Universidad de Valladolid

ESCUELA DE INGENIERÍAS INDUSTRIALES

DEPARTAMENTO DE INGENIERÍA QUÍMICA Y
TECNOLOGÍA DEL MEDIO AMBIENTE

TESIS DOCTORAL:

PRETRATAMIENTO DE BAGAZO DE CAÑA DE AZÚCAR POR OZONÓLISIS PARA OBTENCIÓN DE BIOALCOHOLES: EFECTOS SOBRE LA LIBERACIÓN DE AZÚCARES, LA GENERACIÓN DE INHIBIDORES Y LAS FERMENTACIONES

Presentada por Rodolfo Travaini para optar al grado de doctor por la Universidad de Valladolid

Dirigida por:
Dra. Silvia Bolado Rodríguez

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SECRETARÍA

La presente tesis doctoral queda registrada en el folio número ____ del correspondiente libro de registro número_____.

Valladolid, a ___ de _____ de 2016

Fdo. el encargado del registro.

Dra. Silvia Bolado Rodríguez
Profesora Titular de Universidad
Departamento de Ingeniería Química y Tecnología del Medio Ambiente
Universidad de Valladolid

Certifica que:

RODOLFO TRAVAINI ha realizado bajo su dirección el trabajo *"Pretratamiento de bagazo de caña de azúcar por ozonólisis para obtención de bioalcoholes: efectos sobre la liberación de azúcares, la generación de inhibidores y las fermentaciones"*, en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral, expresa su conformidad con dicha presentación.

Valladolid, a ___ de _____ de 2016

Fdo. Silvia Bolado Rodríguez

Reunido el tribunal que ha juzgado la Tesis Doctoral: *"Pretratamiento de bagazo de caña de azúcar por ozonólisis para obtención de bioalcoholes: efectos sobre la liberación de azúcares, la generación de inhibidores y las fermentaciones"* presentada por el Químico Ambiental Rodolfo Travaini, y en cumplimiento de lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011 acuerda conceder por _____ la calificación de _____.

Valladolid, a ___ de _____ de 2016

PRESIDENTE SECRETARIO

1er VOCAL 2º VOCAL 3er VOCAL

AGRADECIMIENTOS

A Silvia, por su paciencia, dedicación e inagotable energía desde el primer día que me contestó al correo de pedido de estancia con un WELCOME!!! Por haberme demostrado con su exigencia que siempre es posible hacer las cosas mejor, y con su tenacidad que nada es imposible cuando uno se lo propone.

A Enrique Barrado por su gran contribución en los análisis, su ayuda desinteresada y su capacidad de contagiar a los demás con su tranquilidad y buen humor.

A Daniela por su colaboración durante el desarrollo de esta tesis y sobre todo por el apoyo personal y "nossos papos de louco".

A los compis de laboratorio Ana, Judit y Ricardo, por las risas, las discusiones y por el apoyo en los momentos de estrés.

A los compañeros de trabajo (y fiesta) que he tenido el placer de coincidir durante estos años en el departamento: Ieva, María, Rafaella, Alma, Dimas, Sari, Osvaldo, Alija, Javi, Jesús, Mariana, Bruno, Tiago, Josiani, Mayara, Isra, Danielly, Victória, Natália, Ilan, Roxana, Edinéia, Marta Octávio, Silvia... ¡Gracias por los momentos dentro y fuera del departamento!

A todos los profesores y técnicos del Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la UVa, por toda la ayuda, y por formarme como profesional.

A Isidro, por estar siempre a mi lado apoyándome, riéndose de mí y aguantando mis días de poco humor. A toda la gente maravillosa que he conocido aquí "al otro lado del charco", y que aún que alguno se me haya desperdigado por el mundo esté un poco desaparecido, sé que siempre serán parte de mi vida: Jéssica, Paola, Lucía, Marian, Sergio, Rosendo, Misha, Alvaci, Tatjana, Letícia, Emílio, María, Raúl, Belén, Marta, Lenadra, Karin, Mario, Ana Celina, Elisa, Ana, Eva, Jennifer, Marceli, Brunno, Patrick, Andrea, Rober, José, Sunamita, Phillipi, Gil, Arthur.

A mis amigos de siempre, que me hecho ver que es posible estar cerca estando lejos, y que el tiempo es sólo un detalle: Fer, Breno, Alessandra, Dre, Maycon, Luciana, Lilian, Juliana, Samira, Allan, Clarisse, Nayara, Junior, Mila, Janaina, Marcelo, Tiago, Zamoner, Jéssica.

A mi familia. A mis padres pelo apoyo y amor incondicionales, a mi madre por los consejos incontables y por ser mi soporte a todas las horas, a mis hermanas por todo el cariño y pelas risas incontrolables. A mi abuela por oírme siempre y contarme historias. A mis primas por todo el apoyo y buenos momentos. ¡Gracias por vuestro amor incondicional!

A todas las personas que no pude nombrar, más que también me aportaran personalmente de una forma o de otra.

¡A la gran fuerza organizadora del Universo!

Gracias. Obrigado.

ÍNDICE DE CONTENIDOS

RESUMEN

En las últimas décadas, el creciente aumento de la población humana asociado al desarrollo industrial ha llevado al consumo excesivo de combustibles fósiles, que han sido los responsables del incremento en las concentraciones de gases contaminantes en la atmosfera, amenazando la calidad ambiental del planeta. Este panorama ha llevado a la búsqueda de nuevas alternativas para la sustitución de los combustibles fósiles por otras reservas energéticas ambientalmente sostenibles y provenientes de fuentes renovables. Una de las opciones más prometedoras es la producción de biocombustibles líquidos para utilización como carburantes a partir de residuos abundantes como los lignocelulósicos, ricos en azucares. Su ventaja principal es que se trata de residuos que, en vez de ser tratados como tales, pueden ser valorizados generando productos de valor añadido, pero, además, suponen una baja huella de gases del efecto invernadero. Sin embargo, su producción es compleja, y presenta retos tecnológicos que todavía deben de ser superados para llegar a procesos que sean económicamente viables.

La producción de biocombustibles lignocelulósicos, en lo que se denomina combustibles de segunda generación, puede dividirse, de manera simplificada, en tres etapas principales. La primera etapa, el pretratamiento, la clave para la obtención de buenos rendimientos, es la responsable de la desestructuración de la biomasa y liberación de los polímeros de azúcares de su matriz con la lignina que les protege. Una vez liberados los polímeros de azúcares, la etapa siguiente, la hidrólisis enzimática, tiene por objetivo su sacarificación y producción de azúcares más pequeños que puedan ser convertidos por microorganismos en los productos de interés. En una última etapa, los hidrolizados ricos en azúcares simples son

fermentados por microorganismos especializados, convirtiéndolos en biocombustibles.

En esta tesis doctoral se ha estudiado la aplicación del ozono como forma de pretratamiento para producción de biocombustibles a partir de un residuo lignocelulósico muy abundante, el bagazo de caña de azúcar. Este pretratamiento, llamado ozonólisis, es un método químico oxidativo para la remoción de la lignina por degradación y/o solubilización. A pesar de haber sido poco explorado todavía, presenta características muy prometedoras. Comparándole con los pretratamientos tradicionalmente empleados, sus principales ventajas son: la no generación de furfurales o hemifurfurales, sus suaves condiciones de operación (presión y temperatura ambiente), y la ausencia de residuos intermedios, ya que el ozono utilizado se descompone durante el pretratamiento o es fácilmente destruido al final del proceso. Se ha seleccionado la configuración del pretratamiento en lecho fijo, debido a su mejor cinética, mejor aprovechamiento de la corriente de ozono, menor cantidad de reacciones secundarias y ausencia de fase líquida. Se ha utilizado como materia prima el bagazo de caña de azúcar, un residuo generado en grandes cantidades en varios países tropicales, sobre todo Brasil. Este residuo presenta características muy deseables para la aplicación como sustrato, tales como: alta disponibilidad, bajo coste, alto contenido de carbohidratos y bajo contenido de extractivos y cenizas.

En el **Capítulo 1** se presentan la justificación práctica de los estudios de esta tesis doctoral, asimismo los objetivos que se han planteado y su forma de desarrollarse. En el **Capítulo 2** se hace un recogido por la literatura más reciente de la producción de biocombustibles, enfocándolo hacía los objetivos de esta tesis. Se

presentan también su relación temática con los artículos de la tesis, la metodología que se empleó, los resultados alcanzados y las conclusiones más relevantes obtenidas.

El **Capítulo 3** de esta tesis, empleado a modo de presentación de antecedentes o estado del arte, es una revisión ya publicada que recoge los principales aspectos de la utilización de la ozonólisis como pretratamiento: las reacciones involucradas; la generación de compuestos inhibidores; las modificaciones estructurales y morfológicas post pretratamiento; y, los efectos de los parámetros de proceso y de la configuración del reactor empleado.

El **Capítulo 4** se trata del primer estudio realizado sobre la capacidad del pretratamiento por ozonólisis para aumentar la digestibilidad enzimática del bagazo de caña de azúcar. Se exploran las posibilidades de este pretratamiento para la ruptura de lignina y liberación de azúcares y se estudian el efecto de las que se consideraron las principales variables de operación: humedad del bagazo y concentración de ozono en el flujo de aire. Se observó una disminución del tiempo de reacción con el aumento de la concentración de ozono, y el efecto protector de las altas concentraciones de humedad sobre la degradación de los carbohidratos. El poder de delignificación del pretratamiento varió con las combinaciones de humedad y concentración de ozono, con su valor más alto en 66,8%, utilizando 3.44% (mol/mol) de ozono y 40% (m/m) de humedad.

El pretratamiento proporcionó muy reducidos porcentajes de degradación de carbohidratos, con recuperaciones de entre 92,5% y 98,7%. Los principales compuestos inhibidores generados fueron xilitol y ácido acético, satisfactoriamente eliminados por lavado con agua. En la mejor condición de operación aplicada, la

digestibilidad del bagazo aumentó de 6,64% a 41,79% para celulosa y de 2,05 a 52,44% para xilano, comparando bagazo *in natura* y pretratado. Para el bagazo ozonizado-lavado, estos valores fueron de un 46% para celulosa y un 28% para xilano. El aumento en la digestibilidad de la celulosa con el lavado ha sido atribuido a la remoción de compuestos que estarían generando impedimento estérico a las enzimas, mientras que la disminución en el xilano ha sido debido a su lixiviación en el agua del lavado.

Con el objetivo de realizar un estudio sistemático y optimizar las variables de operación, se realizó un estudio estadístico aplicando un diseño experimental con una matriz ortogonal $L_9(3)^4$. Los resultados de este primer estudio estadístico están descritos en **Capítulo 5**, dónde se ha analizado la influencia de los parámetros: humedad, concentración de ozono, flujo de ozono/oxígeno y tamaño de partícula. Los resultados revelaron que la concentración de ozono es el parámetro más importante del pretratamiento para la liberación de azúcares, obteniéndose rendimientos máximos para concentraciones de ozono de 2% (mol/mol). Por otro lado, en cuanto al consumo de ozono, la humedad es el factor más influyente en la producción de azúcares por gramo de ozono gastado en el pretratamiento. La optimización de las condiciones de operación resultó en una combinación similar a la de uno de los experimentos ensayados, donde se obtuvieron un 77,55% y un 56,95% de conversión de celulosa y de xilano, respectivamente.

La fermentación de los hidrolizados por la levadura *Saccharomyces cerevisiae* bakery proporcionó rendimientos de entre 80% y 88% respecto al máximo teórico, y el ANOVA de los resultados demostró ausencia de efecto inhibidor. Por otro lado, la levadura *Pichia stipitis* DSM 3651 fue completamente inhibida, no siendo capaz de

crecer en ningún de los hidrolizados. Estudios preliminares de balance energético entre el ozono gastado y el etanol producido pusieron de manifiesto la necesidad del aprovechamiento de la fracción xilosa.

El estudio del **Capítulo 6** se centra en la producción de inhibidores durante el pretratamiento, y aplica una etapa de detoxificación, mediante lavado con agua, entre el pretratamiento y la hidrólisis enzimática. Este trabajo ha permitido evaluar los efectos de los parámetros de operación sobre la producción de inhibidores, el tipo y concentración de los subproductos generados, el efecto del lavado en la solubilización de la biomasa pretratada y la fermentabilidad de los hidrolizados obtenidos para obtener etanol empleando *Pichia stipitis* y butanol con dos tipos de *Clostridium.*

El proceso de lavado supuso unas pérdidas de materia del bagazo ozonizado de hasta un 30%, debido en gran parte a la lixiviación del xilano y la remoción de los compuestos de degradación de la lignina. Estas pérdidas presentaron relación directa con la cantidad de ozono gastado en el pretratamiento, que afectaron principalmente a la composición de xilano y lignina ácida insoluble. Los rendimientos de conversión de los polímeros de azúcares también se relacionaron directamente con la cantidad de ozono gastado, con ambos aumentando en el mismo sentido.

El análisis de la matriz ortogonal demostró que el ozono y humedad son los factores más importante en la delignificación del bagazo, con sus óptimos en un 2% (mol/mol) y 45% (m/m), respectivamente. En términos de conversión de azúcares, la concentración de ozono fue el parámetro más importante, con su óptimo en un 2% (mol/mol). Por otro lado, la humedad fue el factor determinante en la generación de inhibidores, con mayor generación de compuestos fenólicos para bajas humedades

y mayor formación de ácidos orgánicos para humedades intermedias (con su máximo en 45%, m/m).

El proceso de detoxificación por lavado resultó bastante eficiente, removiendo completamente el ácido fórmico, y hasta un 97%, 82% y 77% de ácido oxálico, ácido acético y compuestos fenólicos, respectivamente. Los inhibidores remanentes en los hidrolizados no afectaron a los microorganismos utilizados para producción de etanol, *Pichia stipitis* DSM 3651, y butanol, *Clostridium acetobutylicum* DSM792 y *Clostridium beijerinckii* DSM6422. Se observó, sin embargo, una relación directa entre la cantidad de azúcar inicial en las fermentaciones y los productos obtenidos, con los rendimientos aumentando con el incremento de la concentración de azúcares iniciales. Para *P. stipitis* se alcanzó hasta un 88% de rendimiento de etanol máximo teórico, mientras que para *C. acetobutylicum* los productos alcanzaron valores de 0,072 $g_{BUTANOL}/g_{AZÚCAR}$ y 0,188 $g_{ABE}/g_{AZÚCAR}$, y, para *C. beijerinckii* 0,165 $g_{BUTANOL}/g_{AZÚCAR}$ y 0,257 $g_{ABE}/g_{AZÚCAR}$. El balance energético para etanol fue favorable, con 1,03 MJ de energía neta generada por kg de bagazo pretratado y fermentado. En cuanto a las fermentaciones para producción de butanol, el balance energético fue negativo para las dos bacterias testadas, resultando un mayor gasto de energía en la producción del ozono que lo obtenido en la combustión teórica de los biocombustibles producidos.

En el **Capítulo 7** se presenta un estudio desarrollado en colaboración con el *Laboratorio de Enzimas Microbianas* del *Instituto de Química* de la *Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP*, campus de Araraquara, Estado de São Paulo, Brasil. En ese trabajo, se evaluó la aplicación de enzimas fúngicas a la hidrólisis del bagazo ozonizado, comparándolas con las enzimas comerciales

tradicionalmente empleadas. Para ello se utilizó un hongo termófilo, *Myceliophtora thermophila* JCP 1-4, aislado en pilas de bagazo de caña de azúcar de factorías de producción de azúcar y alcohol. Las enzimas fúngicas, en hidrólisis a 50 ºC, demostraron resultados ligeramente mejores que las enzimas comerciales. También se encontró en este hongo una característica poco documentada, la capacidad de producir glucosa isomerasa, convirtiendo parte de la glucosa de los hidrolizados en fructosa.

Las enzimas producidas presentaron también buenas características térmicas, con la mejor cinética de producción de glucosa a 60 ºC y 8h de tiempo de hidrólisis. El monitoreo de la actividad enzimática durante las sacarificación del bagazo ozonizado puso de manifiesto, además, que el bagazo ozonizado induce la actividad de las enzimas fúngicas, aumentándola con el tiempo de reacción. Cuando se optimizó la cantidad de FPU por gramo de celulosa, se encontraron los mejores rendimientos de glucosa en 7,5, valor ligeramente más bajo que los convencionalmente utilizados en trabajos para hidrólisis de materiales lignocelulósicos pretratados. Con el objetivo de hidrolizar bagazo en mayores concentraciones, las enzimas producidas fueron concentradas por rota vapor. Si por un lado las enzimas presentaron buena resistencia al proceso de concentración, por otro se encontró un ligero efecto inhibidor trabajando con mayores cargas de materia seca en las hidrólisis, probablemente debido a la inhibición de la β-glucosidasa. Los mejores hidrolizados obtenidos fueron fermentados por *Saccharomyces cerevisiae* bakery para producción de etanol, con rendimientos alrededor del 60% del máximo teórico.

RELACIÓN DE ARTÍCULOS PERTENECIENTES A LA TESIS

Esta tesis doctoral se presenta como un compendio de publicaciones, y los artículos incluidos en ella como capítulos están listados a continuación. Se compone de una revisión del tema principal de la tesis, el pretratamiento por ozonólisis, y cuatro artículos originales de investigación, todos publicados en la revista internacional *Bioresource Technology*, que posee un factor de impacto de 4,917 y está indexada en la base *Journal Citation Report* (JCR), en el primer cuartil, ocupando la primera posición del ranking en la categoría de *Agricultural Engineering*.

Revisión. Travaini, R., Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodríguez, S., 2016. Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited. Bioresour. Technol. 199, 2–12.

Artículo I. Travaini, R., Otero, M.D.M., Coca, M., Da-Silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation. Bioresour. Technol. 133, 332–339.

Artículo II. Travaini, R., Barrado, E., Bolado-Rodríguez, S., 2016. Effect of ozonolysis pretreatment parameters on the sugar release, ozone consumption and ethanol production from sugarcane bagasse. Bioresour. Technol. 214, 150–158.

Artículo III. Travaini, R., Barrado, E., Bolado-Rodríguez, S., 2016. Effect of ozonolysis parameters on generation of inhibitory compounds and on production of ethanol by

Pichia stipitis and Acetona-Butanol-Ethanol by *Clostridium* from ozonated and water washed sugarcane bagasse. Bioresour. Technol. In press.

Artículo IV. de Cassia Pereira, J., Travaini, R., Paganini Marques, N., Bolado-Rodríguez, S., Bocchini Martins, D.A., 2016. Saccharification of ozonated sugarcane bagasse using enzymes from *Myceliophthora thermophila* JCP 1-4 for sugars release and ethanol production. Bioresour. Technol. 204, 122–129.

CONTRIBUCIÓN A LOS ARTÍCULOS INCLUIDOS EN LA TESIS

Revisión. En ese trabajo, fui el responsable de la revisión bibliográfica y organización de todos los datos publicados hasta la fecha de envío a la revista, sobre la utilización de la ozonólisis como pretratamiento de biomasa lignocelulósica para producción de biocombustibles y compuestos de valor industrial. La revisión fue escrita con la colaboración de Judit Martín Juárez y Ana Lorenzo Hernando, bajo la supervisión de la Dra. Silvia Bolado Rodríguez.

Artículo I. Fui el responsable de la realización de los experimentos y análisis de laboratorio con la colaboración de Marian Derly Morales Otero. Realicé los cálculos, balances de materia y escritura del artículo bajo la supervisión de la Dra. Silvia Bolado Rodríguez y del Dr. Roberto da Silvia. La Dra. Mónica Coca colaboró en la forma de organización y presentación del artículo.

Artículo II. En este artículo fui el responsable de los cambios realizados en el montaje experimental respecto a los trabajos anteriores para que fuese posible hacer un análisis estadístico, asimismo realicé los experimentos, análisis de laboratorio y cálculos estadísticos. El diseño de experimento y el análisis estadístico del trabajo fueron ejecutados bajo la supervisión del Dr. Enrique Barrado; los balances de materia, análisis de resultados y escritura del artículo fueron ejecutados bajo la supervisión de la Dra. Silvia Bolado Rodríguez.

Artículo III. Este trabajo fue realizado por mí en términos de experimentación, análisis de laboratorio y análisis estadístico. El estudio estadístico fue desarrollado bajo la

supervisión del Dr. Enrique Barrado, y los balances de materia, análisis de resultados y escritura del artículo bajo la supervisión de la Dra. Silvia Bolado Rodríguez.

Artículo IV. Este trabajo fue realizado en colaboración con el *Laboratorio de Enzimas Microbianas* del *Instituto de Química* de la *Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP*, campus de Araraquara, Estado de São Paulo, Brasil. Fui el responsable de definir los experimentos de este artículo y auxiliar en la ejecución de los experimentos y análisis de laboratorio, asimismo responsable de la supervisión de la alumna Josiani de Cássia Pereira, durante su estancia de investigación en la Universidad de Valladolid. Ambos trabajamos en los cálculos, análisis de resultados y preparación del manuscrito bajo la supervisión de la Dr. Silvia Bolado Rodríguez y de la Dra. Daniela Alonso Bocchini Martins. Natália Paganini Marques contribuyó con el estudio microbiológica de este trabajo.

ALCANCE DE LA TESIS

CAPITULO 1

1.1 JUSTIFICACIÓN DE LA TESIS

El crecimiento de la población mundial, unido al desarrollo industrial en las últimas décadas, ha llevado la humanidad a una dependencia energética de los combustibles fósiles derivados del petróleo. Este elevado nivel de su uso ha sido relacionado con diversos problemas ambientales, sobretodo la emisión de contaminantes y gases del efecto invernadero. A esto hay que unir los problemas de tipo político y económico surgidos debido a la concentración de las reservas de combustibles fósiles en zonas muy limitadas del globo terrestre. Esta situación ha hecho que la búsqueda de sustitutos a estos combustibles fósiles, resulte de gran interés. Dentro de las muchas alternativas propuestas, los biocombustibles de segunda generación han aparecido como una de las opciones más prometedoras. Las principales ventajas atribuidas a estos biocombustibles son la gran disponibilidad de materia prima de bajo coste, su renovabilidad y bajo impacto ambiental, comparados con los combustibles tradicionalmente utilizados. Aunque estos también generan gases del efecto invernadero durante su quema, el carbono liberado proviene de carbono disponible asimilado recientemente en las materias primas, y no de reservas de carbono acumulado, como ocurre con los combustibles fósiles.

Pese a los grandes esfuerzos científicos destinados al desarrollo de combustibles de segunda generación, muchos retos tecnológicos quedan por solucionarse antes de su aplicación industrial. Los procesos de pretratamientos tradicionalmente utilizados en la desestructuración de la biomasa lignocelulósica, a pesar de sus buenos rendimientos de azúcares, generan cantidades de inhibidores

que reducen considerablemente los rendimientos de la etapa de fermentación. Por otro lado, otros pretratamientos que generan inhibidores en menor cantidad proporcionan rendimientos de liberación de azúcares muy reducidos, no resultando viables. De ese modo, se hace necesario el estudio de nuevos métodos de pretratamiento que produzcan buenos rendimientos de azúcares a la vez que generen inhibidores en baja cantidad, o de fácil detoxificación.

Teniendo en cuenta este panorama, ésta tesis doctoral se basa en el estudio de un pretratamiento que ha resurgido en los últimos años con resultados prometedores, la ozonolisis. Varios estudios han sido publicados en la literatura evaluando la aplicación de este pretratamiento con diversos tipos de materias primas, pero muchas cuestiones relativas a su puesta en práctica y mecanismos de actuación permanecían inexploradas. Se ha elegido como materia prima de estudio el bagazo de caña de azúcar, un residuo lignocelulósico generado en grandes cantidades en varios países, con bajo coste y características deseables para la producción de biocombustibles de segunda generación.

1.2 OBJETIVOS DEL ESTUDIO

El objetivo general de este estudio es evaluar la aplicación del pretratamiento por ozonólisis sobre el bagazo de caña de azúcar para la producción de azúcares por hidrólisis enzimática, y su fermentación para la producción de etanol y butanol. Para lograr este objetivo el bagazo de caña de azúcar fue pretratado en un reactor de lecho fijo de escala de laboratorio, variándose los parámetros de proceso. Los sólidos pretratados fueron sometidos a hidrólisis enzimáticas estándar con enzimas

comerciales y los hidrolizados obtenidos fermentados con levaduras para producción de etanol y con bacterias para producción de butanol. Se evaluó también la aplicación de enzimas fúngicas producidas por un hongo termófilo en la sacarificación del bagazo pretratado, y su comportamiento durante las hidrólisis enzimáticas. De esta manera, se plantearon los siguientes objetivos específicos:

1. Estudiar el impacto del pretratamiento por ozonólisis sobre los cambios estructurales del bagazo de caña de azúcar, la generación de inhibidores y los rendimientos de azúcares.
2. Estudiar la etapa de sacarificación del bagazo pretratado por hidrólisis enzimática utilizando enzimas comerciales, celulasa y β-glucosidasa, y un extracto enzimático producido por un hongo termófilo. Se ha planteado también el estudio de la influencia de las condiciones de hidrólisis sobre los rendimientos de las enzimas fúngicas.
3. Evaluar el efecto de los principales parámetros de proceso de la ozonólisis (humedad, concentración de ozono, flujo de ozono/oxígeno y tamaño de partícula) y sus interacciones, sobre los rendimientos de azúcares, consumo de ozono y generación de inhibidores.
4. Optimizar los parámetros de pretratamiento para la máxima producción de azúcares, menor consumo de ozono y mínima generación de inhibidores.
5. Analizar el efecto de la aplicación de una etapa de detoxificación del bagazo pretratado, por lavado con agua, sobre la composición, rendimientos de azúcares y fermentaciones.

6. Estudiar la producción de biocombustibles a partir de los hidrolizados enzimáticos. Para la producción de etanol se utilizó la levadura *Saccharomyces cerevisiae*, capaz de convertir glucosa, y la levadura diaúxica *Pichia stipitis*, capaz de convertir glucosa y xilosa. Para la producción de butanol por fermentación ABE se utilizaron dos bacterias del genero *Clostridia*, capaces de convertir glucosa, xilosa y oligómeros: *Clostridium acetobutylicum* y *Clostridium beijerinckii*.
7. Realizar un estudio preliminar de balance energético entre la cantidad de energía gastada en la producción de ozono y la energía liberada por la combustión de los biocombustibles obtenidos.

3.3 DESARROLLO DEL ESTUDIO

Para lograr los objetivos planteados en esta tesis, el trabajo fue dividido en cuatro estudios publicados como cuatro artículos originales de investigación.

En el **Artículo I**, es un estudio preliminar donde se estudió el efecto de los parámetros de operación humedad y concentración de ozono, sobre:

- la capacidad de la ozonólisis en aumentar la digestibilidad enzimática del bagazo de caña de azúcar;
- el consumo de ozono y el tiempo de finalización de la reacción en el lecho fijo empleado;
- los cambios composicionales ocurridos en el bagazo tras el pretratamiento respecto a lignina ácida soluble, lignina ácida insoluble, celulosa y xilano, y los cambios morfológicos por microscopia electrónica de barrido;

- la generación de inhibidores de fermentación y la capacidad de detoxificación por un lavado con agua.

En el **Artículo II**, se estudiaron estadísticamente los efectos de los parámetros de operación humedad, concentración de ozono, flujo de ozono/oxígeno y tamaño de partícula, y sus interacciones, a través de un diseño experimental fraccionario aplicándose para ello una matriz ortogonal $L_9(3)^4$. El porcentaje de efecto de cada parámetro, y de sus interacciones, fueron cuantificados para rendimiento de azúcares en las hidrólisis enzimáticas y tiempo de reacción. El consumo de ozono fue relacionado con la liberación de azúcares. Los parámetros estudiados fueron optimizados para la máxima producción de azúcares y, también, para la máxima producción de azúcares con el mínimo consumo de ozono. Los hidrolizados obtenidos fueron fermentados con la levadura *Saccharomyces cerevisiae* bakery y *Pichia stipitis* para obtención de etanol, y se realizó un estudio preliminar de balance energético.

