía; Laguarda-Miro, Nicolás; Fito, Pedro
Título: Microwave-Assisted Alkali Pretreatment for Enhancing Pineapple Waste Saccharification
EF. Revista/ Libro (ISBN, ISSN, SUPV): BioResources (ISSN 1930-2126)
Volumen: 16 **Páginas:** **Año:** 2016

Autores: Conesa, Claudia; Seguí, Lucía; Laguarda-Miro, Nicolás; Fito, Pedro
Título: Microwave-Assisted Alkali Pretreatment for Enhancing Pineapple Waste Saccharification
Editorial:
REF. Revista/ Libro (ISBN, ISSN, SUPV): BioResources (ISSN 1930-2126)
Volumen: 11 **Páginas:** 6518-6531 **Año:** 2016

Autores: Conesa, Claudia; Seguí, Lucía; Laguarda-Miro, Nicolás; Fito, Pedro
Título: Microwaves as a pretreatment for enhancing enzymatic hydrolysis of pineapple industrial waste for bioethanol production
Editorial: INST Chemical Engineers, 165-189 Railway Terrace, Davis Bldg, Rugby, England, CV21 3HQ
REF. Revista/ Libro (ISBN, ISSN, SUPV): Food and Bioproducts Processing (ISSN 0960-3085)
Volumen: 100 **Páginas:** 203-213 **Año:** 2016

Autores: Conesa, Claudia; García Breijo, Eduardo; Loeff, Edwin; Seguí, Lucía; Fito, Pedro; Laguarda Miró, Nicolás
Título: An Electrochemical Impedance Spectroscopy-Based Technique to Identify and Quantify Fermentable Sugars in Pineapple Waste Valorization for Bioethanol Production
Editorial: Molecular Diversity Preservation International, Matthaeusstrasse 11, Basel, Switzerland, CH-4057
REF. Revista/ Libro (ISBN, ISSN, SUPV): Sensors (ISSN 1424-8220)
Volumen: 15 **Páginas:** 22941 – 22955 **Año:** 2015

Autores: Conesa Domínguez, Claudia; Fito Suñer, Pedro José; Fito Maupoey, Pedro
Título: Extension of the project for obtaining bioethanol from citrus waste
Editorial: ISHS
REF. Revista/ Libro (ISBN, ISSN, SUPV): Acta Horticulturae (ISSN 0567-7572)
Páginas: 1693 - 1702 **Año:** 2015

Autores: Conesa Domínguez, Claudia; Fito Maupoey, Pedro; Fito Suñer, Pedro José; Conesa Roca; Ernesto
Título: El bioetanol: una alternativa a los residuos industriales agrícolas y agroindustriales de frutas y hortalizas
Editorial:
REF. Revista/ Libro (ISBN, ISSN, SUPV): Revista de Fruticultura (ISSN 2013-5742)
Páginas: 40 - 47 **Año:** 2013

Autores: Conesa Roca, Ernesto; Conesa Domínguez, Claudia
Título: Global Solution for Preserving Citrus Fruit Using Natural Treatments
Editorial: David Pub. Co
REF. Revista/ Libro (ISBN, ISSN, SUPV): Journal of Agricultural Science and Technology. A (ISSN 2161-6256)
Páginas: 503 - 510 **Año:** 2013

Autores: Conesa Roca, Ernesto; Tormo Ases, Daniel José; Argüelles Foix, Ángel Luis; Conesa Domínguez, Claudia
Título: Prolongación del uso de las aguas residuales de tratamiento fitosanitario en la postcosecha de cítricos
Editorial: Ediciones y Promociones LAV, SL
REF. Revista/ Libro (ISBN, ISSN, SUPV): Levante Agrícola (ISSN 0457-6039)
Volumen: 51 **Páginas:** 210 - 214 **Año:** 2012