En el **Artículo III**, se empleó el mismo diseño experimental del Artículo II, pero aplicándose un etapa de detoxificación por lavado con agua entre el pretratamiento y la hidrólisis enzimática. En este estudio, se cuantificaron los efectos de los parámetros de operación sobre: la formación de inhibidores de fermentación; los rendimientos de las hidrólisis realizadas con bagazo detoxificado; y, sobre los cambios de composición y la delignificación del bagazo. Se determinó la eficiencia de la etapa de detoxificación, y se relacionó el consumo de ozono con la generación de inhibidores y los rendimientos de liberación de azúcares. Los hidrolizados obtenidos con las mayores concentraciones de azúcares y con el menor gasto de ozono fueron fermentados para producción de etanol por *Pichia stipitis* DSM3651 y

de acetona-butanol-etanol por *Clostridium acetobutylicum* DSM792 y *Clostridium beijerinckii* DSM6422. Un balance energético preliminar entre la energía gastada para la producción de ozono y la energía teórica desprendida por los combustibles obtenidos fue también realizado.

En el **Artículo IV**, se estudiaron la hidrólisis enzimática y fermentación de bagazo ozonizado, utilizándose para sacarificación las enzimas celulolíticas del hongo termófilo *Myceliophtora thermophila* JCP 1-4 y los resultados obtenidos, comparados con enzimas comerciales. Se estudió la influencia de los parámetros de hidrólisis tiempo, temperatura, carga de enzima y carga de bagazo sobre los rendimientos de glucosa y xilosa. Las actividades enzimáticas de FPAsa y β-glucosidasa fueron monitoreadas durante las hidrólisis, con el objetivo de evaluar efectos de inhibición o inducción del bagazo ozonizado sobre las enzimas. La actividad de glucosa isomerasa también fue monitoreada, una vez que se encontró fructosa en los hidrolizados. Los mejores hidrolizados enzimáticos obtenidos fueron fermentados por *Saccharomyces cerevisiae* bakery para evaluar su conversión a etanol.

INTRODUCCIÓN

CAPITULO 2

2.1 ANTECEDENTES

Haciendo un análisis histórico del desarrollo industrial, los últimos 100 años han sido marcados por grandes avances científicos y tecnológicos, lo que ha permitido incrementar de forma masiva la industrialización en muchos países. El desarrollo industrial, unido al crecimiento de la población mundial ha hecho aumentar exponencialmente la demanda energética, tradicionalmente suministrada por combustibles de matriz fósil (Figura 1).

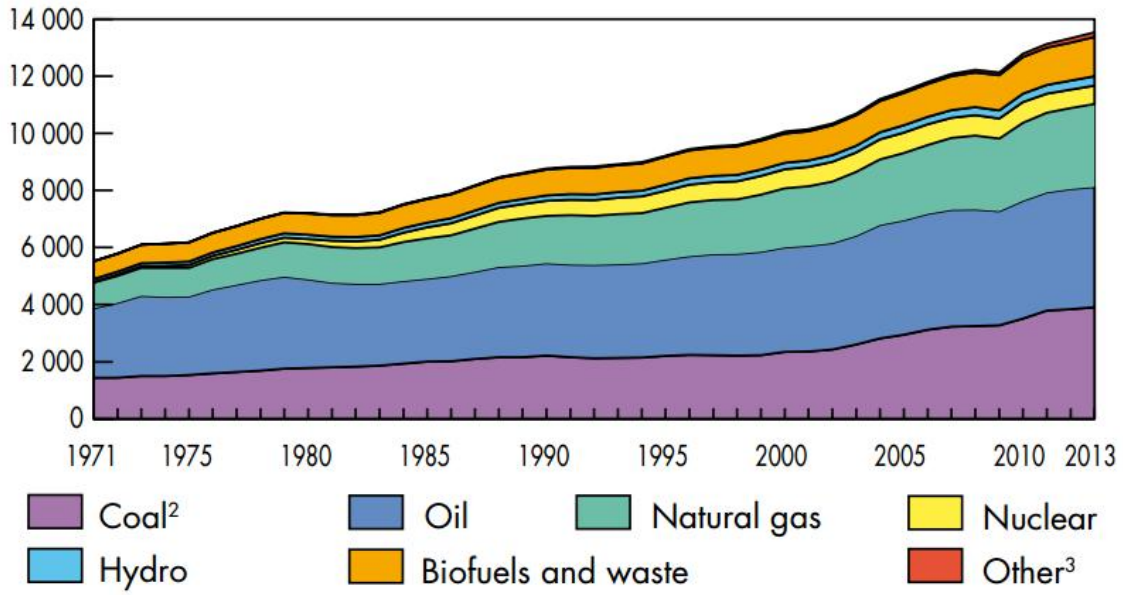

Figura 1: Fuentes de energía primaria totales a nivel mundial (Mtoe) de 1971 a 2013. Mtoe: millones de toneladas equivalentes de petróleo (1toe=11630kWh). Figura adaptada del *Key World Energy Statistics 2015* (IEA, 2015).

Según datos de la *International Energy Agency*, de toda la energía primaria consumida actualmente, un 81.4% proviene de fuentes fósiles no-renovables, de las

cuales un 31.1% es generado de petróleo. Se estima un consumo actual de 94 millones de barriles de petróleo al día, con expectativa de 112 millones barriles al día para 2020 (IEA, 2015).

El consumo de petróleo ha sido relacionado en las últimas décadas con diversos problemas de carácter ambiental, político y económico. Tres crisis en la comercialización del petróleo son descritas históricamente: la primera en 1973, debido a especulaciones económicas entre Occidente y Oriente; la segunda en 1980, debido a la disminución en la producción de barriles por Irán, que pasaba por una revolución; y por fin, el período comprendido entre 2003 y 2007, donde un aumento crecente en el consumo con una producción casi constante ha resultado en las mayores oscilaciones históricas de su precio.

Los problemas ambientales y las crisis económicas y políticas sufridas a causa de la concentración de las extracciones de petróleo en zonas de conflicto ha sido el factor desencadenante para la búsqueda de combustibles que pudiesen reemplazarlo. Dentro de las alternativas que han surgido, la producción de biocombustibles a partir de fuentes renovables rápidamente ha ganado fuerza y se ha expandido por diversos países. El término biocombustible abarca todos los carburantes líquidos y gaseosos producidos a partir de biomasa vegetal. El primer programa de producción masiva de combustibles no-fósiles a partir de biomasa (biocombustible) tuvo lugar en Brasil, en 1975, con el programa de gobierno llamado PROALCOOL. El programa se basó en la producción de etanol a partir de caña de azúcar para su utilización como carburante en motores dedicados, así como para ser utilizado como aditivo en la gasolina de motores convencionales. Con el paso de los años, varios otros programas han surgido en diferentes países y uniones de países.

Dentro de la Unión Europea, la directiva 2009/28/CE establece que el 20% del consumo final de energía deberá provenir de fuentes renovables en 2020. Establece también que de estos 20%, un 10% deberá ser utilizado en el sector de transportes, escenario donde los biocombustibles cobran especial importancia. Ha sido publicada asimismo, en 2012, una propuesta de modificación de la Directiva emitida por la Comisión Europea, dónde se limita a un 5% la utilización de biocombustibles procedentes de cultivos, medida que busca evitar el aumento en los precios de cultivos destinados a fines alimentarios.

La producción de bioetanol a partir de extractos concentrados de azúcares procedentes de cultivos le encuadra dentro los combustibles denominados de primera generación (1G). A pesar de las ventajas ambientales de la utilización de estos combustibles frente a los combustibles fósiles, el uso de tierras para cultivos dedicados a la producción de biocombustibles ha provocado la disminución de la producción de alimentos y su consecuente aumento de precio en muchos países (Alvira et al., 2010; Balat, 2011). Dentro de este contexto, la investigación científica en los últimos años se ha dedicado a la búsqueda de rutas para producción de biocombustibles a partir de los residuos generados por la actividad agrícola y agroindustrial, los llamados residuos lignocelulósicos. Estos residuos, a través de una serie de etapas, son convertidos en los denominados biocombustibles de segunda generación (2G). En diversos países, políticas ambientales han sido desarrolladas fomentando la producción de biocombustibles 2G, como forma de disminuir la emisión de sustancias contaminantes, y también para disminuir su dependencia de los países productores de combustibles fósiles.

Entre los biocombustibles que pueden ser producidos a partir de material lignocelulósico se destacan el etanol y el butanol. El etanol puede ser utilizado directamente como combustible en motores dedicados o en motores denominados flex, capaces de trabajar con gasolina, etanol o cualquier proporción de mezcla entre ellos; ambos motores ya son utilizados en diversos países. El etanol también es utilizado como aditivo a la gasolina para motores convencionales, llegando a mezclas de hasta un 20% sin dañar las maquinarias. El butanol, por otro lado, posee en poder calorífico más alto que etanol, y puede ser utilizado directamente en motores movidos a gasolina sin la necesidad de modificaciones (Dias et al., 2011; Raganati et al., 2015; Soccol et al., 2010; Su et al., 2015).

El biocombustible más utilizado actualmente es el bioetanol 1G procedente mayoritariamente de la caña de azúcar, cultivada en diversos países del mundo, como Brasil, India, China, Tailandia, Colombia, etc. Brasil posee la mayor área cultivada de caña de azúcar del mundo, con aproximadamente 10,87 millones de hectáreas plantadas en 2015, y en 667 millones de toneladas procesadas en la cosecha del año 2015/2016 (UNICA, 2016). Se espera que para los próximos años estos valores sigan aumentando, debido a las políticas públicas del país para producción de bioetanol 1G. El residuo resultante tras la extracción del jugo de la caña de azúcar para la producción de bioetanol 1G y productos alimentarios (azúcar y aguardiente), denominado bagazo, es generado en grandes cantidades, con aproximadamente 250 kg por tonelada de caña procesada. Es un material lignocelulósico fibroso con alto contenido de azúcares y baja composición de cenizas, características que le confieren grandes ventajas frente a otras materias primas para producción de biocombustibles 2G.

2.2 PRODUCCIÓN DE BIOCOMBUSTIBLES DE SEGUNDA GENERACIÓN A PARTIR DE BAGAZO DE CAÑA DE AZÚCAR PRETRATADO POR OZONÓLISIS

La producción de biocombustibles 2G está basada en la utilización de los azúcares contenidos en la biomasa lignocelulósica, y requiere como mínimo tres etapas principales: (i) el pretratamiento, dónde se busca la desestructuración de la biomasa y liberación de los polímeros de azúcares de sus enlaces con la lignina; (ii) la hidrólisis enzimática de los polímeros de azúcares, convirtiéndoles en sus respectivas unidades monoméricas; y (iii) la fermentación de los hidrolizados producidos para su conversión en el biocombustible de interés.

2.2.1 BIOMASA LIGNOCELULÓSICA

La pared celular de la biomasa lignocelulósica está compuesta principalmente de tres polímeros: celulosa, hemicelulosa y lignina. La celulosa es un homopolímero lineal de unidades de D-glucosa, unidas por enlaces glucosídicos β-1,4. Presenta regiones cristalinas (más ordenadas y lineales) y amorfas (desordenada y más susceptible a la degradación enzimática). La hemicelulosa es un heteropolímero ramificado compuesto por unidades de varios tipos de azúcares y ácidos urónicos unidos por enlaces variados. Generalmente presenta un esqueleto principal más o menos lineal de unidades de xilosa unidas por enlaces β1-4 y β1-3. La lignina es un heteropolímero amorfo, tridimensional y altamente ramificado, constituido por unidades monómeras de alcoholes aromáticos enlazados de manera

aleatoria (Haghighi Mood et al., 2013; Harmsen et al., 2010; Tomás-Pejó et al., 2011).

La principal barrera a la utilización de los azúcares contenidos en los materiales lignocelulósicos es la lignina. La estructura de la biomasa (Figura 2), si se observa desde dentro hacía fuera, presenta en la parte más interna las microfibrilas de celulosa, envueltas por polímeros de menor tamaño de hemicelulosa que, a su vez, están ligados covalentemente por carbohidratos mediadores a la lignina. Esta última actúa como el material que confiere resistencia a la pared celular y de protección de los carbohidratos, y es de difícil degradación química y biológica.

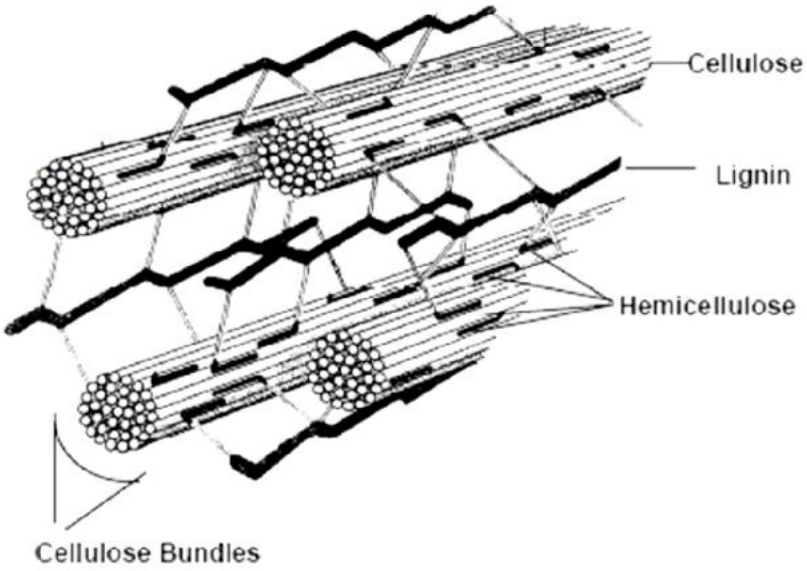

Figura 2: Estructura lignocelulósica de la pared celular vegetal con celulosa, hemicelulosa y lignina (Akerholm and Salmen, 2003).

Diversos tipos de biomasa lignocelulósica han sido estudiados como materias primas para la producción de biocombustibles, desde residuos sólidos urbanos, maderas duras y blandas, residuos agrícolas, herbáceos, y hasta cultivos dedicados

de alto contenido energético. El residuo más estudiado con diferencia es la paja de trigo, producida en muchos países del hemisferio norte (García-Cubero et al., 2009; Tomás-Pejó et al., 2011). El bagazo de caña de azúcar, pese a su gran producción anteriormente citada, todavía se encuentra poco explorado si se compara con otras materias primas. Entretanto, presenta grandes ventajas, como un alto contenido de polímeros de carbohidratos (alrededor de 70%), bajo contenido de cenizas (entre 1-5%) y bajo contenido de extractos solubles (entre 4-10% de acuerdo con las condiciones de cultivo) que podrían interferir en el pretratamiento y favorecer la generación de subproductos tóxicos (Benjamin et al., 2013; Canilha et al., 2012; Driemeier et al., 2011). También es destacable que el bagazo de caña de azúcar post procesamiento presenta un tamaño de partícula bastante menor que aquél encontrado para otros residuos lignocelulósicos, disminuyendo así costes relacionados a procesos de reducción de tamaño de partículas.

El bagazo de caña de azúcar utilizado en los estudios de esta tesis doctoral proviene de factorías de producción de azúcar y alcohol del interior del Estado de São Paulo, Brasil. Una está situada en el municipio de Onda Verde, la Usina Vale, y la otra en el municipio de José Bonifacio, la Usina Virgolino de Oliveira S/A. Ambas factorías utilizan el proceso de extracción de azúcares de la caña por molienda, que consiste en pasar los tallos previamente cortados por una prensa con rodillos a presión controlada. Este método resulta en entre un 94% y 98% de eficiencia de extracción de azúcares, generando un residuo con un 50% de agua, 46% de fibras y un 4% de sólidos solubles.

Para la realización de nuestros estudios, bagazo recién molido era recogido de la parte superficial de las pilas de almacenamiento de la factoría, evitándose así

la utilización de materia prima que hubiese podido haber sido degradada. El bagazo era llevado al laboratorio colaborador en Brasil, dónde era lavado primeramente con agua potable y enseguida con agua destilada, para remoción de partículas de tierra y sólidos solubles remanentes de la extracción. Después del lavado, el bagazo era dispuesto en camadas delgadas en una estufa con ventilación forzada a 37 ºC. El bagazo lavado y seco era entonces almacenado herméticamente cerrado en bolsas de plástico hasta su utilización.

En los **Artículos I y IV**, el bagazo lavado y seco proveniente de la Usina Vale, antes de ser pretratado fue cortado en molienda de cuchillos para reducción de sus partículas hasta un tamaño entre 3-5 mm. Este presentó una composición química en % (m/m): 2,15±0,09 de humedad; 46,21±0,10 de celulosa (como glucosa); 20,86±0,05 de hemicelulosa (como xilosa); 19,54±0,03 de lignina ácida insoluble, 3,13±0,04 de lignina ácida soluble; 1,54±0,08 de extractivos solubles y 1,19±0,10 de cenizas (Travaini et al., 2013).

En los **Artículos II y III**, el bagazo lavado y seco concedido por la Usina Virgolino de Oliveira, S/A, fue tamizado y las partículas mayores que 4,76 mm descartadas, para remoción de las fibras demasiado largas que podrían generar problemas de compactación en el lecho fijo de pretratamiento. La fracción resultante fue utilizada o bien de manera integral, o tamizada una segunda vez con tamiz de 1,25 mm, resultando en tres fracciones de ensayo: Ø<4,76 mm, 4,76 mm<Ø<1,25 mm y Ø<1,25 mm. Las tres fracciones utilizadas presentaron una composición promedia en % (m/m): 44,74±0,47 de celulosa (como glucosa); 25,74±0,26 de hemicelulosa (como xilosa); 21,54±0,44 de lignina ácida insoluble, 4,56±0,12 de lignina ácida soluble y 5,55±0,01 de cenizas (Travaini et al., 2016a).

2.2.2 PRETRATAMIENTO

El principal objetivo de la etapa de pretratamiento es la reorganización de la lignina, y el aumento de la accesibilidad de las enzimas a los carbohidratos en la etapa subsiguiente de hidrólisis enzimática. En esta etapa deben ocurrir modificaciones que transformen la biomasa a nivel macroscópico, microscópico y sub-microscópico, llegando a la desestructuración de la biomasa, por la remoción de compuestos y apertura de sus poros, asimismo alterando la estructura molecular de sus constituyentes, permitiendo un proceso de hidrólisis eficiente. Para que un proceso de pretratamiento sea considerado como prometedor, también se deben de tener en cuenta otras características, tales como: (i) bajo ataque a los polímeros de azúcares; (ii) que no exija etapas previas de preparación de la materia prima demasiado complejas o costosas energéticamente; (iii) que presente baja formación de productos de degradación potencialmente inhibitorios, o en el caso de generación, que sean de fácil detoxificación; y, (iv) presentar fácil recuperación del sólido para las etapas siguientes (Balat, 2011; Hendriks and Zeeman, 2009; Ravindran and Jaiswal, 2016).

Diversos tipos de pretratamientos han sido estudiados, conllevando procesos físicos, químicos, físico-químicos y biológicos, y las combinaciones entre ellos. En la literatura, se encuentran diversos tipos de procesos para el pretratamiento de bagazo de caña de azúcar, destacándose la explosión a vapor sola y combinada (Kaar et al., 1998; Rocha et al., 2012; Wanderley et al., 2013), el organosolv (Mesa

et al., 2016) y la hidrólisis térmica ácida (Chandel et al., 2007; Hernández-Salas et al., 2009; Neureiter et al., 2002).

En los últimos años el pretratamiento químico con ozono, denominado ozonólisis, ha ganado un papel destacado debido a sus ventajas frente a otros pretratamientos tradicionalmente estudiados. Se pueden destacar: (i) la no generación de compuestos de degradación de azúcares, furfural y 5-(hidroximetil)furfural, conocidos como fuertes inhibidores de fermentación; (ii) su selectividad en degradar la lignina debido a su composición rica en dobles enlaces y anillos aromáticos, evitando reacciones secundarias; (iii) reacciones a temperatura y presión ambiente; y, (iv) generación *in situ* del ozono y fácil descomposición al final del proceso cuando necesario (Travaini et al., 2015). Las dos configuraciones más utilizadas para este tipo de pretratamiento son: reactores de mezcla perfecta y reactores de lecho fijo, habiendo trabajos publicados también con variaciones de estos (Travaini et al., 2015). Además de las ventajas generales de la ozonólisis, la operación en lecho fijo conlleva otras como: la ausencia de fase líquida, evitando problemas de separación, reacciones secundarias y perdidas de ozono sin reaccionar; y, una reducción del consumo de ozono, con mejores rendimientos si se compara con reactores mezcla perfecta (Binder et al., 1980; Garcia-Cubero et al., 2012; Li et al., 2015; Neely, 1984). El Capítulo 3 de esta tesis se trata de una revisión sobre la ozonólisis como pretratamiento: Travaini, R., Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodríguez, S., 2016. Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited. Bioresour. Technol. 199, 2–12. En él se discuten las diferentes configuraciones para operar ese pretratamiento; los estudios disponibles sobre el tema; las reacciones químicas involucradas; la

formación de compuestos inhibidores; las modificaciones estructurales y morfológicas post pretratamiento; y en profundidad, el efecto de los parámetros de proceso sobre la materia prima utilizada.

La ozonólisis como pretratamiento ha sido estudiada para diversos tipos de biomasa, principalmente paja de trigo, con estudios estadísticos sistemáticos y de fermentación disponibles (Garcia-Cubero et al., 2010a; García-Cubero et al., 2009; Schultz-Jensen et al., 2011a). Entretanto, para el bagazo de caña de azúcar pocos estudios sobre la ozonólisis se encontraban disponibles, y para sustratos lignocelulósicos, en general, ningún estudio sistemático respecto al efecto de los parámetros de proceso sobre los rendimientos, tema central de esta tesis de doctorado.

En todos los artículos presentados en esta tesis doctoral, se pretrató el bagazo de caña de azúcar en configuración de lecho fijo, utilizándose para ello de un reactor de vidrio con 50 cm de alto y 2,7 cm de diámetro interno. Los pretratamientos se llevaron a cabo siempre a temperatura y presión ambiente. El pretratamiento se realizaba con una corriente de ozono generada por un ozonizador que trabajaba por efecto corona. En los **Artículos I y IV** se alimentó el ozonizador con aire comprimido, mientras que en los **Artículos II y III** se utilizó oxígeno de grado industrial, necesario para llegar hasta la concentración más alta de ozono estudiada (4% mol/mol).

Antes de empezar cada ensayo de pretratamiento, el reactor era cargado con bagazo en el tamaño de partícula y humedad deseadas, el flujo del gas de alimentación del ozonizador se medía con un rotámetro y la concentración de ozono en el flujo regulada por el potenciómetro eléctrico del equipo. La concentración de ozono en el flujo de entrada y salida del reactor era medida por titulación

iodométrica, con una disolución de KI al 20% contenida en botellas lavadoras de gases (APHA-AWWA-WEF, 2005). La reacción era parada 15 min después de que apareciera color en las botellas de la salida del reactor, garantizándose así que la reacción hubiese sido homogénea en los tres cuartos inferiores del lecho (Garcia-Cubero et al., 2012). La cantidad de ozono en el flujo de entrada y la no reaccionada en los 15 minutos finales de reacción eran tenidos en cuenta para los cálculos de la cantidad de ozono gastado durante el pretratamiento.

En el **Artículo I** se estudiaron las variables humedad y concentración de ozono, la primera entre 28% y 80% (m/m), y la segunda entre 1,37% y 3,44% (v/v). Se mantuvieron fijos el flujo, en 60 L/h, y el tamaño de partícula, 3-5 mm. En los **Artículos II y III**, se aplicó un diseño experimental con una matriz ortogonal $L_9(3)^4$, dónde se estudiaron las 4 principales variables según la literatura (Travaini et al., 2016b): la humedad, entre 30-70% (m/m); la concentración de ozono, entre 2-4% (mol/mol), el flujo de ozono oxígeno, entre 30-90 L/h; y, el tamaño de partícula, con las fracciones Ø<4,76 mm, 4,76 mm<Ø<1,25 mm y Ø<1,25 mm. En el **Artículo IV**, se utilizaron las mejores condiciones para rendimientos de azúcares encontradas en el Artículo I, ya que ese trabajo fue realizado antes de la optimización del proceso y se buscaba estudiar el comportamiento de las enzimas en el bagazo pretratado.

Se alcanzó un máximo de 66,8% de delignificación (**Artículo I**), con una composición química final del pretratado en % (m/m): 6,49 de lignina ácida insoluble, 7,21 de lignina ácida soluble, 41,43 de celulosa y 19,57 de xilano. El estudio estadístico (**Artículos II y III**) demostró que la concentración de ozono es el parámetro de proceso más influyente sobre el pretratamiento, con los mejores resultados

obtenidos con un 2% (mol/mol). La humedad por otro lado es el factor determinante sobre el consumo de ozono durante el pretratamiento.

2.2.2.1 COMPUESTOS INHIBIDORES Y DETOXIFICACIÓN

Durante el pretratamiento, aparte de las reacciones esperadas de degradación de la lignina, pueden ocurrir otras reacciones secundarias, incluida la de degradación de carbohidratos, generando todas ellas subproductos. Estos compuestos son denominados de forma genérica de inhibidores, ya que pueden actuar disminuyendo los rendimientos de las hidrólisis enzimáticas, de las fermentaciones o incluso de las dos a la vez. Están divididos en tres grupos principales: (i) ácidos orgánicos, que pueden ser generados por la degradación tanto de la lignina como de los carbohidratos; (ii) compuestos fenólicos, que son resultado de la degradación de la lignina a moléculas de menor peso molecular; y, (iii) furfurales, que son principalmente fufuraldehido y 5-(hidroximetil)furfural, resultado de la degradación de azúcares y su re-condensación (Balat, 2011; Sun and Cheng, 2002; Tomás-Pejó et al., 2011). Algunas veces estos compuestos son formados en concentraciones tan altas durante el pretratamiento, que resultan en rendimientos muy bajos o incluso inhiben totalmente las etapas posteriores de hidrólisis y/o fermentación. Con el objetivo de mitigar estos problemas, una etapa de detoxificación puede ser aplicada al material lignocelulósico pretratado antes de la hidrólisis enzimática.

Los trabajos disponibles en la literatura han demostrado que la mayor parte de los inhibidores formados durante el pretratamiento son ácidos carboxílicos de cadena pequeña, mayoritariamente los ácidos fórmico, acético y oxálico (Travaini et

al., 2016b). Estos ácidos son muy solubles en agua, por lo que en los **Artículos I y III** se seleccionó como método de detoxificación una etapa de lavado con agua a temperatura ambiente. Este método se demostró muy adecuado, con eficiencias de remoción que llegaron al 100% para el ácido fórmico, y variaron entre un 60-82% para el ácido acético, entre 90-97% para el ácido oxálico, entre 60-86% para el xilitol y entre 52-77% para los compuestos fenólicos totales. Por otro lado, el **Artículo III** puso de relieve que el proceso de lavado conlleva unas pérdidas de masa importantes, llegando hasta un 30% (m/m), en gran parte debido a la lixiviación del xilano. En el ensayo optimizado, se obtuvo un sólido pretratado con composición final en % (m/m) de: 12,48 de lignina ácida insoluble, 2,78 de lignina ácida soluble, 53,00 de celulosa y 14,62 de xilano.

2.3.3 HIDRÓLISIS ENZIMÁTICA

Después del pretratamiento, es necesaria una etapa de hidrólisis enzimática para la conversión de los polímeros de azúcares de la biomasa en monómeros, ya que en su forma polimérica no pueden ser asimilados por la mayoría de los microorganismos utilizados en la etapa de fermentación.

La hidrólisis de la celulosa ocurre por de la acción de enzimas conocidas como celulasas, aplicando un proceso sinérgico de al menos tres tipos de ellas (Figura 3), actuando también enzimas accesorias. Las endoglucanasas o endo-1,4-β-D-glucanasas (EC 3.2.1.4) empiezan hidrolizando los polímeros de celulosa de manera aleatoria, actuando sobre zonas cristalinas y amorfas, resultando en una rápida reducción del grado de polimerización y aumentando la cantidad de extremidades

no-reductoras y reductoras. Las exoglucanasas o exo-β-1,4-glucanasas (EC 3.2.1.91) actúan sobre las extremidades no-reductoras de los polímeros, liberando celobiosa, oligómeros de glucosa y celodextrinas. Y, en la etapa final, las β-1,4-glicosidasas o celobiasas (EC 3.2.1.21) hidrolizan las celobiosas, celooligosacarídos y celodextrinas hasta glucosa. Ambas, endoglucanasas y exoglucanasas, sufren inhibición catabólica por la celobiosa, motivo por lo cual las β-glicosidasas desempeñan un papel regulador en la hidrólisis enzimática de la celulosa (Arantes and Saddler, 2010; Leite et al., 2008; Maeda et al., 2013).

La hidrólisis del xilano, aparte de que aporta azúcares a los hidrolizados, es esencial para la sacarificación enzimática de la celulosa, debido a que estos polímeros están ligados por azúcares mediadores generando impedimento estérico a las enzimas celulolíticas (Wang et al., 2016; Zhang et al., 2012). La hidrólisis enzimática del xilano se lleva a cabo a través de por lo menos dos enzimas, la endo-1,4-β-xilanasa, genéricamente denominada xilanasa, y la β-xilosidasa. La primera actúa liberando β-D-xilanopirosil oligómeros cuando no hay enzimas accesorias para la remoción de las cadenas laterales del xilano, cuando estas enzimas están presentes los productos son xilobiosa y xilooligosacarídos. Las β-xilosidasas actúan convirtiendo xilobiosa y xilooligosacáridos en general hasta xilosa (Polizeli et al., 2005).

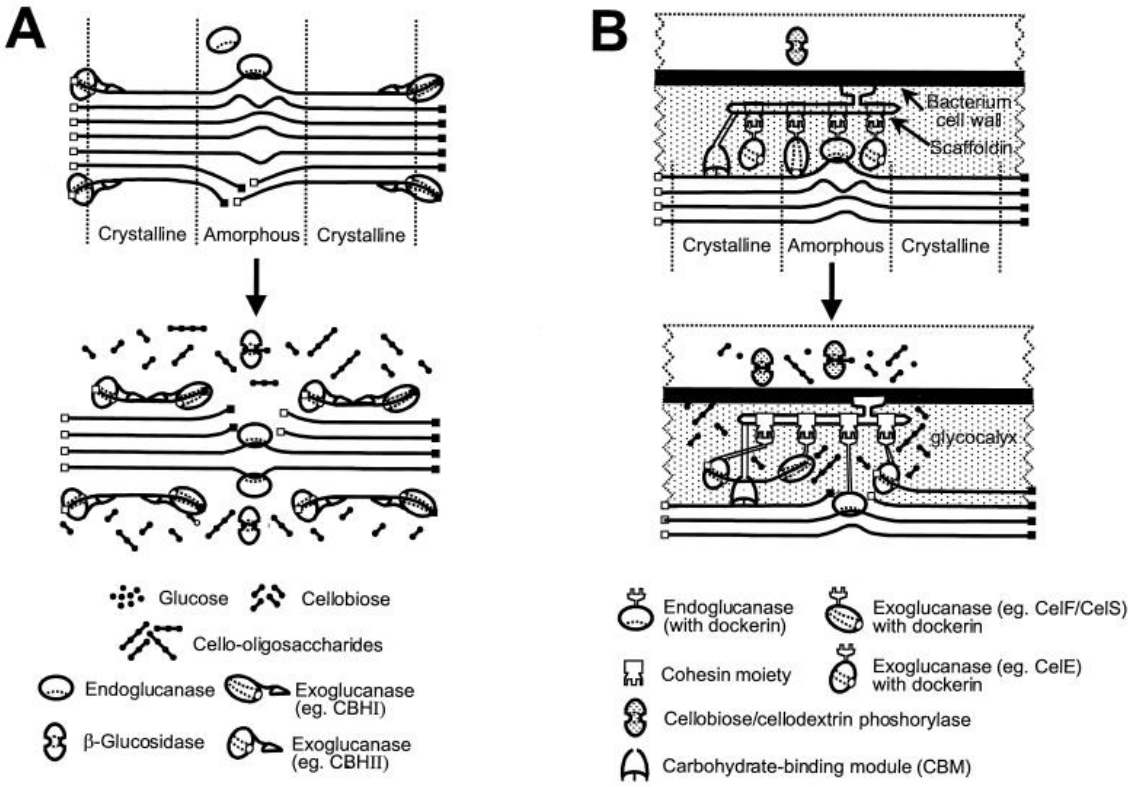

Figura 3: Interacción sinérgica de las celulasas en el proceso de hidrólisis enzimática de la celulosa (Lynd et al., 2002).

En los **Artículos I, II y III** se realizaron hidrólisis estándar al 6% (m/m) de bagazo pretratado con complejos enzimáticos comerciales. En el **Artículo I** se utilizó como fuente de celulasas y xilanasas el complejo NS50013, y como fuente de β-glucosidasa el complejo NS50010, ambas de Novozymes, Dinamarca. En este estudio se fijó la cantidad de enzima en 10 FPU y 10 CBU por gramo de celulosa, y las hidrólisis se llevaron a cabo durante 24 h. En los **Artículos II y III**, se trabajó con hidrólisis de 48 h, con 10 FPU y 30 CBU por gramo de celulosa provenientes de los complejos, Celluclast 1.5L y Novozym188, respectivamente.

En el **Artículo IV** se compararon las enzimas comerciales con las celulasas, xilanasas y β-glucosidasas producidas por el hongo termófilo *Myceliophthora*

thermophila JCP 1-4 por fermentación en estado sólido. En los extractos enzimáticos se obtuvo una proporción de FPU:CBU de 1:3, y se tuvo en cuenta para los ensayos la cantidad de FPU por gramo de celulosa. Para estudiar el efecto de las condiciones de hidrólisis sobre los rendimientos de azúcares, se variaron la temperatura de hidrólisis (40, 50 y 60 ºC), el tiempo de hidrólisis (0, 1, 2, 4, 8, 16, 24 y 48 h), la carga de enzima por gramo de celulosa (2,5, 5, 6, 7,5, 10, 12,5 y FPU/g de celulosa) y la carga de bagazo pretratado-lavado (3,0, 4,5, 6,0, 7,5 y 10 %, m/m).

La optimización de las condiciones de operación del pretratamiento para producción de azúcares proporcionó valores de 50% (m/m) de humedad, 2% (mol/mol) de ozono, 60 L/h de flujo ozono/oxígeno y tamaño de partícula menor de 1,25 mm. En estas condiciones, se obtuvieron hidrolizados con un 78% de conversión de celulosa y un 57% de xilosa para el bagazo ozonizado sin lavar (**Artículo II**), Para el bagazo ozonizado-lavado se obtuvieron hidrolizados con un 84% de conversión de celulosa y un 67% de xilosa (**Artículo III**), con respecto a la composición del bagazo pretratado-lavado, pero un 69% de celulosa y un 27% de xilosa respecto al bagazo *in natura*, debido a las pérdidas de masa durante el lavado. En estos hidrolizados las concentraciones de ácido fórmico, acético y oxálico y compuestos fenólicos totales encontradas fueron de: 2,44 g/L, 1,87 g/L, 4,02 g/L y 585,63 mg/L sin detoxificar, y 0,00 g/L, 0,35 g/L, 0,12 g/L y 133,49 mg/L en los detoxificados.

Los resultados obtenidos en el **Artículo IV** con las enzimas fúngicas fueron más satisfactorios que los obtenidos en condiciones idénticas con las enzimas comerciales. La optimización de las condiciones de hidrólisis estudiadas llevó a la siguiente combinación: 60 ºC, 8 h y 7,5 FPU/g de celulosa. En estas condiciones, se encontró un efecto negativo del aumento de la carga de bagazo, ya que utilizándose

un 3% de materia seca se llegó a 30% de conversión de celulosa (4,86 g/L de glucosa en el hidrolizado) mientras que con un 10% de materia seca se obtuvo un 22% de conversión (10,19 g/L de glucosa).

2.3.4 FERMENTACIONES

El principal biocombustible 2G estudiado hasta hoy es el bioetanol obtenido a través del proceso fermentativo convencionalmente aplicado a la producción de biocombustibles 1G: la conversión de glucosa a etanol por la levadura *Saccharomyces cerevisiae*. Las principales ventajas de la utilización de esa levadura son su elevada cinética de conversión, resistencia a diversos tipos de inhibidores generados en los pretratamientos y el gran conocimiento tecnológico de su aplicación a escala industrial (Kasavi et al., 2012; Sindhu et al., 2011).

Se realizaron fermentaciones estándar del bagazo de caña ozonizado (**Artículos II y IV**) con *Saccharomyces cerevisiae* bakery, obtenida en una panadería local. Las fermentaciones se llevaron a cabo con 5,5% (v/v) de pre-inoculo, con suplemento de sales y nutrientes (Wanderley et al., 2013), durante 24 h a 30 ºC y 175 rpm. No se observó efecto inhibidor utilizándose hidrolizados provenientes de bagazo ozonizado sin lavar, obteniendo hasta un 80% de etanol, respecto al máximo teórico (**Artículo II**). En el estudio con enzimas fúngicas (**Artículo IV**), pese a que se haya utilizado bagazo ozonizado-lavado, el rendimiento máximo obtenido fue un 60% respecto al máximo teórico. Ese rendimiento más bajo puede haber sido debido a la baja concentración de azúcares iniciales en las fermentaciones.

Sin embargo, para la utilización eficiente de los materiales lignocelulósicos es imprescindible la sacarificación del xilano, componente mayoritario de la hemicelulosa, ya que este representa alrededor de un tercio de los azúcares de la biomasa vegetal (Garcia-Cubero et al., 2010b). Por ese motivo, se ha tratado de utilizar otros microorganismos capaces de aprovechar también la fracción de xilosa de los hidrolizados. La levadura diaúxica más empleada es la *Pichia stipitis*, una levadura nativa, capaz de convertir glucosa y xilosa de los hidrolizados en etanol. A pesar de su capacidad de metabolización de la xilosa, rendimientos de etanol más bajos han sido reportados y también ha sido encontrada una mayor inhibición por subproductos de los pretratamientos (Canilha et al., 2010; Sarkar et al., 2012).

Las fermentaciones por *Pichia stipitis* DSM 3651 de los trabajos de esta tesis se realizaron con un 10% (v/v), a 30 ºC y 175 rpm durante 168 h, sin suplementación de sales o nutrientes (Toquero and Bolado, 2014). Los hidrolizados de bagazo ozonizado sin lavar mostraron un fuerte efecto inhibidor sobre la *Pichia stipitis* DSM 3651 utilizada, que no pudo crecer en ninguno de los hidrolizados ensayados (**Artículo II**). No se observó ningún efecto inhibidor en esta levadura durante la fermentación de hidrolizados de bagazo ozonizado-lavado, alcanzando rendimientos de producción de etanol de alrededor del 88% del máximo teórico (**Artículo III**). En este caso, se encontró una relación directa entre el rendimiento y la la cantidad de azúcares iniciales en el medio de fermentación.

Otra alternativa que ha ganado notoriedad en los últimos años es la producción de biobutanol por bacterias anaerobias del genero *Clostridia*, por fermentación ABE. Estas bacterias son capaces de metabolizar pentosas, hexosas y oligosacáridos, convirtiéndolos en una mezcla de acetona-butanol-etanol, con sus

proporciones variando acorde con la cepa utilizada y con los azúcares de partida. En contrapartida, dependiendo de los inhibidores que se hayan podido generar durante el pretratamiento los rendimientos pueden ser muy bajos, e incluso, las reacciones bioquímicas pueden ser paralizadas en la fase de acidogénesis no llegando hasta los productos finales (Jin et al., 2011; Raganati et al., 2015).

En los ensayos de producción de biobutanol a partir de bagazo de caña de azúcar ozonizado-lavado e hidrolizado (**Artículo III**), se utilizaron dos bacterias del genero *Clostridia*: *Clostridium acetobutylicum* DSM792 y *Clostridium beijerinckii* DSM6422. Las dos son descritas en la literatura como capaces de convertir pentosas, hexosas y oligosacarídos en disolventes ABE (Bellido et al., 2014; Pang et al., 2016; Raganati et al., 2015). Los hidrolizados testados fueron suplementados con sales, nutrientes y vitaminas, necesarios para las rutas de acidogénisis y solvatogénisis (Baer et al., 1987; Bellido et al., 2015). Tras la suplementación, los medios de fermentación fueron inoculados con un 10% (v/v) de pre-inoculo conteniendo esporas reactivadas por choque de temperatura a 80 ºC, durante 3,5 min. Las fermentaciones se llevaron a cabo en anaerobiosis a 37 ºC y 120 rpm durante 96 h, Las dos bacterias fueron capaces de crecer en todos los hidrolizados testados, encontrándose una dependencia entre la concentración inicial de azúcares en los hidrolizados y los rendimientos, con ambos creciendo paralelamente. En la fermentación con *C. acetobutylicum* los productos alcanzaron valores máximos de 0,072 $g_{BUTANOL}/g_{AZÚCAR}$ y 0,188 $g_{ABE}/g_{AZÚCAR}$, mientras que para *C. beijerinckii* estos valores llegaron a 0,165 $g_{BUTANOL}/g_{AZÚCAR}$ y 0,257 $g_{ABE}/g_{AZÚCAR}$.

2.3.5 BALANCES ENERGÉTICOS

La generación de ozono a partir de oxígeno es un proceso endotérmico, requiriendo una considerable cantidad de energía, lo que puede poner en duda la viabilidad de este pretratamiento. Sin embargo, el avance tecnológico de los componentes eléctricos utilizados en los equipos de generación de ozono y en los sistemas de disipación de calor ha reducido considerablemente la cantidad de energía necesaria para su producción. En 2011, se publicó que la energía necesaria para la producción de 1 kg de ozono estaba alrededor de 23,8 MJ (Schultz-Jensen et al., 2011b), mientras que en 2016 ese valor ha sido registrado en 16,5 MJ (Travaini et al., 2016b). Este panorama apunta buenas perspectivas futuras en la aplicación del ozono en diversos procesos, incluida su utilización como pretratamiento de la biomasa lignocelulósica.

Como balance energético preliminar de la utilización de la ozonólisis como pretratamiento, se realizaron cálculos de la cantidad de MJ generados y/o consumidos en cada caso. Para ello, se tuvieron en cuenta la energía gastada en la producción del ozono utilizado durante el pretratamiento y la energía liberada por la combustión de los productos de fermentación. No se tuvieron en cuenta la energía gastada en las etapas intermedias y/o secundarias. Se empleó el valor de energía para producción de ozono anteriormente citado, y los valores de energía de combustión de los disolventes: 26,7 MJ/kg de etanol, 33,1 MJ/kg de butanol y 28,6 MJ/kg de acetona (Jin et al., 2011; Schultz-Jensen et al., 2011b).

Cuando se utilizó la levadura *Saccharomyces cerevisiae* bakery, para la conversión de la fracción de glucosa de los hidrolizados hasta etanol, el mejor

balance resultó en 0,76 MJ de energía generada por kg de bagazo pretratado, hidrolizado y fermentado (**Artículo II**). Por otro lado, la fermentación de los hidrolizados por la levadura *Pichia stipitis* DSM 3651, capaz de convertir glucosa y xilosa hacía etanol, supuso la producción de 1,03 MJ por kg de bagazo (**Artículo III**). En cuanto a la producción de disolventes ABE por las bacterias *Clostridia* testadas, la combustión de los disolventes generados tras la fermentación de los hidrolizados no sería suficiente para superar el gasto energético durante la producción de ozono de pretratamiento (**Artículo III**). Sin embargo, en este estudio preliminar no se han tenido en cuenta aspectos que podrían aumentar su viabilidad, como la recuperación del ácido butírico generado, la posible concentración de los hidrolizados de azúcares para aumentar los rendimientos de fermentación, o la utilización de los azúcares lixiviados y de los residuos de fermentación para producción de biogás.

2.3 REFERENCIAS

1. Akerholm, M., Salmen, L., 2003. The oriented structure of lignin and its viscoelastic properties studied by static and dynamic FT-IR spectroscopy. Holzforschung 57, 459–465.

2. Alvira, P., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis : A review. Bioresour. Technol. 101, 4851–4861.

3. APHA-AWWA-WEF, 2005. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, D.C.

4. Arantes, V., Saddler, J.N., 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol. Biofuels 3, 4.

5. Baer, S.H., Blaschek, H.P., Smith, T.L., 1987. Effect of Butanol Challenge and Temperature on Lipid Composition and Membrane Fluidity of Butanol-Tolerant Clostridium acetobutylicum. Appl. Environ. Microbiol. 53, 2854–61.

6. Balat, M., 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Convers. Manag. 52, 858–875.

7. Bellido, C., Infante, C., Coca, M., González-Benito, G., Lucas, S., García-Cubero, M.T., 2015. Efficient acetone-butanol-ethanol production by Clostridium beijerinckii from sugar beet pulp. Bioresour. Technol. 190, 332–8.

8. Bellido, C., Loureiro Pinto, M., Coca, M., González-Benito, G., García-Cubero, M.T., 2014. Acetone–butanol–ethanol (ABE) production by Clostridium beijerinckii from wheat straw hydrolysates: Efficient use of penta and hexa carbohydrates. Bioresour. Technol. 167, 198–205.

9. Benjamin, Y., Cheng, H., Görgens, J.F., 2013. Evaluation of bagasse from different varieties of sugarcane by dilute acid pretreatment and enzymatic hydrolysis. Ind. Crops Prod. 51, 7–18.

10. Binder, A., Pelloni, L., Fiechter, A., 1980. Delignification of straw with ozone to enhance biodegradability. Eur. J. Appl. Microbiol. Biotechnol. 11, 1–5.

11. Canilha, L., Carvalho, W., de Almeida Felipe, M. das G., de Almeida e Silva, J.B., Giulietti, M., 2010. Ethanol Production from Sugarcane Bagasse Hydrolysate Using Pichia stipitis. Appl. Biochem. Biotechnol. 161, 84–92.

12. Canilha, L., Kumar Chandel, A., dos Santos Milessi, T.S., Fernandes Antunes, F.A., da Costa Freitas, W.L., das Graças Almeida Felipe, M., da Silva, S.S., 2012. Bioconversion of sugarcane biomass into ethanol: an overview about composition,

pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. J. Biomed. Biotechnol. 15.

13. Chandel, A.K., Kapoor, R.K., Singh, A., Kuhad, R.C., 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501. Bioresour. Technol. 98, 1947–1950.

14. Dias, M.O.S., Cunha, M.P., Jesus, C.D.F., Rocha, G.J.M., Pradella, J.G.C., Rossell, C.E. V, Filho, R.M., Bonomi, A., 2011. Second generation ethanol in Brazil: can it compete with electricity production? Bioresour. Technol. 102, 8964–71.

15. Driemeier, C., Oliveira, M.M., Mendes, F.M., Gómez, E.O., 2011. Characterization of sugarcane bagasse powders. Powder Technol. 214, 111–116.

16. Garcia-Cubero, M.T., Coca, M., Bolado, S., Gonzalez-Benito, G., 2010a. Chemical oxidation with ozone as pre-treatment of lignocellulosic materials for bioethanol production. Chem. Eng. Trans. 21, 1273–1278.

17. García-Cubero, M.T., González-Benito, G., Indacoechea, I., Coca, M., Bolado, S., 2009. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresour. Technol. 100, 1608–1613.

18. Garcia-Cubero, M.T., Marcos, M., Bolado, S., Coca, M., Gonzalez-Benito, G., 2010b. Optimization of the operating conditions in enzymatic hydrolysis of pretreated lignocellulosic materials. Chem. Eng. Trans. 21, 1285–1290.

19. Garcia-Cubero, M.T., Palacin, L.G., Gonzalez-Benito, G., Bolado, S., Lucas, S., Coca, M., 2012. An analysis of lignin removal in a fixed bed reactor by reaction of cereal straws with ozone. Bioresour. Technol. 107, 229–34.

20. Haghighi Mood, S., Hossein Golfeshan, A., Tabatabaei, M., Salehi Jouzani, G., Najafi, G.H., Gholami, M., Ardjmand, M., 2013. Lignocellulosic biomass to bioethanol,

a comprehensive review with a focus on pretreatment. Renew. Sustain. Energy Rev. 27, 77–93.

21. Harmsen, P., Huijgen, W., Bermudez, L., Bakker, R., 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass.

22. Hendriks, a T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–8.

23. Hernández-Salas, J.M., Villa-Ramírez, M.S., Veloz-Rendón, J.S., Rivera-Hernández, K.N., González-César, R.A., Plascencia-Espinosa, M.A., Trejo-Estrada, S.R., 2009. Comparative hydrolysis and fermentation of sugarcane and agave bagasse. Bioresour. Technol. 100, 1238–1245.

24. IEA, 2015. Key World Energy Statistics 2015, Statistics, Key World Energy Statistics. OECD Publishing.

25. Jin, C., Yao, M., Liu, H., Lee, C.F., Ji, J., 2011. Progress in the production and application of n-butanol as a biofuel. Renew. Sustain. Energy Rev. 15, 4080–4106.

26. Kaar, W.E., Gutierrez, C.V., Kinoshita, C.M., 1998. Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol. Biomass and Bioenergy 14, 277–287.

27. Kasavi, C., Finore, I., Lama, L., Nicolaus, B., Oliver, S.G., Toksoy Oner, E., Kirdar, B., 2012. Evaluation of industrial Saccharomyces cerevisiae strains for ethanol production from biomass. Biomass and Bioenergy 45, 230–238.

28. Leite, R.S.R., Alves-Prado, H.F., Cabral, H., Pagnocca, F.C., Gomes, E., Da-Silva, R., 2008. Production and characteristics comparison of crude β-glucosidases produced by microorganisms Thermoascus aurantiacus e Aureobasidium pullulans in agricultural wastes. Enzyme Microb. Technol. 43, 391–395.

29. Li, C., Wang, L., Chen, Z., Li, Y., Wang, R., Luo, X., Cai, G., Li, Y., Yu, Q., Lu, J., 2015. Ozonolysis pretreatment of maize stover: The interactive effect of sample particle size and moisture on ozonolysis process. Bioresour. Technol. 183, 240–247.

30. Lynd, L.R., Weimer, P.J., Zyl, W.H. Van, Isak, S., 2002. Microbial Cellulose Utilization : Fundamentals and Biotechnology Microbial Cellulose Utilization : Fundamentals and Biotechnology Downloaded from http://mmbr.asm.org/ on February 6 , 2013 by INDIAN INSTITUTE OF TECHNOLOGY MADRAS 66, 506–577.

31. Maeda, R.N., Barcelos, C.A., Anna, L.M.M.S., Pereira, N., 2013. Cellulase production by Penicillium funiculosum and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. J. Biotechnol. 163, 38–44.

32. Mesa, L., López, N., Cara, C., Castro, E., González, E., Mussatto, S.I., 2016. Techno-economic evaluation of strategies based on two steps organosolv pretreatment and enzymatic hydrolysis of sugarcane bagasse for ethanol production. Renew. Energy 86, 270–279.

33. Neely, W.C., 1984. Factors affecting the pretreatment of biomass with gaseous ozone. Biotechnol. Bioeng. 26, 59–65.

34. Neureiter, M., Danner, H., Thomasser, C., Saidi, B., Braun, R., 2002. Dilute-acid hydrolysis of sugarcane bagasse at varying conditions. Appl. Biochem. Biotechnol. 98, 49–58.

35. Pang, Z.-W., Lu, W., Zhang, H., Liang, Z.-W., Liang, J.-J., Du, L.-W., Duan, C.-J., Feng, J.-X., 2016. Butanol production employing fed-batch fermentation by Clostridium acetobutylicum GX01 using alkali-pretreated sugarcane bagasse hydrolysed by enzymes from Thermoascus aurantiacus QS 7-2-4. Bioresour. Technol. 212, 82–91.

36. Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A., Amorim, D.S., 2005. Xylanases from fungi: properties and industrial applications. Appl. Microbiol. Biotechnol. 67, 577–591.

37. Raganati, F., Olivieri, G., Götz, P., Marzocchella, A., Salatino, P., 2015. Butanol production from hexoses and pentoses by fermentation of Clostridium acetobutylicum. Anaerobe 34, 146–55.

38. Ravindran, R., Jaiswal, A.K., 2016. A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: Challenges and opportunities. Bioresour. Technol. 199, 92–102.

39. Rocha, G.J.M., Martín, C., da Silva, V.F.N., Gómez, E.O., Gonçalves, A.R., 2012. Mass balance of pilot-scale pretreatment of sugarcane bagasse by steam explosion followed by alkaline delignification. Bioresour. Technol. 111, 447–452.

40. Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: An overview. Renew. Energy 37, 19–27.

41. Schultz-Jensen, N., Kádár, Z., Thomsen, A.B., Bindslev, H., Leipold, F., 2011a. Plasma-assisted pretreatment of wheat straw for ethanol production. Appl. Biochem. Biotechnol. 165, 1010–1023.

42. Schultz-Jensen, N., Leipold, F., Bindslev, H., Thomsen, A., 2011b. Plasma-Assisted Pretreatment of Wheat Straw. Appl. Biochem. Biotechnol. 163, 558–572.

43. Sindhu, R., Kuttiraja, M., Binod, P., Janu, K.U., Sukumaran, R.K., Pandey, A., 2011. Dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. Bioresour. Technol. 102, 10915–21.

44. Soccol, C.R., Vandenberghe, L.P. de S., Medeiros, A.B.P., Karp, S.G., Buckeridge, M., Ramos, L.P., Pitarelo, A.P., Ferreira-Leitão, V., Gottschalk, L.M.F., Ferrara, M.A.,

2010. Bioethanol from lignocelluloses: Status and perspectives in Brazil. Bioresour. Technol. 101, 4820–4825.

45. Su, H., Liu, G., He, M., Tan, F., 2015. A biorefining process: Sequential, combinational lignocellulose pretreatment procedure for improving biobutanol production from sugarcane bagasse. Bioresour. Technol. 187, 149–60.

46. Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83, 1–11.

47. Tomás-Pejó, E., Alvira, P., Ballesteros, M., Negro, M.J., 2011. Chapter 7 – Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion, in: Pandey, A., Larroche, C., Ricke, S.C., Dussap, C.-G., Gnansounou, E. (Eds.), Biofuels. Elsevier, USA, pp. 149–176.

48. Toquero, C., Bolado, S., 2014. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour. Technol. 157, 68–76.

49. Travaini, R., Barrado, E., Bolado-Rodríguez, S., 2016a. Effect of ozonolysis pretreatment parameters on the sugar release, ozone consumption and ethanol production from sugarcane bagasse. Bioresour. Technol. 214, 150–158.

50. Travaini, R., Marangon-Jardim, C., Colodette, J.L., Morales-Otero, M.D., Bolado-Rodríguez, S., 2015. Ozonolysis, in: Pandey, A., Negi, S., Binod, P., Larroche, C. (Eds.), Pretreatment of Biomass. Elsevier, USA, pp. 105–135.