Autores: Gil, Rebeca; Conesa, Claudia; Besada Ferreiro, Cristina María; Tormo, Daniel; Salvador, Alejandra
Título: Tratamiento mixto para la eliminación de la astringencia de caqui
Editorial: Ediciones Lav
REF. Revista/ Libro (ISBN, ISSN, SUPV): Agrícola Vergel (ISSN 0211-2728)
Volumen: 30 **Páginas:** 351 - 356 **Año:** 2011

A.1.2. Actas de congresos

A.1.2.1. Actas de congresos internacionales publicadas en editorial

Autores: Conesa Domínguez, Claudia; Laguarda Miró, Nicolás; Olguín Pinatti, Cristian Ariel; Loeff, Edwin; Seguí Gil, Lucía; Fito Maupoey, Pedro
Título: An approach to the determination and quantification of sugars in fruits by Impedance Spectroscopy
Editorial: Universidad Nacional de Entre Ríos y Universitat Politècnica de València
Ref. Revista/ libro (ISBN, ISSN, SUPV): International Conference on Food Innovation (FoodInnova 2014) (ISSN 978-950-698-376-5)
Páginas: 409 - 409 **Año:** 2016 **Clave:** PR

Autores: Conesa, Claudia; Gómez-Cocera, Jessica; Laguarda Miró, Nicolás; Olguín Pinatti, Cristian
Título: Monitorización mediante Espectroscopía de Impedancias de la hidrólisis enzimática de los residuos de piña para la obtención de bioetanol de segunda generación
REF. REVISTA/ LIBRO (ISBN, ISSN, SUPV): IX International Workshop on Sensors and Molecular Recognition (ISSN 978-84-608-2360-5)
Páginas: 348 - 351 **Año:** 2015 **Clave:** PR

Autores: Conesa, Claudia; Ferrando, Belén; Laguarda Miró, Nicolás; Seguí, Lucía; Ibáñez-Civera, Javier
Título: Aplicación de la espectroscopía de impedancias para la identificación y cuantificación de bioetanol de segunda generación a partir de residuos de piña
Ref. Revista/ libro (ISBN, ISSN, SUPV): IX International Workshop on Sensors and Molecular Recognition (ISSN 978-84-608-2360-5)
Páginas: 330 - 333 **Año:** 2015 **Clave:** PR

Autores: Conesa Domínguez, Claudia; Fombuena, Vicente; Loeff, Edwin; Olguín Pinatti, Cristian Ariel; Seguí Gil, Lucía; Laguarda Miró, Nicolás
Título: Discriminación de azúcares mono y di-sacáridos presentes en alimentos mediante espectroscopía de impedancias
Editorial: Universitat de València
Ref. Revista/ libro (ISBN, ISSN, SUPV): VIII International Workshop on Sensors and Molecular Recognition (ISSN 978-84-697-1302-0)
Páginas: 198 - 202 **Año:** 2014 **Clave:** PR

Autores: Olguín Pinatti, Cristian Ariel; Laguarda Miró, Nicolás; Conesa Domínguez, Claudia; Ibáñez Civera, Francisco Javier; Loeff, Edwin;
Título: Estudio voltamétrico para el control de biocidas no oxidantes en torres de refrigeración
Editorial: Universitat de València
Ref. Revista/ libro (ISBN, ISSN, SUPV): VIII International Workshop on Sensors and Molecular Recognition (ISSN 978-84-697-1302-0)
Páginas: 193 - 197 **Año:** 2014 **Clave:** PR

Autores: Conesa Domínguez, Claudia; Bernat-Senent, Ignacio; Seguí Gil, Lucía; Fito Maupoey, Pedro;
Título: Pretratamiento con microondas para la obtención de bioetanol a partir de residuos industriales de piña: una propuesta de mejora de la hidrólisis enzimática
Editorial: Universitat Politècnica de València
Ref. Revista/ libro (ISBN, ISSN, SUPV): IX Congreso Iberoamericano de Ingeniería de Alimentos (CIBIA 2014) (ISSN 978-84-9048-168-4)
Páginas: 435 - 443 **Año:** 2014 **Clave:** PR