51. Travaini, R., Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodríguez, S., 2016b. Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited. Bioresour. Technol. 199, 2–12.

52. Travaini, R., Otero, M.D.M., Coca, M., Da-Silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation. Bioresour. Technol. 133, 332–339.

53. UNICA, 2016. Relatório da safra de 2015/2016, UNICADATA, avaliable at http://www.unicadata.com.br/, last accessed june, 2016. São Paulo, Brasil.

54. Wanderley, M.C.D.A., Martín, C., Rocha, G.J.D.M., Gouveia, E.R., 2013. Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. Bioresour. Technol. 128, 448–453.

55. Wang, X., Li, K., Yang, M., Zhang, J., 2016. Hydrolyzability of xylan after adsorption on cellulose: Exploration of xylan limitation on enzymatic hydrolysis of cellulose. Carbohydr. Polym. 148, 362–370.

56. Zhang, J., Tang, M., Viikari, L., 2012. Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. Bioresour. Technol. 121, 8–12.

Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited.

Travaini, R., Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodríguez, S., 2016. Bioresour. Technol. 199, 2–12.

CAPITULO 3

Bioresource Technology 199 (2016) 2–12

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Review

Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited

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HIGHLIGHTS

- Ozonolysis promotes high delignification for diverse types of biomass.
- Lignin removal by O_3 reaction increases sugar release during enzymatic hydrolysis.
- Short-chain carboxylic acids are the main inhibitory compounds produced.
- Moisture and reactor model are key parameters for ozonolysis efficiency.
- Optimization and biorefinery conceptualization are basic in full-scale implementation.

ARTICLE INFO

Article history:
Received 30 June 2015
Received in revised form 24 August 2015
Accepted 25 August 2015
Available online 16 September 2015

Keywords:
Ozone
Pretreatment
Lignocellulosic biomass
Sugar release
Biofuel

ABSTRACT

Ozonolysis, as a lignocellulosic biomass pretreatment, goes back to 80s; however, in the last years it is becoming widespread again owing to its efficiency and mild operation conditions. Ozone reacts preferably with lignin than carbohydrates, promoting biomass destructuration and delignification, and so the sugar release by enzymatic hydrolysis. The hydrolysate from pretreated biomass has being used as sugars source for second-generation fuels production, mainly ethanol, methane and hydrogen. Short-chain carboxylic acids are the main inhibitory compounds generated, being properly removed by water washing. The most common inhibitory compounds reported for other pretreatments, furfural and HMF (5-hydroxymethylfurfural), are not found in ozone-pretreated hydrolysates. Composition of pretreated biomass and ozone consumption depends on several process parameters: reactor design, moisture content, particle size, pH, reaction time, ozone/air flow and ozone concentration. Additional studies are necessary to clarify process parameters effect and to optimize the process to achieve high yields with economic feasibility.

1. Introduction

Fuels production from renewable sources is becoming crucial due to the growing dependence on the oil production, the unavoidable depletion of fossil fuel reservoirs, and the need to reduce greenhouse gases emissions because of its influence on global climate change. In this regard, the biorefinery concept involves the conversion of biomass in different products such as fuels, thermal energy and chemicals. Lignocellulosic biomass is the main potential raw material for biorefinery, because of its sugar content, high availability and low price. It is a complex structure mainly formed by cellulose (40–50%), hemicellulose (25–35%) and lignin (15–35%), and other components such as extractives and several inorganic materials. Lignin is a heterogeneous cross-linked aromatic polymer with a three-dimensional structure that protects and covers cellulose, hindering its biodegradation. Cellulose is a linear polymer formed by glucose units that can form strains or fibrils due to the formation of hydrogen bonds between polymer chains generating recalcitrant structures. Hemicellulose is an amorphous and branched polymer, which may make it less resistant than cellulose, composed by random pentose and hexose units.

The core process in a biorefinery is the production of second-generation biofuels. The main stages involved in this process are a biomass pretreatment, an enzymatic hydrolysis to convert sugar carbohydrates into monomers and finally a fermentation to generate products of interest.

The primary challenge for biomass-based refinery is the efficient, economic and sustainable design of pretreatment in order

Abbreviation: HMF, 5-hydroxymethylfurfural.

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http://dx.doi.org/10.1016/j.biortech.2015.08.143
0960-8524/

Table 1
Advantages and disadvantages of ozonolysis pretreatment for lignocellulosic biomass compared with other pretreatments.

Advantages	Disadvantages
Low generation of inhibitory compounds, and specially the no generation of furfural and HMF (which might hinder following downstream stages)	Highly reactive, flammable, corrosive and toxic characteristics of ozone, leading to potentially dangerous processes
Selective lignin degradation with minimal effects on cellulose and hemicellulose	Exothermic characteristics of process may require cooling systems
Operation at ambient temperature and pressure	Special – construction materials, capable of resisting ozone high-oxidative condition: austenitic stainless steel, glass and other ceramics, Teflon, Hypalon and concrete
On-site ozone generation and direct utilization (avoiding problems related to chemical supply and storage)	
Ability of microorganisms and animals to metabolize most ozonolysis subproducts	High generation costs due to large energy demand (oxygen and electric energy costs around 0.135 € per kg of generated ozone; ozone generator equipment maintenance costs not included)[a]
Reduction of environmental pollution with a suitable process design, decomposing residual ozone at the end of the process	
Absence of liquid phase, avoiding problems related to product dilution	
No requirement of chemical additives during all the pretreatment process	

[a] Information provided by Jose Antonio del Rey Martin. Operation manager of ViAQUA – Gestión de Aguas de Galicia SAU, Agbar group, Santiago de Compostela, Spain.

to break and to open the complex lignocellulosic structure and thus facilitating the enzymes access during hydrolysis, for ultimately enhancing the rate and yield of sugars release. This stage represents up to 20% of the total production costs.

Among all alternatives, ozonolysis is one of the most promising lignocellulosic biomass oxidative pretreatment for lignin degradation with minimal effects on the hemicellulose and cellulose contents. Table 1 resumes the main advantages and disadvantages of ozonolysis compared with other pretreatments (Travaini et al., 2014).

Ozone is one of the strongest oxidizing agents ($E^0 = 2.07$ V, 25 °C), soluble in water (110 mg/L, 25 °C), and readily available for use after its production from oxygen in a strongly endothermic reaction. Due to ozone electron deficiency in the terminal oxygen, it attacks preferably lignin, an electron-rich substrate, than carbohydrates. Most research on ozone pretreatment is related to bleaching in pulp paper industry but its applications have increased principally over the last two decades, such as in ground and industrial wastewaters treatment.

In the search for new alternatives, ozone has been used as delignificant with different types of biomass, such as cereal straw, wood pulp and wood chips, cotton stalk, grass, newsprint and magazine pulps or sugarcane bagasse, among others, and to increase the digestibility of other biomass such as macroalgae or municipal solid waste. Ozonolysis was recently used in the presence of solvents to depolymerize lignin to produce compounds suitable for blending with petrochemical fuels. Hence, the application of this pretreatment could lead to a major improvement in industrial processes that use delignificated biomass as raw material, for example second-generation biofuel production (mainly ethanol and biogas, but also butanol and biohydrogen) or even enzymes production (Travaini et al., 2014).

Binder et al. (1980) delignificated wheat straw with ozone to increase its digestibility achieving a reduction of lignin content of 60%, with 75% of glucose released by enzymatic hydrolysis compared with just 20% for the untreated straw. Neely (1984) demonstrated the enhancement of enzymatic hydrolysis sensitivity for a variety of lignocellulosic biomass (poplar and pine shavings, red oak sawdust, peanut shells, bagasse, wheat straw and green hay) with 1–2 h ozone treatment and a consumption of 4–6 g ozone by 100 g of biomass. Vidal and Molinier (1988) reported an increase from 0% to 57% in the enzymatic hydrolysis yield of ozonized poplar sawdust and a decrease in the percentage of lignin from 29% to 8%.

Silverstein and Chen (2007) applied ozone pretreatment to cotton stalks. They did not obtain the expected effect, probably because of the operation conditions. Nevertheless, Kaur et al. (2012) achieved over 42% reduction of lignin content, 53% glucose release and 89% ethanol yield with the same material. Kojima and Yoon (2008) improved enzymatic hydrolysis from 37% to 58% ozonating newsprint and magazine pulps.

García-Cubero et al. (2010) studied the ozone effect on cereal straws, relating sugar release yield with the acid insoluble lignin fraction. They obtained enzymatic hydrolysis yields up to 88.6% (11.2% acid insoluble lignin) and 57% (12.1% acid insoluble lignin) after ozonolysis compared with 29% (17.1% acid insoluble lignin) and 16% (22.1% acid insoluble lignin) on untreated wheat and rye straws (García-Cubero et al., 2009). Schultz-Jensen et al. (2011a) obtained 52% ethanol yield by saccharification and fermentation of washed pretreated wheat straw for 3 h, reducing the lignin content by 60%. Heiske et al. (2013) investigated ozone pretreatment of wheat straw to produce biogas, attaining a bioconversion to methane of 45%. Wu et al. (2013a,b) investigated ozonolysis pretreatment (from 15 to 90 min of ozonation time) for enhancing biohydrogen production from wheat and barley straws. Optimal hydrogen production in the ozonated samples was obtained for 45 min, with 158% and 166% augmentation for wheat and barley straw respectively, compared to the untreated material.

Mamleeva et al. (2009) achieved residual lignin content as low as 1% after ozonolysis of aspen sawdust. Lee et al. (2010) significantly improved glucose release yield in coastal Bermuda grass from 24% to 53% with a consumption of ozone of 26.4 g of ozone by 100 g of biomass. Yu et al. (2011) reduced the total lignin content on loblolly pine and mixed southern hardwood pulps, with a maximum carbohydrate conversion for the latter of around 80%. Miura et al. (2012) ozonated Japanese cedar chips, finding remarkable hemicellulose removals. Panneerselvam et al. (2013a) tested enzymatic hydrolysis for four types of ozonated energy grass obtaining up to 100% glucose conversion and 60.6% xylan conversion for washed samples.

Travaini et al. (2013) pretreated sugarcane bagasse with ozone to increase lignocellulosic material digestibility. Acid insoluble and total lignin decreased whereas acid soluble lignin increased in all experiments, reaching cellulose and xylan recoveries over 92% after the pretreatment. Yields of sugar enzymatic hydrolysis release increased from 6.64% to 46.06% and from 2.05% to 52.44% for glucose and xylose, respectively, under the best experimental conditions.

Schultz-Jensen et al. (2013) applied ozonolysis to pretreat the macroalgae *Chaetomorpha linum*, increasing ethanol yield from 31 g ethanol/100 g glucan for untreated macroalgae to 41 g ethanol/100 g glucan for ozonated macroalgae. Li et al. (2015) studied

the ozonolysis of maize stover. After pretreatment, both lignin and xylan content decreased while cellulose was only slightly affected. The best result provided 78% lignin removal, increasing the glucose yield after enzymatic hydrolysis from 18.5% to 80%.

Besides these studies, ozonolysis has been used in combination with other pretreatments. For Japanese cypress treated with a combination of ozone-oxidation and dioxane-water extraction (Yokota et al., 2006), mild ozonization for 10–30 min was sufficient to remove lignin from the thin sections of cell walls. Barros et al. (2013) combined ozonolysis with wet disk milling for sugarcane bagasse and straw. The glucose yields for ozonolysis followed by wet disk milling were 89.7% for bagasse and 63.1% for straw, whereas for wet disk milling followed by ozonolysis resulted in 81.1% for bagasse and 92.4% for straw.

Gitifar et al. (2013) studied the ozonization of sugarcane bagasse previously treated with diluted sulfuric acid in autoclave. Delignification and sugar production increased by applying the acid pretreatment; no data about degradation compounds was provided. Karunanithy et al. (2014) explored a sequential extrusion-ozone pretreatment to improve sugar recoveries. When comparing with control samples, glucose, xylose, and total sugar recovery rates attained increases of 3.42, 5.01, and 3.42 times for switchgrass and of 4.5, 2.7, and 3.9 times for big bluestem. Shi et al. (2015) applied ozonolysis and planetary ball milling to promote the enzymatic hydrolysis of corn straw.

Ozonolysis has been also used for other applications, like enzymes production. The suitability of ozonolysis for pretreating wheat straw to be used as substrate for enzymes production by *Trichoderma reesei* was tested elsewhere (Rodriguez-Gomez et al., 2012).

Despite all these laboratory-scale research, full-scale biomass pretreatment with ozone has not been developed yet. Ozone generation is an important drawback considering two factors: its current production costs and the large amount of ozone that process needs. Technological advances are steadily reducing ozone production costs, decreasing a 30% in the last four years. Schultz-Jensen et al. (2011b) estimated an electrical energy requirement of 2.38 MJ per 100 g of ozone and, nowadays, only 1.65 MJ are consumed.[1] Since combustion of 100 g ethanol releases 2.67 MJ, an economically feasible ethanol production by applying ozonolysis requires the optimization of pretreatment conditions minimizing ozone consumption while maximizing efficiency to, finally, reduce costs. Heiske et al. (2013) obtained similar conclusions for biogas production from ozonated wheat straw.

Despite the poor initial energetic balance, many promising alternatives emerge when ozonolysis is integrated in the biorefinery concept. Fermentation of released sugar to other high-value products or the integration of alcohol production with a subsequent methane production process, or even the generation of electricity and heat, from their corresponding residues would improve the economic balance (Papa et al., 2015).

Probably, the most promising alternative would be the recovery of the degradation products from lignin (the unique renewable source of aromatic compounds) and hemicellulose as value-added products, besides biofuel production. The sequential release pattern of various degradation products (e.g. phenols, benzene, fatty acids) during pretreatment suggests that ozonolysis would be an appropriate pretreatment to obtain lignin-derived products (Schultz-Jensen et al., 2011a). Ozonolysis was recently used in the presence of different solvents to depolymerize lignin and produce compounds suitable for petrochemical fuels blending. Ozonolysis using ethanol as solvent resulted in a low conversion of oxygenated aromatics for short reaction times, whereas a range of saturated esters was obtained over 24-h reaction time. Short chain oxygenates can be used as fuel additives, replacing a certain percentage of hydrocarbon fuel while improving some of fuel properties (Chuck et al., 2013).

From this overall perspective, this review describes the main progress in the use of ozonolysis as lignocellulosic biomass pretreatment, the most usual routes of ozone-biomass chemical reactions and inhibitory compounds formation, biomass compositional and structural changes produced by ozone, and a compilation of the effects of ozonation parameters on biofuel production.

2. Chemical reactions and compositional and structural changes

2.1. Chemical reactions

The ozone, a powerful oxidant, is highly reactive toward lignin, but it shows low selectivity. It can therefore react with carbohydrates and other compounds, degrading them and generating byproducts that may act as inhibitory compounds in downstream processes. The most accepted routes of reaction are described below; however, many secondary reactions have not been studied yet and remain unclear.

2.1.1. Reactions between ozone and lignin

Studies have proved the potential of ozone pretreatment for delignification of lignocellulosic biomass, which depends on the substrate and process parameters. Different reaction mechanisms have been proposed: selective reaction with carbon–carbon double bonds, attack to aromatic centers and glycosidic bond cleavage (Bule et al., 2013). Ozone preferentially reacts with olefinic, aromatic and phenolic compounds because of their electron density. The reactivity and the route of reaction of these compounds change according to the substituents.

The reactions of ozone with aromatic compounds involve an initial electrophilic attack followed by a hydroxylation of the aromatic ring, which increases electrophilic substitution reactivity of the ring. Hence, a probable subsequent step is a 1,3-cycloaddition. Ionic 1,3-dipolar cycloaddition occurs by Criegee mechanism (Criegee, 1975; Souza-Correa et al., 2013b), which opens olefinic double bonds, and 1,1-cycloaddition occur via π- and σ-complexes. Another reaction mode is the ozone insertion into carbon-hydrogen bonds in alcohol-, aldehyde- and ether-type structures. In the case of aryl and alkyl ethers, the reaction results in the cleavage of the ether bond (Gierer, 1982; Olkkonen et al., 2000; Ragnar et al., 1999).

The chemical analysis of samples evidences that ozone attacks partially acid insoluble lignin transforming it to acid soluble lignin and degrading a fraction of the total lignin. Experiments with bagasse sugarcane resulted in a 66.8% of acid insoluble lignin reduction, acid soluble lignin increased from 3.13% in the raw material to 7.21% and total lignin decreased 39.6% (Travaini et al., 2013).

2.1.2. Reactions between ozone and carbohydrates

Reactions between ozone and carbohydrates are in the range of 10^6 times slower than those between ozone and lignin, although carbohydrate oxidation may become relevant depending on system parameters. The ozone, in water, generates reactive hydroxyl radicals through the formation of superoxide (the primarily formed radical), which react with carbohydrates resulting in random cleavage of glycosidic bonds (Bule et al., 2013; Gierer, 1982; Staehelin and Hoigne, 1985). Direct ozone attack on carbohydrates may also occur, resulting in the formation of carbonyl and carboxyl groups. The attack of β-glucoside involves the 1,3-dipolar addition

[1] Technical information provided by Jose Antonio del Rey Martin. Operation manager of ViAQUA – Gestión de Aguas de Galicia SAU, Agbar Group, Santiago de Compostela, Spain.

on the anomeric carbon in the C–H bond, generating hydrotrioxide hemiorthoester. Afterward, this ester-type compound undergoes several routes of fragmentation (Olkkonen et al., 2000).

2.2. Inhibitory compounds formation

During ozonolysis pretreatment, initial ozone-oxidized products may react further with extra ozone, generating different inhibitory compounds depending on the reactivity of functional groups.

During ozonolysis, sugar degradation generates mainly oxalic acid, formic acid, acetic acid and levulinic acid. By contrast, furfural and HMF, commonly found as sugar degradation compounds, were not detected (Travaini et al., 2013).

Lignin degradation products include a wide range of aromatic and polyaromatic compounds that may be subsequently converted into carboxylic acids. Various studies found the relation between the production of inhibitory compounds and percentage of biomass delignification (Hoigné and Bader, 1983; Travaini et al., 2013). Oxidation products of p-coumaric and ferulic acids (aromatic aldehyde and acids including p-hydroxybenzaldehyde, vanillin, vanillic acid, caproic acid, azelaic acid, and p-hydroxybenzoic acid) are also found (Quesada et al., 1999; Schultz-Jensen et al., 2011a).

Kádár et al. (2015) found also fatty acids produced by wax degradation in ozonated wheat straw, as a result of pH diminution.

Weak carboxylic acids (mostly acetic, formic and oxalic acid) are reported to be the major inhibitors generated from biomass ozonolysis. Various carboxylic acids, besides formaldehyde and methanol, were found in ozonated wheat straw samples (Binder et al., 1980); for ozonated wood chips, the acids acetic, formic, oxalic, glyoxalic and tartaric were identified (Ben'ko et al., 2013a) while in ozonated macroalgae less than 0.5 g/100 g of formic and acetic acids were detected (Schultz-Jensen et al., 2013). The inhibitory effect of carboxylic acids is pH dependent, since they have to be in a undissociate form to penetrate cell membrane. As intracellular pH is higher, they dissociate intracellularly decreasing internal pH affecting cell functions. Therefore, the fermentability of ozone-pretreated hydrolysates can be improved by raising pH. High oxalic and acetic concentrations (6.5 and 1.5 g/L, respectively) strongly inhibited fermentation of ozonated wheat straw hydrolysates to ethanol by *Pichia stipitis* consuming just a 10% of sugar content (Bellido et al., 2013).

In most publications, water washing of ozonated samples resulted to be an effective detoxification step, removing inhibitory compounds while enhancing glucose release after enzymatic hydrolysis. Simple carboxylic acids and phenolic compounds, as well as nearly 30 lignin degradation products, were found in the washing water of ozonated wheat straw (Schultz-Jensen et al., 2011a).

Nevertheless, it is important to remark that the washing step also leaches xylan, since it becomes partially soluble after ozonolysis oxidation. Water washing of ozonated sugarcane bagasse removed 100% of formic and lactic acids generated during ozonolysis, remaining low concentrations of xylitol and acetic acid. The washing step solubilized up to 2.13% of glucose and 8.16% (w/w) of xylan. For the test where the washing step provided the maximum effect, glucose release increased from 35.22% to 45.39% and xylose release decreased from 52.44% to 26.40% (Travaini et al., 2013). For maize stover, the washing step only increased glucose yield around to ten percentage points, but decreased xylose yield a 42% (Li et al., 2015). Schultz-Jensen et al. (2011a), working with ozonated wheat straw, found that this washing step reduced the hydrolysis duration. In this work, similar xylose yields were obtained for both washed and unwashed material. Ethanol yields for one-hour ozonated wheat straw did not result affected by detoxification step, 45% yield for both, washed and unwashed material. Three-hour ozonated unwashed samples could not be fermented, whereas a 52% ethanol yield was reached for washed ones.

2.3. Structural and morphological changes on ozone pretreated samples

Further understanding and identification of structural and morphological changes of pretreated biomass are crucial for improving the ozonolysis process. Several techniques have been used, besides chemical composition analysis, in order to acquire a broader comprehension of these changes, which are shown in the following subsections.

2.3.1. Structural changes

Bule et al. (2013) investigated the selective modification/degradation of lignin subunits of wheat straw after ozonolysis, finding significant changes on the lignin structure. NMR analysis suggested the degradation of β-O-4 moieties and aromatic ring opening in different lignin subunits. The spectrum showed differences between aromatic structures of the control and ozone pretreated samples, concurrent with a decrease in concentration of the aromatic carbon signal. They observed changes attributed to the methoxy groups, suggesting the possible breakdown of ester-linked structures.

Mamleeva et al. (2009) investigated the conversion of lignin during aspen sawdust ozonation by UV and Fourier transform-infrared (FT-IR) spectroscopy. The study showed the destruction of aromatic rings, and the formation of new ring-conjugated structures (aldehydes, ketones), relatively resistant toward ozone. Aliphatic carbon acids and carbonyl compounds were formed as a result of both lignin and hemicellulose partial oxidative destruction. In addition, the spectra indicated that a part of the softwood lignin is not subjected to ozone action. Souza-Correa et al. (2013b) studied lignin oxidation of sugarcane bagasse, using mass spectrometry and FTIR. Their results supported Criegee's mechanism of ozone attack toward lignin double bonds.

Kádár et al. (2015) applied Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) to study the chemical changes on the surface of ozonated wheat straw. They observed a significant decrease in aliphatic compounds, related with the degradation of the cuticular and epicuticular waxes and a decrease of intensity of the peaks assigned to aromatic skeletal vibrations, attributed to lignin degradation. UV spectrometry of ozone treated thermomechanical paper pulp wastes showed that ozone attacks first the surface of the pulp fiber, namely the cell wall, and then reaction spread into the cell wall (Kojima and Yoon, 2008).

Yu et al. (2011) evaluated changes in crystallinity index for loblolly pine and mixed southern hardwood chips pretreated in alkaline conditions followed by ozonation. They found a general augmentation after pretreatment, attributed to a decrease on the amount of amorphous components (lignin and hemicellulose). In contrast, Ben'ko et al. (2013b) found that crystallinity index varied with the pretreatment intensity: while short times of ozonolysis resulted in no significant changes, longer pretreatment times caused firstly a decrease and ultimately an increase (38% for non-pretreated samples and 37%, 28% and 44% for 15-min, 60-min and 150-min ozonated samples, respectively). These variations were attributed to a cellulose amorphization and a subsequent recrystallization caused by sample processing.

2.3.2. Morphological changes

Scanning Electron Microscopy (SEM) analysis showed a disturbed structure and nanoscopic fibrous morphology in ozone-pretreated biomass. These changes were clear for materials sensitive to the ozone attack, as sugarcane bagasse. Lignin removal

allowed the visualization of biomass microfibers only in some areas, probably from the primary cell wall, where lignin content is lower and delignification could easily release cellulose microfibrills. However, this effect only increases the specific surface area by generating a porous structure, whereas the delignification of the second cell wall allows better saccharification yields (Barros et al., 2013). SEM microscopy of ozonated bagasse after the washing step clearly showed the destructive effect of ozone pretreatment on lignin. The washing step leached acid soluble lignin and hemicellulose, and more grooves were appreciated (Travaini et al., 2013). SEM was also used to observe the degradation of waxes, which appear as flakes at the surface of wheat straw. In can be observed that the stronger the ozone pretreatment, the lower the amount of wax flakes found (Kádár et al., 2015).

The delignification of Japanese cypress by ozonization followed by dioxane-water (9:1 v/v) extraction was studied by microspectrometry, using the Wiesner color reaction. Ozone delignification process started from lumen to middle lamella, with the secondary wall ozonized faster than middle lamella. Therefore, ozone reacted topochemically depending on the tissue lignin content (Yokota et al., 2006).

Accessible pore volume (APV) was measured for alkaline-ozone pretreated wood chips by differential scanning calorimetry (DSC), and the results were interpreted by the accumulation of freezing bond water (Yu et al., 2011). The APV decreased for pretreated samples up to 40% delignification. For higher lignin removal values, the APV gradually increased, although it remained beneath values of the non-pretreated samples. The observed reduction might be an indication of structural collapse of fibers caused by the ozone delignification of surface structures, with fibers becoming twisted and severely shrunken. The ozone penetration into the pulp of highly delignificated samples does not counterbalance the APV reduction by external fibers collapse.

3. Effects of process parameters

As described by different authors, many process factors have influence on the ozonolysis pretreatment efficiency (Table 2), such as: reactor design, moisture, particle size, pH, ozone concentration, ozone/air flow rate and time of ozonolysis. The only study about the temperature influence was conducted by Sugimoto et al. (2009) mentioning experiments at 0 °C and 40 °C, providing only results of ozone consumption and no pretreatment efficiency data. The knowledge about the effect of the most remarkable process parameters on the ozonolysis pretreatment is summarized in this section.

3.1. Effect of reactor design

Reactor design is one of the fundamental parameters in ozonolysis pretreatment as it determines the effective ozone concentration profile, which has a considerable effect on ozone consumption, reactions kinetics, sugar release and interacting with other process parameters. The reactor configuration should provide a contact between ozone and substrate as effective as possible, in order to favor the oxidation reaction. Moreover, it is important to ensure the highest efficiency and lowest residual ozone mass due to the high costs associated to ozone generation.

Theoretically, two different model reactors can be defined: perfect mixed and plug flow. For ideal mixing model, ozone concentration in the reaction medium is homogenous and the same as in the reactor outlet flow; whereas for plug flow model, ozone concentration decreases with the reactor length, from the inlet to the outlet. Different research works have used a variety of reactor design options, from mixed to plug flow models: batch reactor (Vidal and Molinier, 1988), rotatory reactor (Miura et al., 2012), Drechsel trap reactor (Cesaro and Belgiorno, 2013), cylindrical reactor (Neely, 1984), bubble column reactor (Cesaro and Belgiorno, 2013), multi-layer fixed bed reactor (Heiske et al., 2013) or fixed-bed reactor (García-Cubero et al., 2012; Panneerselvam et al., 2013b; Travaini et al., 2013) as showed schematically in Fig. 1.

Cesaro and Belgiorno (2013) tested two different reactors to ozonate the organic fraction of municipal solid waste diluted with distilled water (ratio 1:3 w/w). The first reactor was a Drechsel trap and the second reactor consisted of a bubble column: this last one was more effective, a higher amount of ozone reacted with the substrate and lower residual ozone leaved the reactor.

Neely (1984) evaluated the influence of the reactor design (rotating horizontal cylinder, stirred-bed reactor and fixed- bed reactor) with different types of biomass (pine, oak, poplar, wheat straw, peanut shells, corn stover and bagasse). Its studies concluded rotating cylinder reactor was the most satisfactory for general use. Nevertheless, scarce information about the reactor design was provided and the comparison was not systematic.

Vidal and Molinier (1988) studied the poplar sawdust ozonolysis in both fixed bed column (30% and 75% (w/w) of water in a dry matter basis) and stirred semibatch reactor (50 g/L initial concentration). The number of moles of ozone consumed to oxidize one C9 unit was three in the solid and seven in the slurry. The stirred reactor provided higher ozone concentration at the outlet of the reactor.

In experiments with 5% (w/w) suspension of wheat straw in a tank reactor, 5 h of reaction time were necessary to achieve about 50% delignification with an ozone consumption of 0.2 g O_3 by g of dry wheat straw (Binder et al., 1980). García-Cubero et al. (2012) achieved the same 50% delignification of wheat straw in a fixed bed column with 40% (w/w) moisture and 2 h reaction time. The reaction consumed 100% of fed ozone the first 60 min and 97% of fed ozone in 120 min once the breakthrough time was attained, which corresponded to 0.09 g O_3 by g of dry wheat straw.

These studies showed that mixed reactors work with constant ozone flow and concentration, but the ozone starts to escape from time zero. It occurs due to the mixing in the reactors, the mass transfer barrier between solid and gas phases, and the slight solubility of ozone in experiment with a liquid phase. The fixed bed reactor seemed to be more efficient than the stirred reactor, comparing the ratio ozone consumption/solubilized lignin. In any case, the study of parameters effect should take into account that reactor model and moisture are directly related: low concentration of substrate provides ideal mixing and low moisture provides plug flow reactor.

Nowadays, most of the researchers use plug flow reactors with theirs modifications (Li et al., 2015; Shi et al., 2015). García-Cubero et al. (2012) studied the profile of ozonolysis effect on rye and wheat straw in a fixed bed reactor (50 cm high and 2.7 cm diameter). The delignification and sugars release yields decreased along the column in all the experiments, proving the plug flow model.

Heiske et al. (2013) compared single layer and multiple layer bed reactor configurations to enhance the bioconversion of wheat straw to methane. Reaction goes multi-longitudinally through thin layers from ozone inlet to outlet; the layers became saturated until ozone arrives at the outlet zone, escaping without react. The single layer reactor provided treated straw with 16.2% lignin concentration and 14.52% sugars available. The lignin content decreased in the three layers of the multiple-layer reactor from top (17.5%) over mid (13.0%) to bottom (7.2%), increasing the sugars availability (top 8.8%, mid 16.9%, and bottom 28.4%). Nevertheless, methane production yields indicated little but no substantial difference between the layers. The inhibitory effects of lignin degradation

Table 2
Published ozonolysis process parameters providing the highest delignification and enzymatic hydrolysis yields.

Raw material	Reactor design	Moisture content (%)	Particle size (mm)	Ozone concentration (%)	Flow (L/h)	Time (h)	Ozone consumption[a]	Delignification (%)[b]	Enzymatic hydrolysis (%)[c]		References
									NW	W	
Wheat straw	Batch	95	<0.2	0.44	60	5	30	50	75 G	–	Binder et al. (1980)
	Fixed bed	40	3–5	2.7	60	2.5	–	34	88.6 G	–	García-Cubero et al. (2009)
		50	1	0.6–1	36	3	33	60	–	78 G	Schultz-Jensen et al. (2011a)
		50	1	0.6–1	36	1	–	–	49 G[d]	–	Kádár et al. (2015)
Rye straw	Fixed bed	60	3–5	2.7	60	2.5	–	45	57 G	–	García-Cubero et al. (2009)
Sugarcane bagasse	Fixed bed	80	3–5	3.44	60	1	12	66.8	42 G 33 X	46 G 24 X	Travaini et al. (2013)
Cotton stalks	Fixed bed	35	2	2.1	22.2	2.5		42.38	53 G 74 X	– –	Kaur et al. (2012)
Maize stover	Fixed bed	60	<0.053	2.8	60	1	25.6	78	80 G 54 X	80 G 54 X	Li et al. (2015)
Coastal Bermuda grass	Rotatory	30	<2	25	60	1	26.4	31 TL*	53 G 23 X	– –	Lee et al. (2010)
Energy grass	Fixed bed	30	<2	1.9	15	2	–	>51	– –	100 G 60.6 X	Panneerselvam et al. (2013a)
Spent culture media	Rotatory	40	<5	6	3	1	–	~47% KL	21.8 G[e] 6.5 X[e] 0.3 GL[e]	–	Ueda et al. (2014)
Red oak	Cylindrical	50	0.106	3.37	30	2	–	–	89 G	–	Neely (1984)
Poplar sawdust	Fixed bed	30	2	1.63	60	3	–	72*	57 RS	–	Vidal and Molinier (1988)
Aspen wood chips	Fixed bed	40	0.6–1	2.8	10	1.67	–	–	49 RS	–	Ben'ko et al. (2013a)

* Estimated value from graphic on the referenced article.
[a] g of consumed ozone by 100 g of biomass.
[b] Acid insoluble lignin decreases in regard to the initial biomass content. *Abbreviations:* KL: Klason lignin, TL: total lignin.
[c] Percentage of original polymer converted into its respective monomers. *Abbreviations:* NW: non-washed, G: glucose, X: Xylose, GL: galactose, R: reducing sugars, W: washing step before enzymatic hydrolysis.
[d] Only the lower third of the reactor height was used.
[e] g per 100 g of raw material.

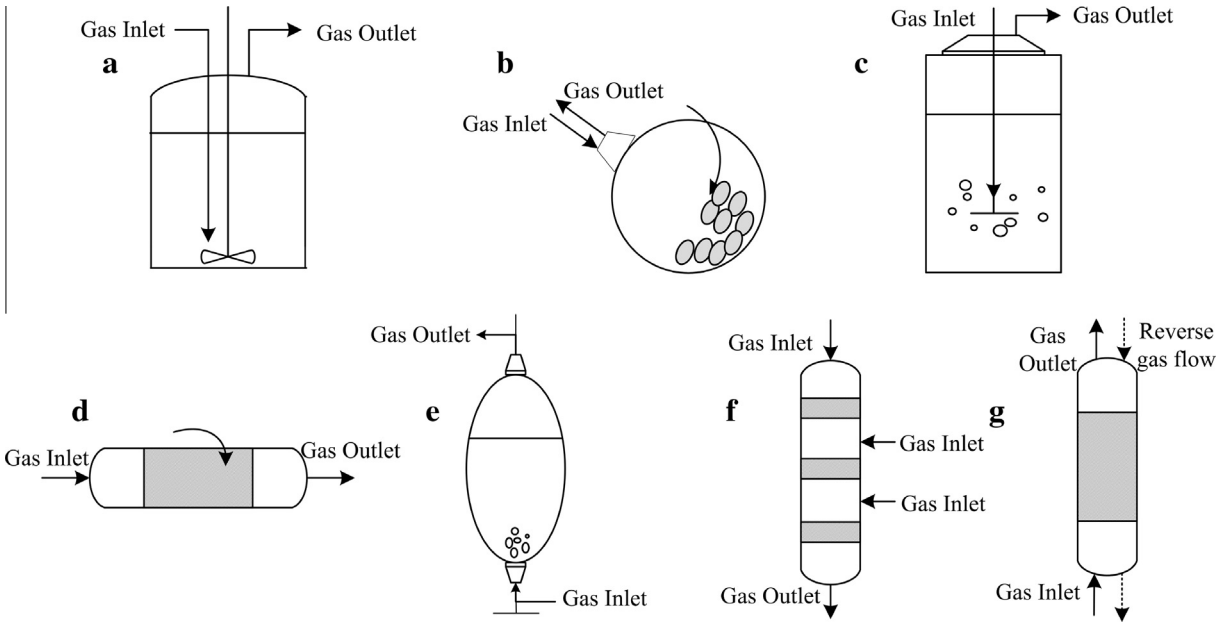

Fig. 1. Different reactor designs used in ozonolysis experiments: (a) batch reactor, (b) rotatory reactor, (c) Drechsel trap reactor, (d) cylindrical reactor, (e) bubble column reactor, (f) multi-layer fixed bed reactor and (g) fixed-bed reactor.

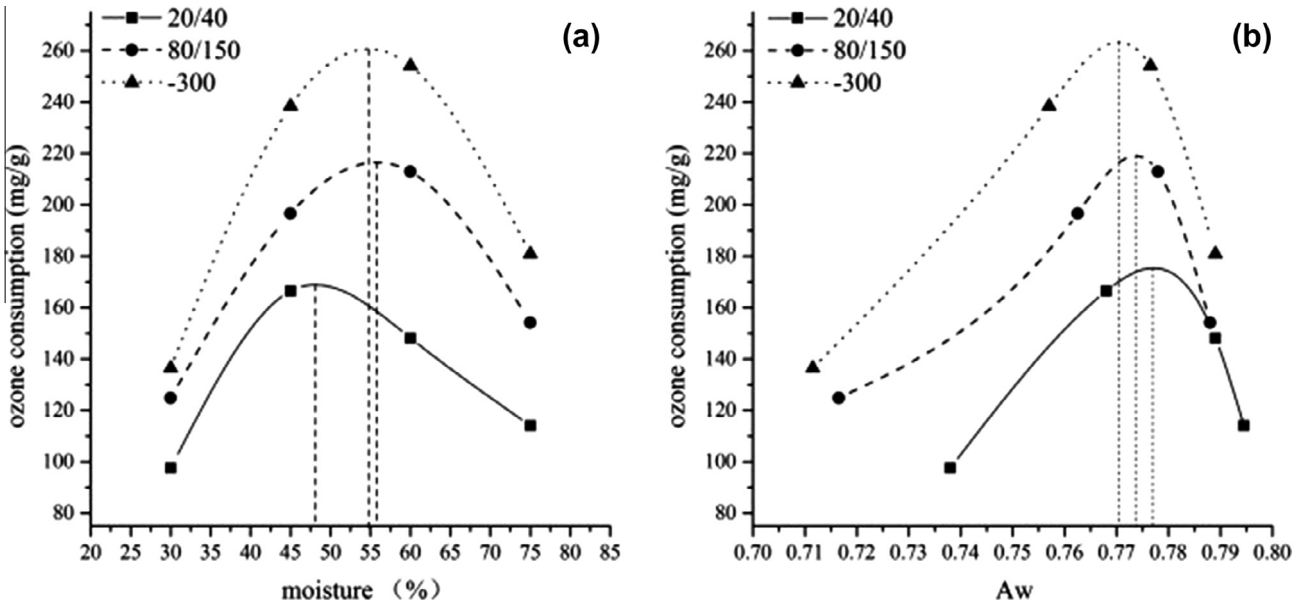

Fig. 2. Adapted from Li et al. (2015). Ozone consumption on maize stover ozonolysis for three particle size ranges (20/40, 80/150 and <300 mesh): (a) as a function of moisture content and (b) as a function of water activity.

products found in the analysis of the pretreated wheat straw washing water balanced out the beneficiary effects of a stronger treatment.

Panneerselvam et al. (2013b) experimented with four varieties of energy grasses in a fixed bed reactor using two different feed flow configurations: firstly, direct ozone flow and secondly, reverse ozone flow by inverting the reactor. Reverse flow configuration showed the highest biomass delignification, the highest sugar recovery, the lowest retention time and the strongest reaction between the bedsides.

3.2. Effect of moisture content

Most authors agree that sample moisture is the most important ozonolysis process parameter, because of its function as mass transport medium, and its effect on ozone and generated radicals' effective concentrations. As described previously, moisture content interacts closely with the reactor model: high water content is related with mixed reactors and, thus, with homogenous ozone concentration, whereas low water content promotes plug flow reactor models.

The reaction starts with ozone transference from gas phase to free water, then from free water to bounded water, and finally the reaction between ozone and lignocellulosic biomass occurs. (Choi et al., 2002; Li et al., 2015). Therefore, at the beginning of pretreatment, virtually no ozone exists in the water reaction face, being all the ozone in the free water. When ozone passes to the bounded water, reaction starts. During pretreatment, reaction rate slows down, being ozone mass transfer the limiting step.

Each type of biomass has its optimal moisture content. According to Neely (1984) this optimal moisture content corresponds to the saturation point of the fibers. At low water concentrations, the ozone mass transfer is limited, and it cannot react properly with all biomass, resulting in shorter breakthrough times. If the water concentration is excessive, a thick film of water blocks biomass pores favoring large residence time of ozone and promoting its decomposition in another hydroxyl radicals. Reactions then follow other non-selective pathways, and an excessive ozone consumption is observed (Mamleeva et al., 2009; Neely, 1984; Puri, 1983; Vidal and Molinier, 1988). Li et al. (2015) studied the interactions between moisture content and particle size in maize stover ozonolysis, finding that water activity (the ratio between free and bounded water) regulates optimal moisture content. For each particle size, different optimal moisture content for delignification were found, but the water activity measured for them was always around 0.775 (Fig. 2).

Usually, the optimal water content for agricultural residues is higher than that observed for woods, due to their capacity to bond water. Souza-Correa et al. (2013a) studied 10%, 25%, 50% and 75% (w/w) moisture content for ozonization of sugarcane bagasse, and found the optimum result at 50% moisture content. Travaini et al. (2013), working with sugarcane bagasse in a fixed bed reactor, achieved a 46% of glucose yield at 80% (w/w) moisture content, six percentage points more than at 40% (w/w) moisture content. Low water contents favored inhibitory compounds formation. Bule et al. (2013), working with wheat straw in a 1 cm high fixed bed reactor and moisture contents between 30% and 90% (w/w), found the optimal glucose release at 90% (w/w). This extra high value can be attributed to the small size of the bed, which may mitigate the thick water layer formation.

Ben'ko et al. (2013a), working in a fixed layer flow unit reactor with aspen chips, studied water contents from 7% (w/w, air-dry wood) to 90% (w/w, water suspension), applying ozone absorption as efficiency criterion. They found that extreme values do not absorb ozone, with the maximum absorption at 40% (w/w) moisture content. This value is higher than that of wood fiber saturation, 20–23% (w/w), at which just cell walls have water, and the cavities are empty. This reinforces the need of free water for great ozonization. For Japanese cedar the optimal moisture content is around 40% (w/w), but after this value free water overload results excessive (Miura et al., 2012; Sugimoto et al., 2009). For red oak treated in a stirred bed reactor, the optimal water content was between 25% and 35% (w/w), with an augmentation of ozone consumption when increasing the water content (Neely, 1984). Ueda et al. (2014) studied the influence of moisture content of shiitake spent culture media (wood meal, *Quercus serrata*). They obtained the best moisture content value between 20% and 40%; whereas working with water content higher than 60%, lignin was not properly degraded.

The air-ozone flow and the increase of temperature caused by the exothermic oxidation reactions can have a remarkable drying effect, decreasing considerably the moisture during the ozonolysis (Mamleeva et al., 2009). Feeding to the reactor an air-ozone flow saturated of water can minimize this moisture reduction, but the increase of temperature in the reactor displaces the equilibrium of water saturation and this drying effect is, in fact, difficult to avoid.

3.3. Effect of particle size

Since ozonolysis pretreatment is a surface reaction, particle size has an important role as process parameter. Nevertheless, any mechanical step will increase the process cost, and an optimal particle size should be pursued, as very small particles promote agglomeration and sugar degradation. A ball mill was used for particle size reduction of corn straw before ozonolysis in a fixed bed reactor with particles from 160 to 21 μm, obtaining the highest glucose conversion with 64 μm particles (Shi et al., 2015). The particle size reduction favored delignification and glucose release, although it decreased xylose yield, probably because of xylose degradation. Schultz-Jensen et al. (2011b), working in a fixed bed reactor with wheat straw, tested the effect of three particle sizes: 0.5, 1 and 2 mm. Glucan and xylan conversion for each particle size were 23%, 50% and 19.4%, and 57.5%, 75% and 45%, respectively. Ozone losses were higher for 2 mm particles, probably due to a decrease on reaction kinetic caused by a lower surface area, promoting the unreacted ozone going out from the reactor. For 0.5 mm particles, the decrease of sugars conversion was attributed to agglomeration effect.

For grinded sieved maize stover pretreated in a fixed bed reactor (particle sizes fractions between 20/40 and 80/150 mesh, and less than 300 mesh, the smaller the particle size, the greater delignification was achieved, with the optimal moisture depending on particle size (Fig. 2). Around 75% delignification and 80% glucose conversion were achieved for particle size lower than 300 mesh (Li et al., 2015).

The combination of wet disk milling and ozonolysis allowed a less expensive process from an energetic point of view, when compared to individual pretreatments, obtaining glucose release yields of 81.1% for bagasse and 92.4% for straw (Barros et al., 2013). Working with red oak in a stirred bed reactor with 50% (w/w) moisture content to obtain 80% digestibility, the reaction time decreased four times when reducing the particle size from 1000 to 106 μm. However, a reduction below 0.5 mm provided small advantage (Neely, 1984).

García-Cubero et al. (2009), studying the particle size effect (<1 and 3–5 mm) for wheat and rye straw in a fixed bed reactor, found that particle size does not influence significantly on delignification and sugars yields. Vidal and Molinier (1988) had the same conclusion with 1 and 2 mm particle of poplar sawdust. Souza-Correa et al. (2014) working with sugarcane bagasse, found small improvements on delignification efficiency (from 75% to 80%) reducing particle size (from 2 to 0.08 mm). In contrast, cellulose conversion ratio increased (from 61% to 79%) as the particle size diminished, observing the most significant change for particles below 0.5 mm. The way in which particle size affects ozonolysis depends on the type of biomass studied, the range of particle sizes and their relation to moisture content.

3.4. Effect of pH

The pH of water used for adjust biomass moisture content can influence the pretreatment. Additionally, pH decreases during ozonolysis process by formation of organic acids from degradation reactions (Binder et al., 1980; García-Cubero et al., 2009; Yu et al., 2011). Alkaline media favors the delignification of biomass, because it removes lignin bonded to carbohydrates. However, at pH greater than 4 ozone destruction is favored, and another reactive species are generated that can attack carbohydrates (Pan et al., 1984).

Aspen wood chips ozonization, neutral and with 2% and 12% NaOH, showed a decrease of sugar release with the increase of NaOH concentration, attributed to cellulose decomposition (Ben'ko et al., 2013a). García-Cubero et al. (2009) studied the ozonolysis of wheat and rye straw in a fixed bed reactor moisturized with water and 20% NaOH solution. The alkaline media resulted in carbohydrate degradation and lower delignification. Binder et al. (1980), working in 5% liquid media suspension of wheat straw, found a higher ozone consumption in alkaline media. No ozone was found in outlet gas at initial times, indicating ozone degradation. A higher delignification was achieved for green liquor

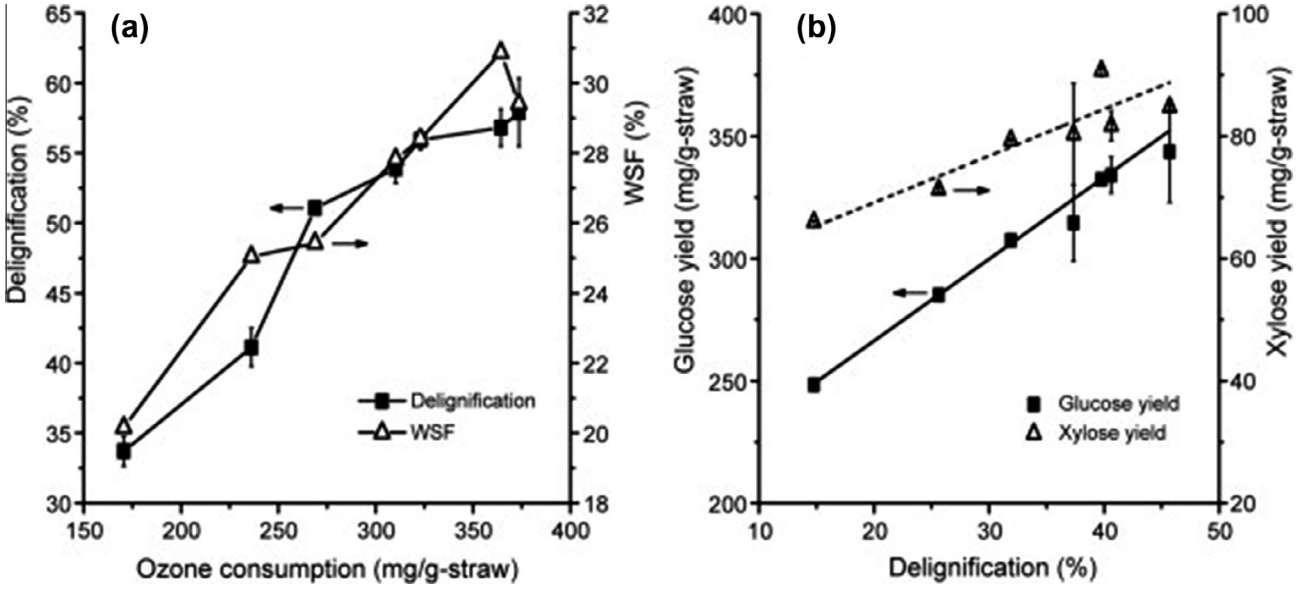

Fig. 3. Adapted from Shi et al. (2015). Ozonolysis of corn straw: (a) effect of ozone consumption on delignification and water-soluble fraction release and (b) correlation between delignification and enzymatic digestibility.

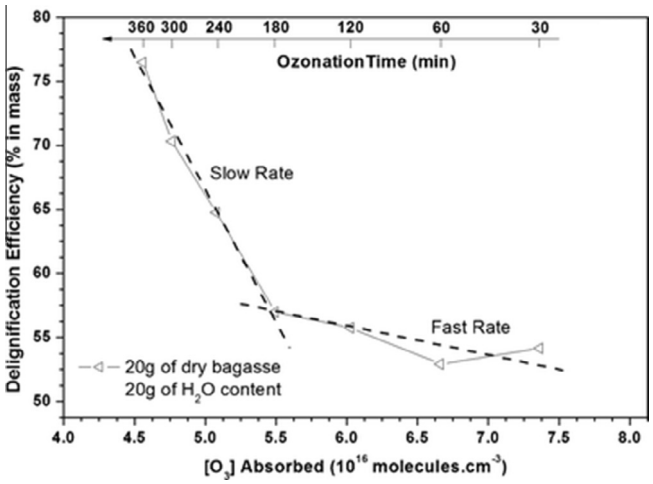

Fig. 4. Adapted from Souza-Correa et al. (2013a). Delignification efficiency as a function of ozone concentration absorbed on the sugarcane bagasse surface, working with 50% moisture and average size of 0.5 mm. The standard deviations on both axes were equal to or less than 5%.

of mixed wood chips when ozonated in pH 2 than compared to neutral medium (Yu et al., 2011). In conclusion, the effect of pH should be counterbalanced between ozone destruction and delignification.

3.5. Ozone consumption: effect of reaction time, ozone concentration and ozone flow

The most important variable of ozonolysis pretreatment, from an economic point of view, is the ozone consumed by gram of dry biomass pretreated. Ozone consumption is expected to be closely related to delignification and hence, to sugar hydrolysis yield (Fig. 3) (García-Cubero et al., 2009; Shi et al., 2015; Travaini et al., 2013). Nevertheless, excessive ozone consumption promotes the production of inhibitory compounds by sugar degradation and by reaction with low molecular lignin compounds (Binder et al., 1980; Neely, 1984).

Ozone consumption is the most complex variable to compare, since it is directly dependent on many process parameters as reaction time, ozone concentration and inlet gas flow; and influenced by the other process parameters discussed before. Li et al. (2015) pretreated maize stover in a fixed bed reactor, varying the ozone consumption from 100 to 250 mg of ozone by g of biomass. They did not find a relation between ozone consumption and delignification and sugar yield because of the effect of moisture, as water content could influence the ozone decomposition rate. Travaini et al. (2013), ozonated sugarcane bagasse in a fixed bed reactor. An experiment with 3.07% (v/v) ozone concentration, 40% (w/w) moisture content and 180 min of reaction time with a consumption of 0.32 g of ozone by g of dry bagasse resulted in 55% delignification and 23% enzymatic hydrolysis glucose release. Another experiment using 3.44% (v/v) ozone concentration and 80% (w/w) moisture, 45 min of reaction time and a consumption of 0.12 g of ozone by g of dry bagasse, provided 33% of delignification but enzymatic hydrolysis glucose release yield of 45%.

The ozone consumption varies with the reaction time in a similar way for all types of biomass. Initially, the preferential reaction is between ozone and lignin: ozone consumption is very fast, and the highest delignification and enzymatic hydrolysis sugar rates are achieved. After this time, a stabilization period begins, when ozone consumption quickly decreases, and delignification and sugar release yields increase very slowly. In a last step, sugar polymers start to be degraded and the hydrolysis sugar release diminishes (Fig. 4). Partial and global reaction times are specific for each combination of process parameters and type of biomass (Binder et al., 1980; García-Cubero et al., 2009, 2012; Neely, 1984; Schultz-Jensen et al., 2011b).

The relation between ozone concentration and sugar release yield is not clear. In several studies, when using a fixed bed reactor, the increase of ozone concentration does not result in a proportional sugar yields increment. For red oak, an increase of ozone from 3% to 3.37% (v/v) augmented digestibility from 64% to 89%, whereas just 91% digestibility was found with 6.08% ozone concentration (Neely, 1984). For sugarcane bagasse, the sugar release increased with ozone concentration but not proportionally, from 16% working with 1.37% (v/v) ozone concentration, 105 min reaction and 0.09 g of ozone by g of biomass to 46% with a 3.44% (v/v), 60 min reaction and 0.12 g of ozone by g of biomass (Travaini

et al., 2013). For wheat and rye straw, ozone concentrations of 2.7% and 3% (v/v) provided similar results for delignification and sugar release (García-Cubero et al., 2009). With energy grasses, ozone concentrations of 40, 50 and 58 g/L provided similar results of delignification and sugar release too (Panneerselvam et al., 2013b).

The increase in ozone/air flow reduces the reaction time, but increases ozone consumption. Inlet overflow provides an excess of ozone, which is going to be available for reaction, obtaining a virtually constant ozone concentration in the reactor. Working with pine shavings, ozonization with 3% (mol/mol) ozone concentration, 17 L/min flow and 10 min of reaction time provided around 60% digestibility, and the experiment with the same ozone concentration, 0.5 L/min flow and 90 min reaction provided 65% of digestibility. In the first case, 0.066 g ozone by g of biomass was consumed, while the consumption was just 0.024 g ozone by g of biomass for the second case. Large flows promote faster reactions with higher ozone consumption, what could make process more expensive and generate inhibitors, as discussed before (Neely, 1984). When reaction time was fixed, larger flows resulted in lower sugar yields for wheat and rye straw in a fixed bed reactor (García-Cubero et al., 2009), but the optimal reaction time for glucose yield (2 h) decreased two times working with wheat straw in a fixed layer reactor (Schultz-Jensen et al., 2011b).

4. Conclusions

Ozonolysis has proved its efficiency as pretreatment for diverse lignocellulosic biomass providing high delignification (≈80%) and total sugar release yields (≈75%) with very low carbohydrate losses. The low generation of inhibitory compounds enables subsequent enzymatic hydrolysis and fermentation steps for biofuels production. The best results, in terms of ozone consumption and efficiency, were obtained working with fixed bed reactors. Moisture is the most important parameter and passes through a maximum depending on the material. Key factor in global process economy is biorefinery conceptualization, by valorizing the whole biomass residues to produce other energy forms, hemicellulose-derived compounds and lignin ozone-oxidized products.

Acknowledgements

The authors thank the "Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria – INIA" and "Ministerio de Economía y Competitividad – MINECO", Spain (RTA2013-00056-C03-02), and "Junta de Castilla y León – JCyL", Spain (VA094U14) for financially support this work. Rodolfo Travaini is also grateful to "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq", Brazil (238059/2012-0) for providing him Doctorate Scholarship.