A.1.2.2. Actas de congresos nacionales

Autores: Conesa Domínguez, Claudia; Tirone, Alfonso; Seguí Gil, Lucía; Fito Maupoey, Pedro
Título: Efecto del pretratamiento con microondas en el contenido en aceites esenciales y en la hidrólisis enzimática de los residuos cítricos para la obtención de bioetanol
Editorial: Universidad de Córdoba; Don Folio
Ref. Revista/ libro (ISBN, ISSN, SUPV): VII Congreso Nacional de Ciencia y Tecnología de los Alimentos (CyTA 2013) (ISSN 978-84-15105-95-4)
Páginas: 153 - 154 **Año:** 2013 **Clave:** PR

Autores: Conesa Domínguez, Claudia; Bernat-Senent, Ignacio; Seguí Gil, Lucía; Fito Maupoey, Pedro;
Título: Efecto del pretratamiento con microondas en la hidrólisis enzimática de los residuos industriales de la piña para la obtención de bioetanol
Editorial: Universidad de Córdoba; Don Folio
Ref. Revista/ libro (ISBN, ISSN, SUPV): VII Congreso Nacional de Ciencia y Tecnología de los Alimentos (CyTA 2013) (ISSN 978-84-15105-95-4)
Páginas: 152 - 152 **Año:** 2013 **Clave:** PR

A.1.2.3. Actas de congresos publicadas sin ISBN

Autores: Conesa Domínguez, Claudia; Bernat-Senent, Pedro; Seguí Gil, Lucía; Fito Maupoey, Pedro
Título: Microwave-assisted pretreatments for enhancing enzymatic hydrolysis of industrial pineapple waste for bioethanol production
Ref. Revista/ libro (ISBN, ISSN, SUPV): EFFoST Annual Meeting (EFFoST 2013)
Páginas: 1 – 2 **Año:** 2013 **Clave:** PR

Autores: Conesa Domínguez, Claudia; Hurtado Abad, Ana Luz; Seguí Gil, Lucía; Fito Maupoey, Pedro;
Título: Enzymatic hydrolysis of industrial pineapple waste with commercial enzyme mixtures for bioethanol production
Ref. Revista/ libro (ISBN, ISSN, SUPV): International Conference of Food Science and Technology Innovation (FoodInnova 2012)
Páginas: 51 - 51 **Año:** 2012 **Clave:** PR

Autores: Conesa Domínguez, Claudia; Bernat-Senent, Ignacio; Seguí Gil, Lucía; Fito Maupoey, Pedro
Título: Saccharification of Industrial Pineapple Waste with Aspergillus niger Enzymes and the effect of pH and Temperature for obtaining Bioethanol
Ref. Revista/ libro (ISBN, ISSN, SUPV): International Conference of Food Science and Technology Innovation (FoodInnova 2012)
Páginas: 197 - 198 **Año:** 2012 **Clave:** PR

Autores: Conesa Domínguez, Claudia; Fito Suñer, Pedro José; Fito Maupoey, Pedro
Título: Extension of the project for obtaining bioethanol from citrus waste
Ref. Revista/ libro (ISBN, ISSN, SUPV): 12th International Citrus Congress
Páginas: 362 - 362 **Año:** 2012 **Clave:** PR

Autores: Conesa Domínguez, Claudia; Seguí Gil, Lucía; Fito Maupoey, Pedro
Título: Saccharification of industrial pineapple waste with commercial enzyme mixtures
Ref. Revista/ libro (ISBN, ISSN, SUPV): EFFoST Annual Meeting (EFFoST 2012)
Páginas: 19 - 19 **Año:** 2012 **Clave:** PR

A.1.3. Participación en proyectos de investigación

Título: Desarrollo de alimentos funcionales por incorporación o sustitución del azúcar común por azúcares de caña no refinados. (GV/2013/047)
Entidad Financiadora: Generalitat Valenciana
Duración desde: 01/01/2013 **Hasta:** 01/01/2015
Investigador principal: Seguí Gil, Lucía
Importe de la subvención: 12.000,00
Nº total de investigadores: 3