References

de Barros, R.D.R.O., Paredes, R.D.S., Endo, T., Bon, E.P.D.S., Lee, S.H., 2013. Association of wet disk milling and ozonolysis as pretreatment for enzymatic saccharification of sugarcane bagasse and straw. Bioresour. Technol. 136, 288–294.

Bellido, C., González-Benito, G., Coca, M., Lucas, S., García-Cubero, M.T., 2013. Influence of aeration on bioethanol production from ozonized wheat straw hydrolysates using *Pichia stipitis*. Bioresour. Technol. 133, 51–58.

Ben'ko, E.M., Manisova, O.R., Lunin, V.V., 2013a. Effect of ozonation on the reactivity of lignocellulose substrates in enzymatic hydrolyses to sugars. Russ. J. Phys. Chem. A 87, 1108–1113.

Ben'ko, E.M., Manisova, O.R., Murav'eva, G.P., Lunin, V.V., 2013b. Structural changes in wood during ozonation. Russ. J. Phys. Chem. A 87, 1097–1101.

Binder, A., Pelloni, L., Fiechter, A., 1980. Delignification of straw with ozone to enhance biodegradability. Eur. J. Appl. Microbiol. Biotechnol. 11, 1–5.

Bule, M.V., Gao, A.H., Hiscox, B., Chen, S., 2013. Structural modification of lignin and characterization of pretreated wheat straw by ozonation. J. Agric. Food Chem. 61, 3916–3925.

Cesaro, A., Belgiorno, V., 2013. Sonolysis and ozonation as pretreatment for anaerobic digestion of solid organic waste. Ultrason. Sonochem. 20, 931–936.

Choi, H., Lim, H.N., Kim, J., Hwang, T.M., Kang, J.W., 2002. Transport characteristics of gas phase ozone in unsaturated porous media for in-situ chemical oxidation. J. Contam. Hydrol. 57, 81–98.

Chuck, C.J., Parker, H.J., Jenkins, R.W., Donnelly, J., 2013. Renewable biofuel additives from the ozonolysis of lignin. Bioresour. Technol. 143, 549–554.

Criegee, R., 1975. Mechanism of ozonolysis. Angew. Chem. Int. Ed. 14, 745–752.

García-Cubero, M.T., González-Benito, G., Indacoechea, I., Coca, M., Bolado, S., 2009. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresour. Technol. 100, 1608–1613.

García-Cubero, M.T., Coca, M., Bolado, S., González-Benito, G., 2010. Chemical oxidation with ozone as pre-treatment of lignocellulosic materials for bioethanol production. Chem. Eng. Trans. 21, 1273–1278.

García-Cubero, M.T., Palacín, L.G., González-Benito, G., Bolado, S., Lucas, S., Coca, M., 2012. An analysis of lignin removal in a fixed bed reactor by reaction of cereal straws with ozone. Bioresour. Technol. 107, 229–234.

Gierer, J., 1982. The chemistry of delignification – A general concept – Part II. Holzforschung 36, 55–64.

Gitifar, V., Eslamloueyan, R., Sarshar, M., 2013. Experimental study and neural network modeling of sugarcane bagasse pretreatment with H_2SO_4 and O_3 for cellulosic material conversion to sugar. Bioresour. Technol. 148, 47–52.

Heiske, S., Schultz-Jensen, N., Leipold, F., Schmidt, J.E., 2013. Improving anaerobic digestion of wheat straw by plasma-assisted pretreatment. J. At. Mol. Phys. 2013, 1–7.

Hoigné, J., Bader, H., 1983. Rate constants of reactions of ozone with organic and inorganic compounds in water—II. Water Res. 17, 185–194.

Kádár, Z., Schultz-Jensen, N., Jensen, J.S., Hansen, M.A.T., Leipold, F., Bjerre, A.-B., 2015. Enhanced ethanol production by removal of cutin and epicuticular waxes of wheat straw by plasma assisted pretreatment. Biomass Bioenergy 81, 26–30.

Karunanithy, C., Muthukumarappan, K., Gibbons, W.R., 2014. Sequential extrusion-ozone pretreatment of switchgrass and big bluestem. Appl. Biochem. Biotechnol. 172, 3656–3669.

Kaur, U., Oberoi, H.S., Bhargav, V.K., Sharma-Shivappa, R., Dhaliwal, S.S., 2012. Ethanol production from alkali- and ozone-treated cotton stalks using thermotolerant *Pichia kudriavzevii* HOP-1. Ind. Crops Prod. 37, 219–226.

Kojima, Y., Yoon, S.L., 2008. Improved enzymatic hydrolysis of waste paper by ozone pretreatment. J. Mater. Cycles Waste Manage. 10, 134–139.

Lee, J., Jameel, H., Venditti, R., 2010. Effect of ozone and autohydrolysis pretreatments on enzymatic digestibility of coastal Bermuda grass. BioResources 5, 1084–1101.

Li, C., Wang, L., Chen, Z., Li, Y.Y., Wang, R., Luo, X., Cai, G., Yu, Q., Lu, J., 2015. Ozonolysis pretreatment of maize stover: the interactive effect of sample particle size and moisture on ozonolysis process. Bioresour. Technol. 183, 240–247.

Mamleeva, N.A., Autlov, S.A., Bazarnova, N.G., Lunin, V.V., 2009. Delignification of softwood by ozonation. Pure Appl. Chem. 81, 2081–2091.

Miura, T., Lee, S.H., Inoue, S., Endo, T., 2012. Combined pretreatment using ozonolysis and wet-disk milling to improve enzymatic saccharification of Japanese cedar. Bioresour. Technol. 126, 182–186.

Neely, W.C., 1984. Factors affecting the pretreatment of biomass with gaseous ozone. Biotechnol. Bioeng. 26, 59–65.

Olkkonen, C., Tylli, H., Forsskåhl, I., Fuhrmann, A., Hausalo, T., Tamminen, T., Hortling, B., Janson, J., 2000. Degradation of model compounds for cellulose and lignocellulosic pulp during ozonation in aqueous solution. Holzforschung 54, 397–406.

Pan, G.Y., Chen, C.L., Chang, H.M., Gratzl, J.S., 1984. Studies on ozone bleaching. I. The effect of pH, temperature, buffer systems and heavy metal-ions on stability of ozone in aqueous solution. J. Wood Chem. Technol. 4, 367–387.

Panneerselvam, A., Sharma-Shivappa, R.R., Kolar, P., Clare, D.A., Ranney, T., 2013a. Hydrolysis of ozone pretreated energy grasses for optimal fermentable sugar production. Bioresour. Technol. 148, 97–104.

Panneerselvam, A., Sharma-Shivappa, R.R., Kolar, P., Ranney, T., Peretti, S., 2013b. Potential of ozonolysis as a pretreatment for energy grasses. Bioresour. Technol. 148, 242–248.

Papa, G., Rodriguez, S., George, A., Schievano, A., Orzi, V., Sale, K.L., Singh, S., Adani, F., Simmons, B.A., 2015. Comparison of different pretreatments for the production of bioethanol and biomethane from corn stover and switchgrass. Bioresour. Technol. 183, 101–110.

Puri, V., 1983. Ozone pretreatment to increase digestibility of lignocellulose. Biotechnol. Lett. 5, 773–776.

Quesada, J., Rubio, M., Gómez, D., 1999. Ozonation of lignin rich solid fractions from corn stalks. J. Wood Chem. Technol. 19, 115–137.

Ragnar, M., Eriksson, T., Reitberger, T., 1999. Radical formation in ozone reactions with lignin and carbohydrate model compounds. Holzforschung 53.

Rodriguez-Gomez, D., Lehmann, L., Schultz-Jensen, N., Bjerre, A.B., Hobley, T.J., 2012. Examining the potential of plasma-assisted pretreated wheat straw for enzyme production by *Trichoderma reesei*. Appl. Biochem. Biotechnol. 166, 2051–2063.

Schultz-Jensen, N., Kádár, Z., Thomsen, A., Bindslev, H., Leipold, F., 2011a. Plasma-assisted pretreatment of wheat straw for ethanol production. Appl. Biochem. Biotechnol. 165, 1010–1023.

Schultz-Jensen, N., Leipold, F., Bindslev, H., Thomsen, A., 2011b. Plasma-assisted pretreatment of wheat straw. Appl. Biochem. Biotechnol. 163, 558–572.

Schultz-Jensen, N., Thygesen, A., Leipold, F., Thomsen, S.T., Roslander, C., Lilholt, H., Bjerre, A.B., 2013. Pretreatment of the macroalgae *Chaetomorpha linum* for the

production of bioethanol-comparison of five pretreatment technologies. Bioresour. Technol. 140, 36–42.
Shi, F., Xiang, H., Li, Y., 2015. Combined pretreatment using ozonolysis and ball milling to improve enzymatic saccharification of corn straw. Bioresour. Technol. 179, 444–451.
Silverstein, R., Chen, Y., 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresour. Technol. 98, 3000–3011.
Souza-Correa, J.A., Oliveira, C., Wolf, L.D., Nascimento, V.M., Rocha, J.G.M., Amorim, J., 2013a. Atmospheric pressure plasma pretreatment of sugarcane bagasse: the influence of moisture in the ozonation process. Appl. Biochem. Biotechnol. 171, 104–116.
Souza-Correa, J.A., Ridenti, M.A., Oliveira, C., Araújo, S.R., Amorim, J., 2013b. Decomposition of lignin from sugar cane bagasse during ozonation process monitored by optical and mass spectrometries. J. Phys. Chem. B 117, 3110–3119.
Souza-Correa, J.A., Oliveira, C., Nascimento, V.M., Wolf, L.D., Gómez, E.O., Rocha, G.J. M., Amorim, J., 2014. Atmospheric pressure plasma pretreatment of sugarcane bagasse: the influence of biomass particle size in the ozonation process. Appl. Biochem. Biotechnol. 172, 1663–1672.
Staehelin, J., Hoigne, J., 1985. Decomposition of ozone in water in the presence of organic solutes acting as promoters and inhibitors of radical chain reactions. Environ. Sci. Technol. 19, 1206–1213.
Sugimoto, T., Magara, K., Hosoya, S., Oosawa, S., Shimoda, T., Nishibori, K., 2009. Ozone pretreatment of lignocellulosic materials for ethanol production: improvement of enzymatic susceptibility of softwood. Holzforschung 63, 537–543.
Travaini, R., Otero, M.D.M., Coca, M., Da-Silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment: effect on enzymatic digestibility and inhibitory compound formation. Bioresour. Technol. 133, 332–339.
Travaini, R., Marangon-Jardim, C., Colodette, J.L., Morales-Otero, M.D., Bolado-Rodríguez, S., 2014. Chapter 7 – ozonolysis. In: Pandey, A., Negi, S., Binod, P., Larroche, C. (Eds.), Pretreatment of Biomass – Processes and Technologies. Academic Press, USA, pp. 105–135.
Ueda, C., Takashima, Y., Ishiguri, F., Iizuka, K., Yoshizawa, N., Yokota, S., 2014. Ozone oxidation pretreatment for enzymatic saccharification of spent culture media after *Lentinula edodes* cultivation. J. Wood Sci. 61, 65–69.
Vidal, P.F., Molinier, J., 1988. Ozonolysis of lignin—improvement of in vitro digestibility of poplar sawdust. Biomass 16, 1–17.
Wu, J., Ein-Mozaffari, F., Upreti, S., 2013a. Effect of ozone pretreatment on hydrogen production from barley straw. Bioresour. Technol. 144, 344–349.
Wu, J., Upreti, S., Ein-Mozaffari, F., 2013b. Ozone pretreatment of wheat straw for enhanced biohydrogen production. Int. J. Hydrogen Energy 38, 10270–10276.
Yokota, S., Iizuka, K., Ishiguri, F., Abe, Z., Yoshizawa, N., 2006. Ozone–dioxane delignification from the cell walls of Japanese cypress (*Chamaecyparis obtusa* Endl.). J. Mater. Cycles Waste Manage. 8, 140–144.
Yu, Z., Jameel, H., Chang, H.-M., Park, S., 2011. The effect of delignification of forest biomass on enzymatic hydrolysis. Bioresour. Technol. 102, 9083–9089.

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CAPITULO 4

Bioresource Technology 133 (2013) 332–339

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Sugarcane bagasse ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation

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HIGHLIGHTS

- Ozonolysis in fixed bed is an efficient sugarcane bagasse pretreatment.
- Sample moisture and ozone concentration have a major impact on ozonolysis.
- Ozonolysis causes low carbohydrate degradation and few inhibitory compounds.
- Water washing is an effective detoxification alternative for bagasse ozonolysis.
- Electronic microscopy cleared morphological changes in bagasse.

ARTICLE INFO

Article history:
Received 8 November 2012
Received in revised form 11 January 2013
Accepted 13 January 2013
Available online 4 February 2013

Keywords:
Ozonolysis
Pretreatment
Sugarcane bagasse
Enzymatic hydrolysis
Inhibitory compounds

ABSTRACT

Sugarcane bagasse was pretreated with ozone to increase lignocellulosic material digestibility. Bagasse was ozonated in a fixed bed reactor at room temperature, and the effect of the two major parameters, ozone concentration and sample moisture, was studied. Acid insoluble and total lignin decreased whereas acid soluble lignin increased in all experiments. Pretreatment barely attacked carbohydrates, with cellulose and xylan recovery rates being >92%. Ozonolysis increased fermentable carbohydrate release considerably during enzymatic hydrolysis. Glucose and xylose yields increased from 6.64% and 2.05%, for raw bagasse, to 41.79% and 52.44% under the best experimental conditions. Only xylitol, lactic, formic and acetic acid degradation compounds were found, with neither furfural nor HMF (5-hydroxymethylfurfural) being detected. Washing detoxification provided inhibitor removal percentages above 85%, increasing glucose hydrolysis, but decreasing xylose yield by xylan solubilization. SEM analysis showed structural changes after ozonization and washing.

1. Introduction

Due to the economic, political, and environmental problems related to the use of fossil fuels, in recent years the search for and use of new fuel sources has been a recurrent theme at an international scale. According to *International Energy Agency* data (OECD, 2011), total final energy consumption increased from 4676 Mtoe in 1973 to 8353 Mtoe in 2009, a rise of more than 78% in a little over three decades. Moreover, in 2009, 41.3%, 15.2%, and 10.0% of this amount came directly from oil, gas and coal/peat respectively. Because of the problems related to the use of fossil fuels, action has been taken worldwide at an international, national and local scale to replace fossil fuels in an effort to cut these figures.

Among the many new energy production alternatives, the use of renewable sources proves the most promising. Due to their cost, abundance and environmental friendly nature, the production of second generation biofuels from lignocellulosic materials, such as agricultural and forest waste, provides an excellent substitute. In Brazil, which has the world's largest commercial biomass exploration program, *PROÁLCOOL* (*National Alcohol Program* – for sugarcane ethanol production), waste from the sugarcane industry is abundant. In the 2010 agricultural year, Brazil processed 719.1 million tons of sugarcane, 625 million tons for sugar and first generation ethanol, and the remainder for *cachaça* (white rum) and *rapadura* (a traditional Brazilian sweet), producing some 201

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0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.01.133

million tons of sugarcane bagasse (Hofsetz and Silva, 2012). This bagasse is sometimes used for generating electricity by combustion or as animal feedstock. Yet, in certain cases it remains stockpiled in the field or in factories, and its degradation could pose an environmental problem (Cardona et al., 2010).

The use of lignocellulosic biomass to produce second generation biofuels requires at least three main steps: (1) pretreatment, to open the biomass lignocellulosic structure, releasing sugar polymers from their ligations to lignin, (2) enzymatic hydrolysis, to break sugar polymers down into their monomeric fermentable units, and (3) fermentation of hydrolysates to ethanol or other biorefinery products (FitzPatrick et al., 2010; Hendriks and Zeeman, 2009; Mosier et al., 2005).

The key factor in pretreatment is chemical and sub-microscopic transformation of bagasse, releasing cellulose and hemicelullose from lignin, opening pores, and thereby improving subsequent hydrolysis, whilst generating the smallest possible amount of anti-fermentative compounds (Martín et al., 2007b). Many types of pretreatment have been proposed, including physical, chemical, physical–chemical, biological, and sometimes a combination thereof (Carrasco et al., 2010; Gámez et al., 2006; Kaar et al., 1998; Martín et al., 2007a; Monte et al., 2011; Mosier et al., 2005).

In this work, chemical pretreatment of sugarcane bagasse, ozonization, was proposed for increasing enzymatic hydrolysis yields. Differences in the electronic characteristics between lignin and carbohydrates, mainly the double bonds and electric donor centers present in lignin, make ozonization a selective process, since ozone reacts 10^6 times faster with lignin than with carbohydrates (Maia and Colodette, 2003). Ozone attacks the double bonds, releasing soluble compounds of lower molecular weight, such as formic and acetic organic acids, changing the material pH from 6.5 to 2. Although organic acids can be formed, the scant generation of the inhibitors furfural and HMF is the most appealing feature of this pretreatment (Contreras Iglesias, 2003). Most references to ozone pretreatment are related to pulp paper industry bleaching experiments. However, the use of ozone as a lignocellulosic pretreatment to release fermentable sugars remains scarce.

Vidal and Molinier (1988) reported an increase from 0% to 57% in enzymatic hydrolysis yield of poplar sawdust after ozonolysis. Kojima and Yoon (2008) studied ozonolysis of newsprint and magazine pulps, and reported a significant decrease in lignin as well as improved enzymatic hydrolysis from 37% to 58%. Garcia-Cubero et al. (2009) increased enzymatic hydrolysis after ozonolysis of wheat and rye straw, with no furfural and HMF being detected. Working with wood pulp ozonolysis, Yu et al. (2011) reduced the total lignin content with loblolly pine and mixed southern hardwood pulps, with a maximum carbohydrate conversion in the latter of around 80%. Kaur et al. (2012) achieved a reduction of over 42% in cotton stalk lignin content using ozone pretreatment.

Hydrolysis of cellulose by cellulases acts synergistically with xylan hydrolysis, the most abundant hemicellulose sugar polymer, through xylanase enzymes. The action of the two groups is important vis-à-vis the overall efficiency of the saccharification process, and also because xylose, a product of hydrolysis, is an important biorefinery sugar (Buaban et al., 2010; FitzPatrick et al., 2010).

The aim of the current work was to evaluate ozonolysis as a pretreatment to enhance enzymatic hydrolysis of sugarcane bagasse. Two different operational parameters, bagasse moisture and ozone concentration, were evaluated working in a biomass fixed bed reactor. The impact of these parameters on ozone consumption, lignin and carbohydrate composition of pretreated bagasse, and enzymatic hydrolysis yield, was studied. The formation of acids and inhibitors was also analyzed, and a simple washing detoxification process was proposed to gauge their impact on sugar release yields. Electronic microscopy was used for qualitative evaluation of ozone attack on bagasse.

2. Methods

2.1. Materials

Sugarcane bagasse (surplus after milling) was donated by Usina Vale, city of Onda Verde, São Paulo State, Brazil. It was washed for particulate material removal, dried in a ventilated oven at 42 °C and ground in an agricultural crusher to a size of between 3 and 5 mm. The chemical composition of the sugarcane bagasse used in this study, analyzed following NREL (National Renewable Energy Laboratory – USA) laboratory analytical procedures (Sluiter et al., 2008), was 2.15 ± 0.09 moisture; 46.21 ± 0.10 cellulose (as glucose); 20.86 ± 0.05 hemicellulose (as xylose); 19.54 ± 0.03 acid insoluble lignin, 3.13 ± 0.04 acid soluble lignin; 1.54 ± 0.08 extractives (waxes, fats, non-structural carbohydrates, resins, tannins and colored substances) and 1.19 ± 0.10 ash. All the results are expressed as mass percentage.

Enzymatic complexes NS50013 (cellulase, xylanase) and NS50010 (β-glucosidase) were kindly provided by Novozymes (Denmark).

All chromatograph standards were analytical grade, and MilliQ Ultrapure water was used.

2.2. Ozonolysis

The raw material was ozonized in a fixed bed reactor (glass column 50 cm in height and 2.7 cm in diameter) under room conditions. In each test, 35 g of bagasse was adjusted to the required moisture value, mixing weighted amounts of bagasse and distilled water. The column was then filled with the moisturized bagasse and exposed to the air/ozone gas stream. Ozone was produced by a corona effect ozone generator, Sander 301, fed by dry air. Ozone concentration in gas phase was regulated by the electrical supply, and measured before starting each test with iodometric titration applying the APHA 2350 E method (APHA-AWWA-WEF, 1995). Air-flow rate remained constant at 60 L/h in accordance with previous findings (Garcia-Cubero et al., 2009). Residual ozone concentration in the reactor outlet was also measured by iodometric titration during the reaction. To standardize the ozonolysis time, each test was stopped 15 min after ozone breakthrough, that is, 15 min after ozone began to emerge from the fixed bed ozonization column. Experimental conditions are summarized in Table 1.

In tests where a detoxification washing process was applied, ozonated bagasse was shaken with distilled water (6% w/w) for 1 h, at 25 °C and 300 rpm.

Ozone-treated bagasse, washed or unwashed, was dried in a ventilated oven at 37 °C, and stored in a freezer at −18 °C until enzymatic hydrolysis or composition analysis.

All experiments were carried out twice.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis of dried bagasse was performed in Erlenmeyer flasks with 6% (w/w) raw or pretreated sugarcane bagasse, washed or unwashed, and sodium citrate buffer 0.1 M (pH 4.8), containing an enzyme dosage per gram of cellulose (dry basis) of 10 FPU g^{-1} (NS50013) and 10 CBU g^{-1} (NS50010). Hydrolysis was performed at 50 °C, 300 rpm for 24 h. After hydrolysis, samples were withdrawn and the supernatant was put through a 0.22 μm filter and stored for analysis of sugars and other compounds (e.g. inhibitors and acids).

Xylanase supplementation was not investigated, since the enzyme complex NS50013 is produced by *Trichoderma reesei*, known to be a xylanase producer. Some works have reported xylanase activity of this complex as 55 U/mL with beechwood xylan as

Table 1
Ozonolysis experiment operation parameters.

Test	Ozone % (v/v)	Moisture % (w/w)	Ozonolysis time (min)	g of O_3 fed/g of bagasse
A[a]	–	–	–	–
B	1.37 ± 0.03	80 ± 0.32	105 ± 0.02	0.09 ± 0.01
C	3.07 ± 0.07	40 ± 0.16	195 ± 0.02	0.32 ± 0.04
D	3.44 ± 0.06	28 ± 0.11	45 ± 0.02	0.11 ± 0.03
E	3.44 ± 0.04	40 ± 0.16	120 ± 0.02	0.21 ± 0.05
F	3.44 ± 0.11	80 ± 0.32	60 ± 0.02	0.12 ± 0.01

[a] Control: raw sugarcane bagasse.

substrate (Zhao et al., 2011), and previous works have shown that xylanase supplementation of NS50013 leads to no significant difference in sugar yields (Wang and Cheng, 2011).

2.4. Analytical methods

Moisture, extractives, ash, lignin (acid insoluble lignin and acid soluble lignin), cellulose (as glucose) and hemicellulose (as xylose) in the raw and pretreated materials were analyzed following NREL (*National Renewable Energy Laboratory* – USA) laboratory analytical procedures (Sluiter et al., 2008). Sugars and degradation compounds (xylitol, lactic acid, formic acid, acetic acid, HMF, furfural, vanillin, syringaldehyde, coumaric acid and ferulic acid), in hydrolysates and washing water were measured by HPLC as previously described (Garcia-Cubero et al., 2009).

2.5. Electronic microscopy

Electronic micrographs were taken in a Jeol JSM-820 scanning electronic microscope, with 50X and 500X magnifications.

3. Results and discussion

To evaluate the use of ozone as a pretreatment to enhance sugarcane enzymatic hydrolysis, three moisture and ozone concentration treatment conditions were initially combined (B, C, and D, Table 1) based on previous results with grain straws (Garcia-Cubero et al., 2009; García-Cubero et al., 2012). Experiment A (Table 1) was a control with raw sugarcane bagasse. These experiments showed that higher ozone feed concentrations led to higher sugar releases, as will be explained later. A new experimental set was thus planned, operating with the highest ozone concentration, and varying bagasse moisture (experimental runs D, E, and F, Table 1).

3.1. Ozonolysis time

As can be seen in Table 1, ozonolysis time decreases with inlet ozone concentration. Comparing experiments C and E (40% moisture), an ozone concentration increase from 3.07% to 3.44% (v/v) decreases ozonolysis time from 195 to 120 min, resulting in lower ozone consumption. For experiments B and F (80% moisture), an ozone concentration increase from 1.37% to 3.44% (v/v) decreases ozonolysis time from 105 to 60 min.

Ozonolysis time was influenced by moisture in an unusual way. The low and high moisture experiments (D, B, and F) evidenced a shorter ozone breakthrough time than experiments with medium moisture content (C, E). Ozonolysis breakthrough time thus presents a maximum versus sample moisture. This is probably due to the type of reactive species generated in each case, their concentration in the aqueous phase, the reaction rate, and the porosity of the resulting moisturized material. The reaction rate depends on ozone concentration at the reactor inlet as well as on the type and concentration of the oxidants available to react in the aqueous phase. Ozone reaction is a multistage process which includes ozone solubilization, formation of reactive species, and finally the reaction with bagasse. The major species generated by ozone solubilization are its own ozone and hydroxyl radicals, although other radicals and even peroxide may be generated (Cogo et al., 1999).

3.2. Lignin changes after pretreatments

The two parameters tested, moisture and ozone concentration, proved to be related to the type and quantity of lignin resulting after pretreatment.

As can be seen in Fig. 1, all ozone pretreatments led to a decrease in acid insoluble lignin (AIL) content, resulting in a maximum decrease from 19.54% in the raw material to 6.49% in experiment E, a 66.8% reduction. By increasing ozone concentration, the AIL pretreated bagasse content decreases, with moisture having a significant impact on final AIL concentration. In pretreatments with 40% moisture (C and E), a slight increase in ozone concentration from 3.07% to 3.44% (v/v) at the reactor inlet reduces pretreated bagasse AIL content to 8.75% and 6.49% respectively, a 25.8% difference. Working with 80% moisture (B and F), and very different inlet ozone concentrations, 1.37% and 3.44% (v/v), pretreated bagasse AIL content differs by only 9.6% (14.37% and 12.99%, respectively). These results show that water probably protects lignin during ozonolysis.

As expected, the quantity of acid soluble lignin (ASL) increases due to ozone reaction with lignin, converting an AIL fraction into ASL by inserting hydrophilic functional groups (Garcia-Cubero et al., 2009; Yu et al., 2011). ASL bagasse pretreated content increased with the fed ozone concentration, reaching the maximum in experiment E, where ASL increased from 3.13% in the raw material to 7.21%. Moisture has a similar effect on ASL increase for different ozone concentrations. Using 40% moisture, a small increase in ozone concentration increases ASL contents from 6.51% to 7.21% in experiments C and E. Operating with 80% moisture and very different ozone concentration inlets, experiments B and F gave a similar increase in ASL with pretreated bagasse contents of 5.55% and 6.22% (Fig. 1).

Total lignin (TL), the sum of AIL and ASL content, can be compared in two groups, one tested with high moisture (B and F), with a mean TL value of 19.5%, and another with lower moisture content (C, D, and E), with a mean TL of 14.5%. This is around a 25% difference between the two groups. Maximum reduction was achieved in experiment E, where TL content fell from 22.67% in the raw material to 13.70%, a 39.6% reduction. LT decrease is related to the lignin fraction oxidized to low molecular compounds (Vidal and Molinier, 1988), and so, a higher organic acid concentration is expected in these low moisture experiments.

These results agree with the idea of water acting as a protector, and so lower moisture contents cause higher lignin solubilization (transformation of AIL to ASL) as well as higher lignin oxidation (TL decrease). From these results, a higher sugar release yield in the enzymatic hydrolysis step for the low moisture experiments (Neely, 1984) should be expected, although this should be coupled

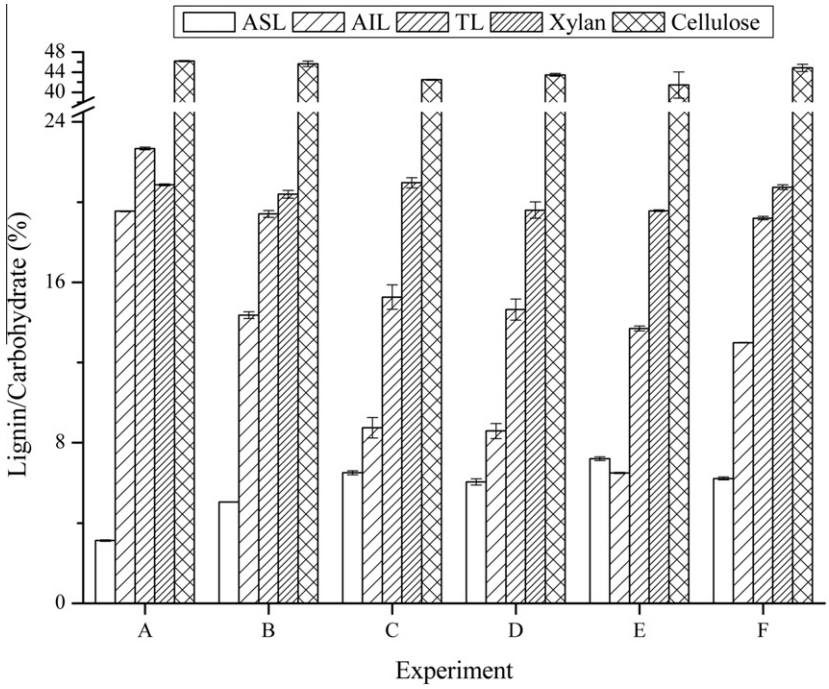

Fig. 1. Lignin and carbohydrate mass percentage content in the raw material and in pretreated solids.

with a higher low molecular weight degradation compound concentration, able to inhibit hydrolysis and/or fermentation steps.

3.3. Pretreatment effect on carbohydrates

Pretreatment using ozone resulted in low sugarcane bagasse carbohydrate degradation. Cellulose suffered most attacks, and showed a 92.5% recovery rate for the most severe ozonolysis conditions (experiment E). Xylan degradation was virtually negligible, with the lowest recovery rate being 98.7% in experiment E (Fig. 1). These carbohydrate recovery data are higher than other published sugarcane bagasse pretreatments such as wet oxidation (Martín et al., 2007a), acid, alkali, alkali-peracetic acid (Zhao et al., 2011), acid-organosolv (Mesa et al., 2011), diluted mixed-acid (Rocha et al., 2011), diluted acid microwave assisted (Rocha et al., 2011), standard steam explosion (Kaar et al., 1998), and steam explosion with alkaline delignification (Rocha et al., 2012).

In sugarcane bagasse pretreatments, solubilization and degradation of sugars generates inhibitors such as furfural, from degradation of pentoses, and 5-hydroxymethylfurfural (HMF) from hexoses (Gámez et al., 2006; Pattra et al., 2008). Low degradation results should thus lead to high hydrolysis yields as well as low furfural and HMF generation.

3.4. Inhibitory compound production

Effective pretreatments normally generate degradation compounds with a noticeable inhibition effect at the fermentation stage. This is a major constraint in alcohol production from lignocellulosic material processes due to the impossibility of fermenting the hydrolysate obtained, or the high cost and complexity of the detoxification stages. As reported before (García-Cubero et al., 2012; Vidal and Molinier, 1988), the ozonolysis process does not usually generate furfural, HMF and other common inhibitors, although it may generate organic acids, mainly acetic acid (Contreras Iglesias, 2003).

Inhibitory compounds were analyzed in the hydrolysates of the three experiments performed with high ozone concentration (D, E, and F), those with the highest sugar release yields. Ozonolysis of sugarcane bagasse generated acids and byproducts (Fig. 2) that were also detected in grain straw ozonolysis (Garcia-Cubero et al., 2009), although in lower concentrations than other published sugarcane pretreatments (Carrasco et al., 2010; Chandel et al., 2007; Kaar et al., 1998). The most common inhibitors referenced for sugarcane bagasse pretreatments are furfural and HMF (Gámez et al., 2006; Pattra et al., 2008). Furfural, HMF, and other common inhibitory compounds such as vanillin, syringaldehyde, coumaric acid and ferulic acid were not detected in our ozonolysis pretreatment experiments. Organic acids, mainly acetic acid, were the main degradation compounds found in these experiments. Even in the experimental run that led to the highest acetic acid formation (D, 2.98 g/L in the hydrolysate), the concentration was lower than reported for acidic pretreatments of sugarcane bagasse, including the least aggressive (Chandel et al., 2007; Cheng et al., 2008; Martín et al., 2002).

Total inhibitor production seems to be related to the TL decrease profile shown in Fig. 1. The highest inhibitory compound production was obtained in experiment E where the highest total lignin degradation was found. Experiment F, with low lignin degradation, yielded the lowest concentration for all the analyzed inhibitory compounds. The individual compound concentrations in experiments D and E displayed different behavior (Fig. 2). Xylitol and formic acid increased with total lignin reduction, although not in the same proportion, with the highest concentrations being reached for the 40% moisture experiment. Nevertheless, lactic and acetic acid generation decreased with sample moisture increase, providing the maximum values in experiment D (28% moisture) despite its lower total lignin degradation.

3.5. Ozone pretreated bagasse washing

In order to remove cheaply and simply the inhibitory compounds generated, pretreated bagasse was washed with water

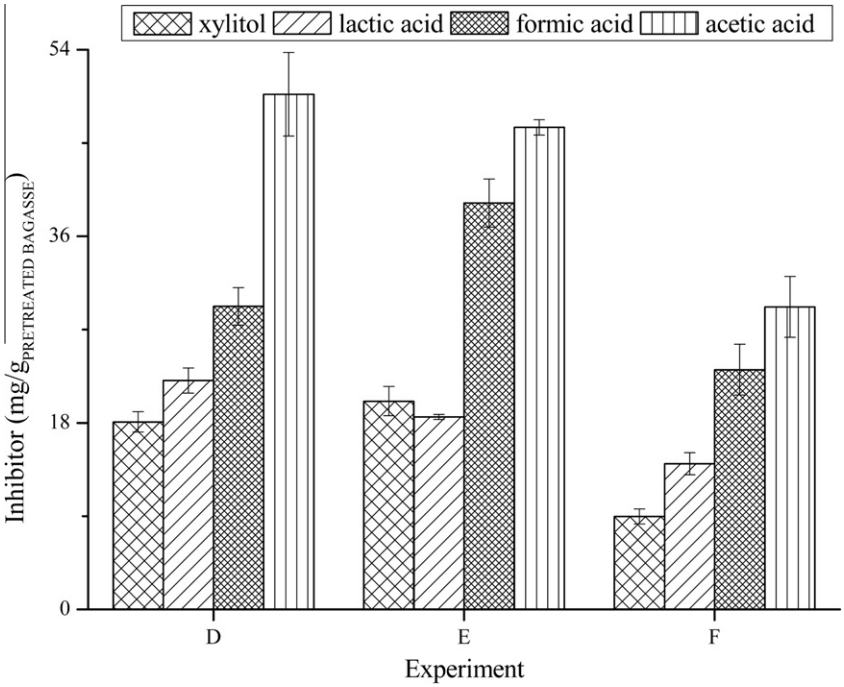

Fig. 2. Inhibitory compound production by ozonolysis pretreatment calculated as mg per g of ozonated bagasse.

Table 2
Washing removal efficiency and final hydrolysate inhibitory compound concentration.

Test	Removal (%)			Final concentration (g/L)	
	Xylitol	Acetic acid	Total inhibitors	Xylitol	Acetic acid
D	85.50	72.70	90.26	0.14	0.64
E	87.95	91.67	95.96	0.13	0.21
F	59.67	57.21	85.91	0.16	0.52

prior to hydrolysis. The effect of washing on inhibitory compound removal and sugar loss was analyzed for the experiments with the highest inlet ozone concentration and, therefore, the highest enzymatic hydrolysis yields (D, E, and F). Washing with water provided extremely high detoxification values, reaching 100% removal efficiency for formic and lactic acids in the three experiments analyzed. Washing proved more efficient for higher initial inhibitor concentrations (Table 2), reaching 95.96% total inhibitory compound removal in the pretreatment that generated most inhibitors, experiment E. 85.91% inhibitor removal was obtained by washing the ozonolysis pretreated solid of experiment F. This provided the lowest inhibitor concentration and the highest cellulose enzymatic hydrolysis yield. Detoxification efficiency is higher than other published values, and extremely low inhibitor concentrations in the hydrolysis liquid were obtained, the lowest value being obtained in experiment E, with 0.34 g/L, with the highest washing efficiency (Table 2). Working with bagasse acid hydrolysis, Chandel et al. (2007) reported an 85.2% and 46.8% reduction in acetic acid with ion exchange treatment and activated charcoal, respectively, no alteration being found with overliming and laccase detoxification. Martín et al. (2002), reported 18% acetic acid removal with overliming.

Sugar loss due to washing was lower than published results for other pretreatments with a liquid phase, or that include washing (Carrasco et al., 2010; Kaar et al., 1998; Martín et al., 2007a). Cellulose losses were extremely low for all the experiments analyzed, with higher values for xylan solubilization being obtained. Sugar losses ranged between 2.13% and 8.16% (w/w) of cellulose and xylan solubilization, respectively, for experiment D, to 1.38% cellulose and 4.75% (w/w) xylan for experiment F.

3.6. Pretreated bagasse hydrolysis

All the treatment conditions tested enhanced enzymatic hydrolysis of bagasse (Fig. 3). Pretreated sample digestibility increased with inlet ozone concentration.

Enzymatic hydrolysis yield of cellulose increased with ozone concentration and sample moisture, improving from 6.64% (g of released glucose/100 g cellulose in the material) of untreated raw material to 35.22% and 41.79% in experiments E (40% moisture) and F (80% moisture), respectively. Enzymatic hydrolysis glucose yields were correlated versus acid insoluble lignin, acid soluble lignin, and total lignin content in the hydrolyzed solid (Fig. 4) as proposed by Garcia-Cubero et al. (2009). A close correlation between glucose yield and lignin content was found for most of the assays except for the results of experiment F, where the highest glucose release yield was obtained despite its high acid insoluble lignin and lignin total content. Experiment F results were therefore not used in the proposed linear trends in Fig. 4.

No clear relation between ozone concentration/sample moisture and xylose release was observed. The highest xylose yields were obtained operating with 40% moisture and 3.44% ozone (experiment E) increasing from 2.05% (g of xylose released/100 g xylan in the material) in the raw material to 52.44%.

The sugar hydrolysis yields obtained are similar but relatively lower than previously published values working with other lignocellulosic materials, further sugarcane bagasse ozonolysis parameter optimization thus proving necessary. Ozonolysis pretreatment has provided maximum enzymatic hydrolysis yields of 57% for poplar sawdust (Vidal and Molinier, 1988) and 58% for newsprint and magazine pulps (Kojima and Yoon, 2008). García-Cubero et al. (2012) obtained a 50% glucose yield for rye straw and 39% for wheat straw, whereas xylose yields were about 30% for both cereal straws after 120 min ozonolysis pretreatment. Enzymatic hydrolysis of ozone pretreated cotton stalk resulted in about 60%

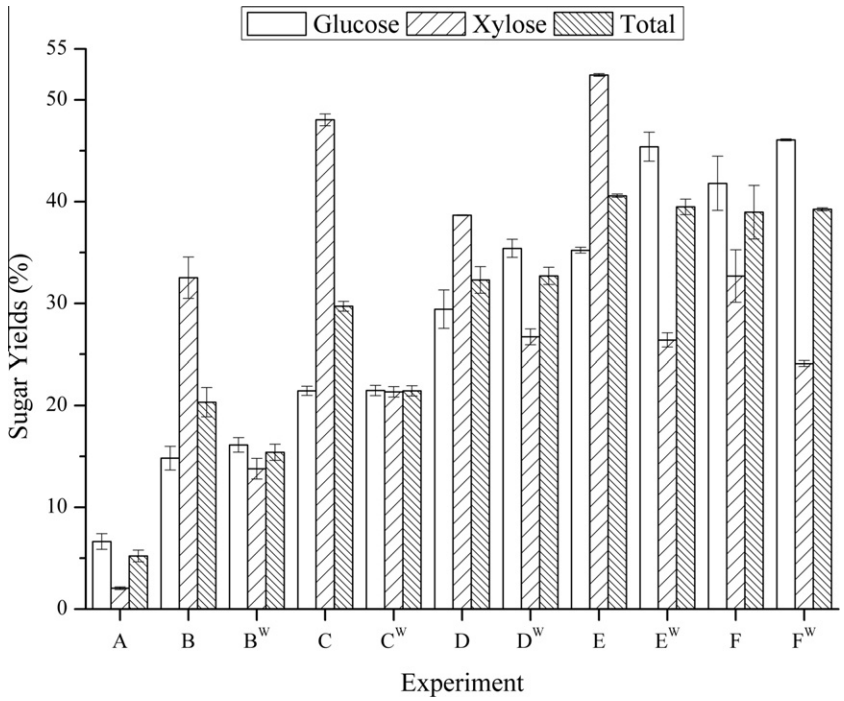

Fig. 3. Sugar release yield after enzymatic hydrolysis of raw material, pretreated solid and washed pretreated solid (superscript w), cellulose (as glucose) and xylan (as xylose).

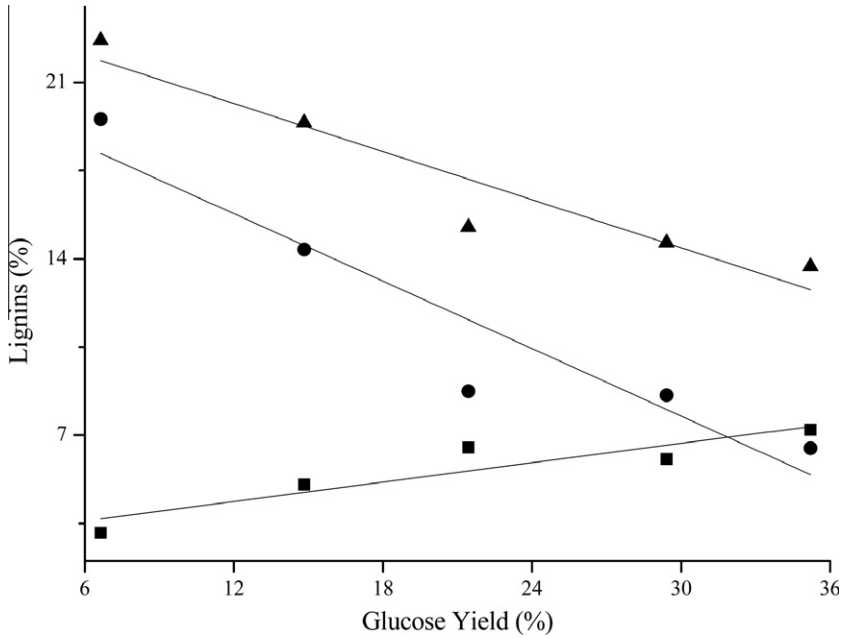

Fig. 4. Enzymatic hydrolysis glucose yield as a function of the ASL (■), AIL (●) and TL (▲) ozonolysis pretreated solid contents.

conversion in glucan and 73.71% xylan conversion (Kaur et al., 2012).

Ozone consumption, related to operation time and inlet ozone concentration, is shown in Table 1, expressed in g of O_3 fed by g of pretreated sugarcane bagasse (dry basis), the highest consumption being in experiment C (0.32 $gO_3/g_{BAGASSE}$), and the lowest in B, with 0.09 $gO_3/g_{BAGASSE}$. Ozone consumptions were 0.21 and 0.12 $gO_3/g_{BAGASSE}$ for experiments E and F, where the best hydrolysis yields were obtained. Ozone consumption thus has no clear impact on pretreatment performance. Differences in ozone pretreatment yield do not seem to be related to different reaction times or ozone consumption, but to the different reactive species generated using different ozonolysis conditions (Cogo et al., 1999).

3.7. Enzymatic hydrolysis of washed ozonolysis pretreated bagasses

Enzymatic hydrolysis of water washed pretreated bagasse was conducted for all the experiments. Washing had a significant effect on sugar yields, as shown in Fig. 3. Cellulose hydrolysis improved, and xylan global hydrolysis yield fell sharply in all experiments. Similar results have been reported in other works (Carrasco et al., 2010; Martín et al., 2007a). Many factors are responsible

for enhanced cellulose hydrolysis, a very important one being the removal of xylose present in the ozonated bagasse, which decreases β-glucosidase activity, and makes the overall process slow and less efficient (Leite et al., 2007). Other key factors are removal of soluble lignin oligomers that function as cellulase adsorbents, as well as other generated by-products that inhibit enzymes (Martín et al., 2007a).

Experiments performed with higher ozone concentrations provided higher glucose yields after washing, and reached values around 46% of the maximum theoretical yield based on the initial dry matter in experiments E and F. These values were achieved in 6% (w/w, dry basis) hydrolysate, high water-insoluble solid (WIS) content, with a final glucose concentration of 12.7 g/L glucose being obtained. Monte et al. (2011) reported a maximum cellulose conversion of 63% bagasse pretreated with alkaline peroxide in 5% WIS content hydrolysis. Working with SO_2-catalyzed steam explosion of bagasse and hydrolysis of washed and unwashed fibers, Carrasco et al. (2010) found higher glucose conversion in the washed fibers, which reached a maximum of around 45% with 2% WIS. By contrast, xylan hydrolysis yield was greatly affected. A considerable fraction of the monomers, oligosaccharides and solubilized xylan released by pretreatment were leached by washing. Nevertheless, total sugar yield remained constant, since cellulose improvement compensates for the lost xylan. This is of particular interest if global biofuel production only considers fermentation of the hexose fraction. The washing step would need to be optimized considering xylan solubilization and inhibitory effect if diauxic microorganisms are used for biofuel production.

3.8. Electronic micrographs

To elucidate physical changes in sugarcane bagasse fiber by ozonolysis and washing, its morphology was studied using SEM. Samples of the raw material, as well as the unwashed and washed pretreated material, were scanned. In each test, one matchstick was carefully chosen, attempting to use the material closest as possible, and identical magnifications were applied for all the samples. In the panoramic pictures with 50× magnification (e-supplement Fig. 1), it is possible to see the non-fragmented raw material (a) and the destructive effect of pretreatment (b), (d), and how, after washing, more grooves appear (c), (e). In pictures with 500× magnification (e-supplement Fig. 2), destruction is clearer, and it can be seen how the fiber covers up removal by washing, resulting in a more open fiber. This effect can be attributed to the hemicellulose fraction solubilized after pretreatment (Rocha et al., 2011).

4. Conclusions

This study showed that ozonolysis is a promising sugarcane bagasse pretreatment, providing high glucose and xylose release yields. The parameters tested, moisture and ozone concentration, influence bagasse digestibility and ozone consumption. Pretreatment was seen to attack carbohydrates slightly, and the strongest fermentation inhibitors, furfural and HMF, found in other sugarcane treatments were not detected. Pretreated bagasse washing with water proved a suitable detoxification alternative, removing inhibitory compounds and increasing cellulose hydrolysis yield, although xylan lost in the washing step reduces final xylose concentration. SEM analysis showed that washing improves hydrolysis by removing material deposited on the fibers.

Acknowledgements

The authors wish to thank the FAPESP (São Paulo State Foundation for Research Support, Brazil), AIUP (Latin-American Academic Association of Postgraduates, Spain), and the *Fundación Carolina* (Spain) for providing financial support for this research. The authors would also like to thank Philip Jaggs for his help in checking the language.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.01.133.

References

APHA-AWWA-WEF, 1995. Standard methods for the examination of water and wastewater, 19th ed. American Public Health Association.

Buaban, B., Inoue, H., Yano, S., Tanapongpipat, S., Ruanglek, V., Champreda, V., Pichyangkura, R., Rengpipat, S., Eurwilaichitr, L., 2010. Bioethanol production from ball milled bagasse using an on-site produced fungal enzyme cocktail and xylose-fermenting *Pichia stipitis*. J. Biosci. Bioeng. 110, 18–25.

Cardona, C.A., Quintero, J.A., Paz, I.C., 2010. Production of bioethanol from sugarcane bagasse: status and perspectives. Bioresour. Technol. 101, 4754–4766.

Carrasco, C., Baudel, H.M., Sendelius, J., Modig, T., Roslander, C., Galbe, M., Hahn-Hägerdal, B., Zacchi, G., Lidén, G., 2010. SO_2-catalyzed steam pretreatment and fermentation of enzymatically hydrolyzed sugarcane bagasse. Enzyme Microb. Technol. 46, 64–73.

Chandel, A.K., Kapoor, R.K., Singh, A., Kuhad, R.C., 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresour. Technol. 98, 1947–1950.

Cheng, K.-K., Cai, B.-Y., Zhang, J.-A., Ling, H.-Z., Zhou, Y.-J., Ge, J.-P., Xu, J.-M., 2008. Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochem. Eng. J. 38, 105–109.

Cogo, E., Albet, J., Malmary, G., Coste, C., Molinier, J., 1999. Effect of reaction medium on ozone mass transfer and applications to pulp bleaching. Chem. Eng. J. 73, 23–28.

Contreras Iglesias, S., 2003. Degradation and biodegradability enhancement of nitrobenzene and 2,4-dichlorophenol by means of Advanced Oxidation Processes based on ozone. Departament d'Enginyeria Química i Metal·lúrgia, Universitat de Barcelona.

FitzPatrick, M., Champagne, P., Cunningham, M.F., Whitney, R.A., 2010. A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products. Bioresour. Technol. 101, 8915–8922.

Gámez, S., González-Cabriales, J.J., Ramírez, J.A., Garrote, G., Vázquez, M., 2006. Study of the hydrolysis of sugar cane bagasse using phosphoric acid. J. Food Eng. 74, 78–88.

Garcia-Cubero, M.T., González-Benito, G., Indacoechea, I., Coca, M., Bolado, S., 2009. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresour. Technol. 100, 1608–1613.

García-Cubero, M.T., Palacín, L.G., González-Benito, G., Bolado, S., Lucas, S., Coca, M., 2012. An analysis of lignin removal in a fixed bed reactor by reaction of cereal straws with ozone. Bioresour. Technol. 107, 229–234.

Hendriks, A.T., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18.

Hofsetz, K., Silva, M.A., 2012. Brazilian sugarcane bagasse: energy and non-energy consumption. Biomass Bioenergy. http://dx.doi.org/10.1016/j.biombioe.2012.06.038.

Kaar, W.E., Gutierrez, C.V., Kinoshita, C.M., 1998. Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol. Biomass Bioenergy 14, 277–287.

Kaur, U., Oberoi, H.S., Bhargav, V.K., Sharma-Shivappa, R., Dhaliwal, S.S., 2012. Ethanol production from alkali- and ozone-treated cotton stalks using thermotolerant *Pichia kudriavzevii* HOP-1. Ind. Crops Prod. 37, 219–226.

Kojima, Y., Yoon, S.-L., 2008. Improved enzymatic hydrolysis of waste paper by ozone pretreatment. J. Mat. Cycles Waste Manag. 10, 134–139.

Leite, R.S.R., Gomes, E., da Silva, R., 2007. Characterization and comparison of thermostability of purified β-glucosidases from a mesophilic *Aureobasidium pullulans* and a thermophilic *Thermoascus aurantiacus*. Process Biochem. 42, 1101–1106.

Maia, E.P., Colodette, J.L., 2003. Efeito do conteúdo e da natureza da lignina residual na eficiência e na seletividade do branqueamento com ozônio. Rev. Árvore 27, 217–232.

Martín, C., Galbe, M., Wahlbom, C.F., Hahn-Hägerdal, B., Jönsson, L.J., 2002. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilising *Saccharomyces cerevisiae*. Enzyme Microb. Technol. 31, 274–282.

Martín, C., Klinke, H.B., Thomsen, A.B., 2007a. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme Microb. Technol. 40, 426–432.

Martín, C., Marcet, M., Almazán, O., Jönsson, L.J., 2007b. Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour. Technol. 98, 1767–1773.

Mesa, L., González, E., Romero, I., Ruiz, E., Cara, C., Castro, E., 2011. Comparison of process configurations for ethanol production from two-step pretreated sugarcane bagasse. Chem. Eng. J. 175, 185–191.

Monte, J.R., Brienzo, M., Milagres, A.M.F., 2011. Utilization of pineapple stem juice to enhance enzyme-hydrolytic efficiency for sugarcane bagasse after an optimized pre-treatment with alkaline peroxide. Appl. Energy 88, 403–408.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96 (6), 673–686.

Neely, W., 1984. Factors affecting the pretreatment of biomass with gaseous ozone. Biotechnol. Bioeng. 26, 59–65.

OECD, 2011. Key world energy statistics 2011. OECD Publishing.

Pattra, S., Sangyoka, S., Boonmee, M., Reungsang, A., 2008. Bio-hydrogen production from the fermentation of sugarcane bagasse hydrolysate by *Clostridium butyricum*. Int. J. Hydrogen Energy 33, 5256–5265.

Rocha, G.J.M., Martin, C., Soares, I.B., Souto Maior, A.M., Baudel, H.M., Moraes de Abreu, C.A., 2011. Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production. Biomass Bioenergy 35, 663–670.

Rocha, G.J.M., Martín, C., da Silva, V.F.N., Gómez, E.O., Gonçalves, A.R., 2012. Mass balance of pilot-scale pretreatment of sugarcane bagasse by steam explosion followed by alkaline delignification. Bioresour. Technol. 111, 447–452.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in biomass (LAP). NREL, Golden, Co.

Vidal, P.F., Molinier, J., 1988. Ozonolysis of lignin — improvement of in vitro digestibility of poplar sawdust. Biomass 16, 1–17.

Wang, Z., Cheng, J.J., 2011. Lime pretreatment of coastal bermuda grass for bioethanol production. Energy Fuels 25, 1830–1836.

Yu, Z., Jameel, H., Chang, H.-m., Park, S., 2011. The effect of delignification of forest biomass on enzymatic hydrolysis. Bioresour. Technol. 102, 9083–9089.

Zhao, X., Song, Y., Liu, D., 2011. Enzymatic hydrolysis and simultaneous saccharification and fermentation of alkali/peracetic acid-pretreated sugarcane bagasse for ethanol and 2,3-butanediol production. Enzyme Microb. Technol. 49, 413–419.

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Travaini, R., Barrado, E., Bolado-Rodríguez, S., 2016. Bioresour. Technol. 214, 150–158.

CAPITULO 5

Bioresource Technology 214 (2016) 150–158

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of ozonolysis pretreatment parameters on the sugar release, ozone consumption and ethanol production from sugarcane bagasse

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HIGHLIGHTS

- Ozone concentration is the fundamental parameter on sugar release yield from SCB.
- Moisture is the fundamental parameter on ozone consumption per gram of sugar released.
- Ozone consumption presented good linear fitting with sugars release yields.
- Degradation compounds inhibited *P. stipitis* but not fermentation by *S. cerevisiae*.
- Fermentation of glucose and xylose is necessary to achieve viable ethanol production.