Título: Aplicación de la metodología SAFES para la modelización de la deshidratación y de los cambios estructurales asociados a la conservación frigorífica de cítricos (FPA/2011/059)
Entidad Financiadora: Generalitat Valenciana
Duración desde: 01/06/2011 **Hasta:** 01/12/2011
Investigador principal: Fito Maupoey, Pedro
Importe de la subvención: 9.300,00

A.1.4. Participación en comités de congresos

Autores: Conesa Domínguez, Claudia
Título: IX Congreso Iberoamericano de Ingeniería de Alimentos (CIBIA 9)
Tipo de participación: Miembro del comité de Organización
Congreso: IX Congreso Iberoamericano de Ingeniería de Alimentos (CIBIA 2014)
Publicación: Libro de actas: Congreso Iberoamericano de Ingeniería de Alimentos. Cibia 9
Lugar de celebración: Valencia, España
Año: 2014

A.1.5. Estancias de investigación

Centro: CIRI-Agroalimentare UNIVERSITÀ DI BOLOGNA
Localidad: Cesena **País:** Italia
Año: 2014 **Duración:** 3 meses
Tema: Estancia Predoctoral
Proyecto: Obtención de bioetanol y de productos de alto valor añadido a partir de los residuos de la IV gama del melón cantalupo (*Cucumis melo* var. cantalupo)

A.2. PRODUCTIVIDAD DOCENTE

A.2.1. Actas de congresos

A.2.1.1. Actas de congresos internacionales publicadas en editorial

Autores: Ibáñez Civera, Francisco Javier; Laguarda-Miro, Nicolás; Gil Sánchez, Luis; Conesa Domínguez, Claudia; Montes-Robles, Roberto; Garcia-Breijo, Eduardo
Título: Assessing competences: innovation, creativity and entrepreneurship. The case of the competition car
Editorial: IATED Digital Library
Ref. Revista/ Libro (ISBN, ISSN, SUPV): 11th International Technology, Education and Development Conference (INTED 2017) (ISSN 978-84-617-8491-2)
Páginas: 2905 - 2910 **Año:** 2017

Autores: Ibáñez Civera, Javier; Laguarda Miró, Nicolás; Conesa Domínguez, Claudia; Olguín Pinatti, Cristian; Montes Robles, Roberto; García Breijo, Eduardo; Gil Sánchez, Luis; Sánchez Díaz, Carlos
Título: Integrating electronics in product design
Editorial: IATED
Ref. Revista/ Libro (ISBN, ISSN, SUPV): 10th International Technology, Education and Development Conference (INTED 2016) (ISSN 978-84-608-5617-7)
Páginas: 8329 – 8332 **Año:** 2016

Autores: Laguarda Miró, Nicolás; Ibáñez Civera, Javier; Conesa Domínguez, Claudia; Olguín Pinatti, Cristian; Montes Robles, Roberto; García Breijo, Eduardo; Gil Sánchez, Luis
Título: Assessing transversal competences in sustainable development and environmental ethics. Environmental sensitization under review
Editorial: IATED
Ref. Revista/ Libro (ISBN, ISSN, SUPV): 10th International Technology, Education and Development Conference (INTED 2016) (ISSN 978-84-608-5617-7)
Páginas: 8347 – 8352 **Año:** 2016

Autores: Ibáñez Civera, Francisco Javier; Laguarda Miró, Nicolás; García Breijo, Eduardo; Gil Sánchez, Luís; Conesa Domínguez, Claudia; Olguín Pinatti, Cristian Ariel; Loeff, Edwin
Título: Importance of attendance in achieving the competency objectives
Editorial: IATED
Ref. Revista/ Libro (ISBN, ISSN, SUPV): 9th International Technology, Education and Development Conference (INTED 2015) (ISSN 978-84-606-5763-7)
Páginas: 4465 – 4468 **Año:** 2015 **Clave:** PR

Autores: Laguarda Miró, Nicolás; Conesa Domínguez, Claudia; Ibáñez Civera, Francisco Javier; García Breijo, Eduardo; Gil Sánchez, Luís; Montes Robles, Roberto
Título: Promoting transversal competences in final degree projects: some experiences at the UPV
Editorial: IATED
Ref. Revista/ Libro (ISBN, ISSN, SUPV): 9th International Technology, Education and Development Conference (INTED 2015) (ISSN 978-84-606-5763-7)
Páginas: 4531 – 4535 **Año:** 2015

Autores: Laguarda Miró, Nicolás; García Breijo, Eduardo; Ibáñez Civera, Francisco Javier; Gil Sánchez, Luís; Conesa Domínguez, Claudia; Olguín Pinatti, Cristian Ariel
Título: From sustainable development and environmental ethics to environmental technology: adapting sensitization subjects to the new degrees
Editorial: IATED
Ref. Revista/ Libro (ISBN, ISSN, SUPV): 8th International Technology, Education and Development Conference (INTED 2014) (ISSN 978-84-616-8412-0)
Páginas: 4196 – 4202 **Año:** 2014

A.2.1.2. Actas de congresos nacionales

Autores: Laguarda Miró, Nicolás; Ballester Sarrias, Enrique; Álvarez Valenzuela, Bernardo; Pérez Herrerías, Ricardo; Conesa Domínguez, Claudia
Título: La evaluación continua en la ETSID
Ref. Revista/ Libro (ISBN, ISSN, SUPV): XXIII Congreso Universitario de Innovación Educativa en las Enseñanzas Técnicas (CUIEET 2015) (ISSN 978-84-606-5611-1)
Páginas: 565 - 577 **Año:** 2015 **Clave:** PR

Autores: Castelló Gómez, María Luisa; Cháfer Nácher, María Teresa; Conesa Domínguez, Claudia; Ortolá Ortolá, Mª Dolores
Título: Uso del screencast y la e-evaluación en las en las prácticas de la asignatura tecnología postcosecha en la Universitat Politècnica de València
Editorial: Editorial Universitat Politècnica de València
Ref. Revista/ Libro (ISBN, ISSN, SUPV): Jornadas de Innovación Educativa y Docencia en Red (IN-RED 2014) (ISSN 9788490482711)
Páginas: 251-259 **Año:** 2014 **Clave:** PR

A.2.2. Asignaturas impartidas

Curso: 2015/2016
Departamento: Tecnología de alimentos
Centro: E.T.S.I. Agronómica y del Medio Natural
Grado: Biotecnología
Asignatura: Ingeniería de Procesos Biotecnológicos I
Código: 13685 **Total de horas de docencia:** 5

Curso: 2015/2016
Departamento: Tecnología de alimentos
Centro: E.T.S.I. Agronómica y del Medio Natural
Grado: Biotecnología
Asignatura: Procesos y Productos Biotecnológicos
Código: 11135 **Total de horas de docencia:** 22,5

Curso: 2015/2016
Departamento: Ingeniería Química y Nuclear
Centro: Escuela Técnica Superior de Ingeniería del Diseño
Grado: Grado en Ingeniería en Diseño Industrial y Desarrollo del Producto, Grado en Ingeniería Aeroespacial, Grado en Ingeniería Mecánica, Grado en Ingeniería Eléctrica, Grado en Ingeniería Eléctronica Industrial y Automática
Asignatura: Desarrollo Sostenible y Ética Ambiental
Código: 13658 **Total de horas de docencia:** 22,5

Curso: 2015/2016
Departamento: Ingeniería Química y Nuclear
Centro: Escuela Técnica Superior de Ingeniería del Diseño
Grado: Ingeniería Mecánica
Asignatura: Tecnología Medioambiental
Código: 12574 **Total de horas de docencia:** 10

Curso: 2013/2014
Departamento: Tecnología de alimentos
Centro: E.T.S.I. Agronómica y del Medio Natural
Grado: Ciencia y Tecnología de Alimentos
Asignatura: Operaciones Básicas en la Industria Alimentaria II
Código: 11203 **Total de horas de docencia:** 10

Curso: 2013/2014
Departamento: Tecnología de alimentos
Centro: E.T.S.I. Agronómica y del Medio Natural
Grado: Biotecnología
Asignatura: Fundamentos de Ingeniería de Procesos Biotecnológicos
Código: 11132 **Total de horas de docencia:** 30

Curso: 2013/2014
Departamento: Tecnología de alimentos
Centro: E.T.S.I. Agronómica y del Medio Natural
Ingeniería: Ingeniero Agrónomo (Acceso 2º ciclo)
Asignatura: Tecnología Postcosecha
Código: 3509 **Total de horas de docencia:** 10

A.2.3. T.F.C. / T.F.G. dirigidos y Tesinas de máster

Trabajo Final de Carrera
Titulación: Ingeniero Técnico Industrial esp. Química Industrial – UPV
Título: Aprovechamiento de los residuos industriales de caqui (IV gama) para la obtención de bioetanol y de otros productos de alto valor añadido
Alumna: Ferrando Giménez, Amparo Belén
Fecha: 30/09/2015 **Nota:** 9,2

Trabajo Final de Carrera
Titulación: Ingeniero Técnico Industrial esp. Química Industrial – UPV
Título: Monitorización de la hidrolisis enzimática de los residuos de piña y cuantificación del contenido en etanol mediante espectroscopia de impedancias electroquímicas
Alumna: Gómez Cocera, Jessica
Fecha: 28/09/2015 **Nota:** 8,5

Trabajo Final de Grado

Titulación: Grado en Ingeniería Agroalimentaria y del Medio Rural – UPV

Título: Estudio de la aplicación de microondas en medio alcalino como pretratamiento para la mejora de la hidrólisis enzimática del residuo de industrialización de piña para la producción de bioetanol

Alumna: Mesa Navarro, Andrea

Fecha: 09/2015 **Nota:** 9,5

Trabajo Final de Grado

Titulación: Grado en Ingeniería Eléctrica – UPV

Título: Diseño de un sensor para la medición de temperaturas en el interior de un campo microondas en muestras de residuos frutícolas destinadas a la obtención de bioetanol de segunda generación

Alumno: Francés Abarca, Aitor

Fecha: 24/09/2015 **Nota:** 9,0

Trabajo Final de Carrera

Titulación: Ingeniero Agrónomo– UPV

Título: Identificación y cuantificación de azúcares fermentables en residuo de piña mediante espectroscopía de impedancias para la monitorización de la hidrólisis enzimática en el proceso de obtención de bioetanol

Alumno: Fombuena Alonso, Vicente

Fecha: 20/02/2015 **Nota:** 9,5

Trabajo Final de Carrera

Titulación: Ingeniero Técnico Industrial esp. Química Industrial – UPV

Título: Cuantificación de azúcares en piña mediante el uso de un aparato de espectroscopía de impedancias

Alumna: Jaunarena González, Daniela Alejandra

Fecha: 12/12/2014 **Nota:** 9,5

Trabajo Final de Carrera
Titulación: Ingeniero Agrónomo – UPV
Título: Pretratamiento con microondas para la obtención de bioetanol a partir de residuos industriales de piña: una propuesta de mejora de la hidrólisis enzimática
Alumno: Masiá Romero, Arturo
Fecha: 29/09/2014 **Nota:** 9,0

Trabajo Final de Máster
Titulación: Máster en Ciencia e Ingeniería de los Alimentos – UPV
Título: Efecto del pretratamiento con microondas en la hidrólisis enzimática de los resíduos industriales de la piña para la obtención de bioetanol.
Alumno: Bernat Senent, Ignacio Gerardo
FECHA: 30/09/2013 **Nota:** 10,0

Trabajo Final de Carrera
Titulación: Ingeniero agrónomo - UPV
Título: Hidrólisis enzimática de los residuos industriales de piña para la obtención de bioetanol
Alumna: Hurtado Abad, Ana Luz
Fecha: 19/07/2013 **Nota:** 10,0