ARTICLE INFO

Article history:
Received 2 March 2016
Received in revised form 19 April 2016
Accepted 20 April 2016
Available online 22 April 2016

Keywords:
Ozonolysis pretreatment
Sugarcane bagasse
Experimental design
Enzymatic hydrolysis
Ethanol

ABSTRACT

A $L_9(3)^4$ orthogonal array (OA) experimental design was applied to study the four parameters considered most important in the ozonolysis pretreatment (moisture content, ozone concentration, ozone/oxygen flow and particle size) on ethanol production from sugarcane bagasse (SCB). Statistical analysis highlighted ozone concentration as the highest influence parameter on reaction time and sugars release after enzymatic hydrolysis. The increase on reaction time when decreasing the ozone/oxygen flow resulted in small differences of ozone consumptions. Design optimization for sugars release provided a parameters combination close to the best experimental run, where 77.55% and 56.95% of glucose and xylose yields were obtained, respectively. When optimizing the grams of sugar released by gram of ozone, the highest influence parameter was moisture content, with a maximum yield of 2.98 $g_{SUGARS}/g\ O_3$. In experiments on hydrolysates fermentation, *Saccharomyces cerevisiae* provided ethanol yields around 80%, while *Pichia stipitis* was completely inhibited.

1. Introduction

With the aim of developing new environmental-friendly sources of fuels and chemical platforms, research groups around the world have focused their efforts on the development of procedures for production of these compounds from renewable sources. Within this context, and among the many production alternatives, the biorefinery concept has appeared. Like a traditional refinery, the core process of a biorefinery is the production of biofuels, but using biomass as raw material. The main biofuel being studied is bio-ethanol, produced from polymeric sugars present in lignocellulosic biomass. It has many advantages over other biofuels, such as the ability to blend with petroleum for its use on traditional engines; the use of its pure anhydrous form in dedicated engines; its high octane number and high vaporization heat; it is considered a clean, renewable and green combustible; etc (Thangavelu et al., 2016).

The production of second-generation ethanol requires a pretreatment step, to degrade lignin and liberate the polymeric sugars (Garcia-Cubero et al., 2010b). Among the different types of pretreatments, the ozonolysis has regained visibility in the last decade (Garcia-Cubero et al., 2010a; Kojima and Yoon, 2008; Mamleeva

Abbreviations: AIL, acid insoluble lignin; ANOVA, analysis of variance; ASL, acid soluble lignin; OA, orthogonal array; SCB, sugarcane bagasse.

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http://dx.doi.org/10.1016/j.biortech.2016.04.102
0960-8524/

et al., 2009). This chemical pretreatment consists in the use of ozone to oxidize, solubilize and degrade biomass lignin, generating a pretreated material with great characteristics for enzymatic hydrolysis. The main advantages that make ozonolysis a promising pretreatment include: low inhibitory compounds formation, generating mainly weak carboxylic acids; minimal effects on sugars polymers, by selective lignin degradation; operation at ambient pressure and temperature, with on-site ozone generation; and the development of new technologies for ozone generation, reducing production costs (Travaini et al., 2016). Plug flow are the reactors most commonly used for ozonolysis pretreatment, mainly as fixed beds, where moisturized lignocellulosic biomass is packed in a tubular reactor and ozone is fed in a gas flow. These dynamics, when applied with the optimal conditions, result in a process with minimal ozone waste (Bhattarai et al., 2015; Garcia-Cubero et al., 2012; Li et al., 2015).

Many works applying ozonolysis pretreatment have been reported with a wide variety of lignocellulosic materials, like agricultural residues, wood chips, municipal solid waste and microalgae (Travaini et al., 2015). Agricultural residues have focused the research, which has most commonly explored wheat straw, on studies about compositional changes, enzymatic hydrolysis yields, fermentation for biofuels and biogas production, etc.

Sugarcane is used in many countries for alimentary sugar and first-generation ethanol production, and sugarcane bagasse (SCB) is its main residue. In Brazil, for example, 200 million tons of SCB were generated in the 2014–2015 harvest, producing 27 billion liters of ethanol and 37 millions tons of sugar (UNICA, 2015). It presents important advantages over other lignocellulosic residues for second-generation ethanol production, highlighting its low ash content, high sugar polymer content, low cost, high viability and the possibility to integrate second-generation ethanol production into the first generation factories (Soccol et al., 2010). Some studies on SCB ozonolysis pretreatment have recently been carried out (Barros et al., 2013; Souza-Correa et al., 2014, 2013; Travaini et al., 2013); nevertheless, they are just exploratory studies. In the first study about SCB ozonolysis, Travaini et al. (2013) observed how the pretreatment can affect the structural composition of the pretreated biomass and increase enzymatic hydrolysis efficiency, when compared with the raw material. Barros et al. (2013) studied the use of SCB ozonolysis pretreatment combined with wet disk milling, and found that the association provided better results than the individual pretreatments. The works of Souza-Correa et al. (2014,2013) studied SCB ozonolysis pretreatment process parameters one factor at a time, applying a NaOH wash as a detoxification step that can influence the results and interpretations. To this day, no systematical and statistical studies about SCB ozonolysis process parameters and their optimization are available; neither has the fermentability of hydrolysates from ozonated SCB been studied, and there is just one study about the fermentation of wash-detoxified ozone pretreated SCB (de Cassia Pereira et al., 2016).

In this work, an OA $L_9(3)^4$ experimental design was performed in order to study the main ozonolysis process parameters (moisture content, ozone concentration, ozone/oxygen flow and SCB particle size). The applied orthogonal array of this experimental design enables the study of, firstly, the way that process parameters individually affect sugar release yields after enzymatic hydrolysis, ozone consumption and time of reaction, and also, the influence of the interactions between process parameters. The results obtained were model-fitted to predict the combination of parameters that optimizes sugar release yields and the ozone consumption. The most concentrated hydrolysates were fermented for ethanol production by the yeasts *Saccharomyces cerevisiae* bakery's strain and *Pichia stipitis* DSM 3651. Finally, a preliminary energy consumption assessment was made.

2. Material and methods

2.1. Sugarcane bagasse

SCB was kindly donated by Usina Virgolino de Oliveira S/A Açúcar e Álcool, José Bonifácio, São Paulo State, Brazil. It was washed with distilled water to remove sugar residues and particulate material, dried in a ventilated oven at 37 °C and stored in hermetic plastic bags until use.

The washed and dried SCB was sieved manually using, initially, a n° 4 mesh (4.76 mm) sieve to remove extra-large fibers that can interfere in pretreatment, and top particles were discarded (particle size P1). A portion of the resulting sieved SCB was used for experiments (particle size P2); while another portion of SCB particle size P2 was sieved again with a n° 120 mesh (0.125 mm) sieve, and both top (particle size P3) and bottom (particle size P4) sieved materials were used for experiments. The percent yield of sieving and the chemical composition (acid insoluble lignin (AIL), acid soluble lignin (ASL), cellulose, xylan and ash) of each particle size sample are shown in Table 1.

2.2. Ozonolysis pretreatment

Ozonolysis pretreatment experiments were conducted in a fixed bed reactor (glass column 50 cm in height and 2.7 cm in diameter), as described before by Travaini et al. (2013), but with some modifications as indicated below.

Before each experimental run, the reactor was filled with 24 ± 1 g (dry basis) of SCB previously moisturized with distillated water to the required value and gently mixed to homogenize the mixture. The ozone generator was fed with industrial grade oxygen. Before each test, the inlet oxygen flow was measured with an oxygen rotameter, the ozone production was controlled by energy supply and the ozone concentration in the generated gas flow was determined using iodometric titration (APHA-AWWA-WEF, 2005). The ozone/oxygen stream was saturated with water passing through a scrubber bottle before entering the reactor in order to minimize moisture losses. Outlet reactor gas flow was passed through 2% KI solution, and the absorbance periodically determined each 30 s, after color appearance, by spectrophotometry at 464 nm. Ozonolysis pretreatment experiments were stopped 15 min after KI solution reached an absorbance of 0.800 (corresponding to 0.028 g of O_3 escaped). The ozone concentration and ozone/oxygen flow in each experiment were used for calculating the ozone expended during the reaction time. The quantity of non-reacted ozone leaving the reactor during the additional 15 min was iodometrically measured (APHA-AWWA-WEF, 2005) and subtracted from the expended ozone to calculate the grams of ozone reacted in each experiment.

The resulting ozone pretreated SCB in the reactor was divided into four parts within the length of the reactor, and the one just before the gas outlet was discharged in order to avoid non-homogeneous pretreated samples (García-Cubero et al., 2009). The other three quarters were gently mixed, the moisture content measured by gravimetry, and stored hermetically in a freezer until enzymatic hydrolysis occurred.

2.3. Enzymatic hydrolysis

Ozone pretreated SCB was enzymatically hydrolyzed in Erlenmeyer flasks (6% w/w, dry basis) in sodium citrate buffer pH 4.80 (0.05 M), with 10 FPU and 30 CBU per gram of cellulose. Enzyme dosages were calculated based on the raw material's cellulose content, using the commercial enzyme cocktails Celluclast 1.5 L as cellulase and Novozym 188 as β-glucosidase sources, both from

Table 1
Sieving yields and chemical composition of sugarcane bagasse raw material used in the ozonolysis pretreatment orthogonal array experimental design $L_9(3)^4$.

Particle size code	Particle size (mm)	Sieving yield% (w/w)[a]	Chemical composition% (w/w, dry basis)				
			AIL[b]	ASL[c]	Cellulose	Xylan	Ash
P1	Ø > 4.76	28.82 ± 0.44	21.25 ± 0.28	4.85 ± 0.16	44.85 ± 0.06	26.72 ± 0.02	3.13 ± 0.19
P2	Ø < 4.76	70.41 ± 0.88	21.54 ± 0.14	4.51 ± 0.26	44.96 ± 1.16	25.93 ± 0.22	5.48 ± 0.01
P3	4.76 > Ø > 1.25	34.20 ± 0.76	20.21 ± 0.46	4.76 ± 0.05	45.45 ± 0.05	27.02 ± 0.00	5.38 ± 0.01
P4	Ø < 1.25	34.48 ± 0.45	22.19 ± 0.71	4.69 ± 0.05	43.82 ± 0.19	24.27 ± 0.56	5.79 ± 0.02

[a] Sieving yield percentages were calculated respect to 100 g of initial dry sugarcane bagasse, i.e., P1 is the percentage of sugarcane bagasse discarded and P2 is the usable fraction. Finally, P3 and P4 yields include the previous P2 sieving yield.
[b] AIL: acid insoluble lignin.
[c] ASL: acid soluble lignin.

Novozymes, Denmark. No xylanase complex addition was required since the cocktail is produced by *Trichoderma reesei*, known as xylanase producer (Kovacs et al., 2009; Wang and Cheng, 2011).

Hydrolysis were performed at 50 °C, 300 rpm for 48 h. The hydrolysates were centrifuged at 20,000×*g* for 5 min, supernatant filtered through 0.22 μm filters and stored for chromatography analysis. For yield calculations, the mass of released sugars was divided by the initial mass of the corresponding sugar polymer in the raw material, and then multiplied by 100. Hence, any potential sugar degradation or loss during the process from raw material to hydrolysates was considered into sugar release yield. For selected experiments, whole slurries from enzymatic hydrolysis were fermented.

2.4. Alcoholic fermentations

2.4.1. Pre-inoculums

A fresh bakery strain of *S. cerevisiae* acquired in a local grocery shop was grown aerobically on a rotatory shaker at 30 °C, 175 rpm for 24 h on commercial YEPD (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose), and it was used as a pre-inoculum.

The strain *P. stipitis* DSM 3651 obtained from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Germany, was maintained in YEPX agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose, and 20 g/L agar) at 4 °C. For the pre-inoculum, the yeast was grown aerobically in YEPX liquid medium Erlenmeyer flasks (10 g/L yeast extract and 20 g/L peptone previously autoclaved at 121 °C, 20 min; and 20 g/L of sterile filtered xylose – 0.22 μm filters – added later at room temperature to avoid sugar degradation) at 30 °C, 175 rpm, 24 h.

2.4.2. Fermentation experiments

Whole slurries of hydrolysates of ozonated SCB or model solution were transferred to penicillin flasks. For *S. cerevisiae* experiments, they were supplemented with 4 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4 and 0.75 g/L $MgSO_4{\cdot}7H_2O$ and inoculated with 5.5% (v/v) of pre-inoculum (Wanderley et al., 2013). For *P. stipitis* experiments, 20 g/L peptone, 10 g/L yeast extract, 12.8 g/L KH_2PO_4, 0.51 g/L Na_2HPO_4, 0.47 g/L $(NH_4)_2SO_4$ and 0.47 g/L $MgSO_4{\cdot}7H_2O$ and 10% (v/v) of pre-inoculum were added to each flask (Toquero and Bolado, 2014). Model solutions were 15 g/L of glucose for *S. cerevisiae* experiments and 15 g/L of glucose and 4 g/L of xylose for *P. stipitis* experiments.

Fermentations occurred semi-anaerobically (with the oxygen present in the empty flask space), incubated at 30 °C under 175 rpm, for 24 h for *S. cerevisiae* and 168 h for *P. stipitis*. All the experiments were conducted in duplicate. Fermentations were centrifuged at 20,000×*g* for 5 min, supernatants were filtered through 0.22 μm and analyzed by chromatography for sugars and ethanol content.

2.4.3. Ethanol yield calculation

In addition to the results obtained for ethanol production in g/L by chromatography, the yield of glucose-to ethanol and sugars-to-ethanol conversion, with respect to the maximum theoretical value, was calculated according to Eqs. (1) and (2):

$$\text{Glucose-to-ethanol conversion yield}(\%) = [(\text{g/L of ethanol obtained})/((\text{g/L of initial glucose}) * 0.511)] * 100 \quad (1)$$

$$\text{Sugars-to-ethanol conversion yield}(\%) = [(\text{g/L of ethanol obtained})/((\text{g/L of initial glucose} + \text{xylose}) * 0.511)] * 100 \quad (2)$$

2.5. Analytical methods

The chemical compositional analysis of biomass: AIL, ASL, cellulose (as glucose), xylan (as xylose) and ashes were performed following NREL (*National Renewable Energy Laboratory*, USA) laboratory analytical procedures: *Determination of structural carbohydrates and lignin in biomass* and *Determination of ash in biomass* (Sluiter et al., 2012, 2008). Sugars and ethanol were measured by HPLC with a Phenomenex® HPLC column Rezex™ RPM-Monosaccharide Pb + 2 (8%), 300 × 7.8 mm, at 80 °C, with MilliQ water as the eluent, at 0.6 mL/min, in a Waters Alliance e2695 HPLC system equipped with a refractive index detector, processed by Empower 3 software (Waters).

2.6. Design of experiments using orthogonal array

The effect of four process parameters, considered in recent literature as the major factors in ozonolysis pretreatment: biomass moisture, ozone concentration, ozone/oxygen flow and particle size (Travaini et al., 2016, 2015) has been studied, applying three levels for each parameter. The parameters levels were selected according to our previous work with SCB ozonolysis (Travaini et al., 2013). An OA experimental design $L_9(3)^4$ was used to select the combinations of operation parameters, assigned as design factors levels of the experimental runs. The factors, factors levels and the OA matrix are shown in Tables 2 and 3, respectively. Each experiment was realized in duplicate, and the realization order of the experiments was randomized. The results obtained were statically treated using the software STATGRAPHICS® Centurion XVI Version 16.2.04 (64-bit). The effect of analyzed parameters on the sugars release yields after enzymatic hydrolysis, both regarding the time of reaction, and the grams of sugar released by g of ozone expended, was studied, applying the analysis of variance (ANOVA), and the factors levels calculated to optimize sugar yields.

Table 2
Orthogonal array experimental design $L_9(3)^4$ factors (ozonolysis parameters for sugarcane bagasse pretreatment) and their levels.

Factors		Levels		
Code	Parameter	1	2	3
A	Sugarcane bagasse moisture (%, w/w)	30	50	70
B	Ozone concentration (%, mol/mol in ozone/oxygen)	2	3	4
C	Ozone/oxygen flow (L/h)	30	60	90 (72)*
D	Particle size (mm)	Ø < 4.76	4.76 > Ø > 1.25	Ø < 1.25

* For experiment number 7, the initially assigned factors and levels of the OA resulted in high pressure drops, appearing hydrodynamic problems, and causing the ozone to scape unreacted since time zero; making the experiment in these operation conditions useless for statistical analysis. To complete the OA matrix, this experiment 7 was repeated changing the flow from 90 L/h (level 3) to 72 L/h, providing a C factor level higher than 2, and these results are shown in the Table 3.

Statistical analysis of the data from the alcoholic fermentations included a one-way ANOVA followed by Tukeýs test with a confidence level of 95.00%.

3. Results and discussion

3.1. OA analysis

Table 3 shows the results of the experimental design used to study the effect of the four operation parameters: A (SCB moisture content, % (w/w)), B (Ozone concentration (%, mol/mol in ozone/oxygen), C (Ozone/oxygen flow (L/h)) and D (Particle size (mm)) on yields of glucose and xylose release after enzymatic hydrolysis, ozone reacted, ozone expended and ozonolysis reaction time. For experiment number 7, the initially assigned factors and levels of the OA resulted in high pressure drops, appearing hydrodynamic problems, and causing the ozone to scape unreacted since time zero; making the experiment in these operation conditions useless for statistical analysis. To complete the OA matrix, this experiment 7 was repeated changing the flow from 90 L/h (level 3) to 72 L/h, providing a C factor level higher than 2, and these results are shown in the Table 3.

Glucose yields ranged between 32.42% and 77.55%, and xylose yields between 19.42% and 56.94%, with both sugar yields increasing in parallel. For all the experiments, an evident direct correlation between sugar release and ozone consumption was found. For reacted ozone, the linear fits of glucose and xylose release yields provided R^2 of 0.9627 and 0.9826, respectively, showing that the ozone reacted during SCB pretreatment is directly related to the yields obtained in each experimental condition. For ozone expended, the linear fit provided R^2 of 0.9739 and 0.9605, respectively, for glucose and xylose, with values in the range of 0.336–0.439 g of ozone by g of total sugars released. The outlet value for non-reacted ozone ranged from 0.003 to 0.030 g/g of biomass.

Differences in reaction time are remarkable, ranging from 24 min to 180 min. The changes in reaction time partially counterbalance the variation of other ozonolysis parameters such as ozone flow or ozone concentration; providing values of reacted ozone by gram of SCB restricted to a narrow range (except for experiment 7).

The sugar yields found in the OA experiments are very high when compared with our first work (Travaini et al., 2013), where an ozone consumption similar to the experimental condition 4 (0.21 g by g of SCB) provided 35.22% (2.2-fold less) and 52.44% of glucose and xylose release yields, respectively, in a 120 min ozonolysis pretreatment using a higher ozone concentration of 3.44% v/v.

3.1.1. ANOVA analysis of the effect of ozonolysis process parameters on glucose/xylose release and reaction time

The experimental design applied, with 9 degrees of freedom, made it possible to estimate, beyond the individual effect of separated parameters, the quadratic terms AA and BB, and the interactions AB and AC. The ANOVA total sum of squares was used to estimate the percentage of influence of each factor on sugar yields and reaction time (Table 4). The ANOVA results for reacted ozone are not shown since they were very similar to those of glucose release, because of the almost linear relation between them. Only the values with $p < 0.05$ were considered, since they indicated a significant difference at a 95.00% confidence level.

The ANOVA analysis of the OA model fitted for glucose yields resulted in a coefficient of determination R^2 that explains 99.09% of the variability. Based on the ANOVA sum of squares of estimated effects (Table 4), the individual parameters had an influence on enzymatic hydrolysis glucose yields in the following order: $B \gg A > C \geqslant D$. Previous works showed moisture content as the most important parameter on ozonolysis pretreatment (Li et al., 2015; Neely, 1984; Travaini et al., 2016). García-Cubero et al. (2009) varied moisture content at 20% and 40%, and the ozone

Table 3
Orthogonal array experimental design $L_9(3)^4$ matrix and experimental results (sugar yields, ozone consumption and time of reaction).

Orthogonal array matrix[a]					Experimental results				
Exp. No.	Parameters and their levels				Sugars yields (%)[b]		Ozone consumption (g by g of biomass)		Time (min)[e]
	A	B	C	D	Glucose	Xylose	Expended[c]	Reacted[d]	
1	1	1	1	1	56.03 ± 0.19	41.15 ± 1.52	0.134 ± 0.005	0.130 ± 0.005	180 ± 4
2	1	2	2	2	50.09 ± 0.44	31.50 ± 0.23	0.105 ± 0.002	0.095 ± 0.007	45 ± 1
3	1	3	3	3	65.85 ± 0.74	42.91 ± 0.78	0.172 ± 0.011	0.142 ± 0.012	38 ± 3
4	2	1	2	3	77.55 ± 1.63	56.94 ± 0.63	0.222 ± 0.005	0.217 ± 0.004	150 ± 7
5	2	2	3	1	62.82 ± 2.05	46.25 ± 2.10	0.160 ± 0.005	0.151 ± 0.001	48 ± 2
6	2	3	1	2	60.94 ± 2.09	43.82 ± 1.73	0.143 ± 0.001	0.136 ± 0.002	89 ± 4
7	3	1	3*	2	32.42 ± 0.06	19.42 ± 0.49	0.042 ± 0.000	0.035 ± 0.001	24 ± 0
8	3	2	1	3	70.83 ± 0.67	47.03 ± 1.79	0.168 ± 0.004	0.165 ± 0.003	146 ± 11
9	3	3	2	1	50.30 ± 0.75	31.42 ± 0.60	0.106 ± 0.005	0.097 ± 0.005	33 ± 1

[a] Orthogonal array matrix used in the experimental design $L_9(3)^4$, parameters studied and their levels values are presented in Table 2.
[b] For yield calculations the weight of released sugars was divided by the initial mass of each sugar in the raw material, and then multiplied by 100. Any potential sugar degradation or lost between raw material and hydrolyzate was considered into the sugar release yield.
[c] Ozone consumed from time zero to the end of pretreatment, divided by g of sugarcane bagasse pretreated (dry basis).
[d] Ozone expended minus ozone escaped, divided by g of sugarcane bagasse pretreated (dry basis).
[e] Time of reaction between experiment begin and ozone unreacted scape, plus additional 15 min of reaction security.
* For experiment number 7, factors and levels of the OA provided a combination where unreacted ozone escaped from reactor since time zero making impossible its use for statistical analysis. To complete the OA matrix, the assay was conducted changing the flow from 90 L/h (level 3) to 72 L/h, providing a C factor level higher than 2.

Table 4
ANOVA sum of squares, *p*-value and percentage of influence of parameters and interactions on sugarcane bagasse sugars release yields and time.

Parameter	Glucose			Xylose			Time		
	Sum of squares	*p*-value	% of influence[a]	Sum of squares	*p*-value	% of influence[a]	Sum of squares	*p*-value	% of influence[b]
A:Moisture	113.0	0.0001	6.54	104.4	0.0003	6.21	1200	0.006	2.78
B:Ozone	292.5	0.0000	16.92	278.1	0.0000	16.53	13975	0.0000	32.36
C:Flow	32.41	0.0086	1.87	49.1	0.0037	2.92	11563	0.0000	26.77
D:Particle Size	29.15	0.0113	1.69	51.8	0.0031	3.08	2458	0.0000	5.69
AA	660.3	0.0000	38.19	721.4	0.0000	42.87	1272	0.0005	2.95
AB	5.44	0.2036	–	0.08	0.8786	–	521	0.0076	1.21
AC	444.8	0.0000	25.72	330	0.0000	19.61	6718	0.0000	15.55
BB	156.9	0.0000	9.07	147.8	0.0001	8.79	5482	0.0000	12.69
ANOVA total sum of squares:	1729	–	–	1682	–	–	43191	–	–

[a] The percentage of influence was calculated by the individual sum of squares of each factor or interactions, divided by the total sum of squares of significant factors of each sugar multiplied by 100.
[b] The percentage of influence was calculated by the individual sum of squares of each factor or interactions, divided by the total sum of squares of significant factors of time multiplied by 100.

concentration at 2.7% and 3.0%, and found that ozone concentration has a low impact on the sugars release from wheat straw. Panneerselvam et al. (2013) observed that, for energy grasses moisturized to 30% water content, 1.85%, 2.33% and 2.71% (mol/mol) of ozone concentration provided similar sugar releases. Nevertheless, a statistical analysis of our experimental results reveals the important effect of ozone concentration. This difference can probably be attributed to the wider range of ozone concentration studied in this work. The scarce influence of ozone/oxygen flow on the glucose release yield may indicate no limitation by mass transfer, with kinetics being the controlling step of the global ozonolysis process (Travaini et al., 2016).

The ANOVA analysis for xylose yield resulted in a R^2 that explains 98.52% of the variability, with very similar results to those for glucose yield. Separate factors affecting xylose yields after enzymatic hydrolysis according to the sum of squares were: $B \gg A > D \geqslant C$ (Table 4). The influence of moisture and ozone concentration was practically identical for both monomeric sugars, and only small differences were found for flow and particle size, changing the order of these parameters. A previous work from Souza-Correa et al. (2014), working with a particle size of between 0.08 mm and 2 mm showed no effect of particle size on glucose release, but some influence on xylose release from ozonated SCB. The xylose release increased from a xylose concentration of 0.65 mg/mL for 2.0 mm particles to 0.95 mg/mL for 0.08 mm particles.

The ANOVA analysis for reaction time resulted in a R^2 that explains 99.30% of the variability, with very different results for the parameter influences with respect to the sugar yields. Ozone concentration and ozone/oxygen flow showed a high effect on reaction time, with a final influence order of: $B > C \gg D > A$. Thus, ozone concentration was concluded to be the parameter that had the greatest influence on all the studied experimental conditions, probably because of the high variation in reaction times with the ozone/oxygen flow softening its effect on sugar release yields. Despite the scarce references in literature about the influence of flow on digestibility, studies using fixed bed reactors with other kinds of lignocellulosic biomass point out that excessive flow, in general, results in shorter reaction times and larger ozone consumption (García-Cubero et al., 2009; Neely, 1984; Schultz-Jensen et al., 2011b). Particle size was shown to have a limited influence on the ozonization process in the studied operation range. Neither differences in surface area nor differences in composition for each particle size fraction (Table 1) had a reliable influence on the ozonolysis operation. Moisture certainly affected sugar release yields, but its effect on reaction time was limited.

3.1.2. Individual effects and interactions of process parameters

The effect of individual parameters on glucose and xylose release yields and reaction time are shown in Fig. 1. The tendency was similar for the three types of experimental results, but as explained previously, there were found to be different degrees of influence on the parameters analyzed (Table 4).

The moisture content plot had the most complex aspect, going through a maximum close to 1.5 level (corresponding to 40% (w/w) moisture content). Whereas low water content seems to limit the ozone mass transfer, excessive water content provides a thick water film that blocks biomass pores, leading to secondary, less-effective reactions (Li et al., 2015).

The effect of ozone concentration, the parameter with the highest influence, reaches a minimum close to the highest ozone concentration analyzed, with a slight increase at the end. This curve shape is identical for all the analyzed results, with the highest gradient for reaction time. The effect of ozone concentration on ozonolysis pretreatment is not clear in literature, probably because of the ozone concentration range studied, and its complex interaction with other operation parameters. In previous works, it was reported as a non-influential parameter on the sugars release for wheat straw (García-Cubero et al., 2009) and energy grasses (Panneerselvam et al., 2013).

As individual factors, both ozone/oxygen flow and particle size caused a linear decline in the analyzed variables within the studied range. As mentioned, only the slope of the reaction time with ozone/oxygen flow was remarkable with a $P < 0.05$. Nevertheless, Barros et al. (2013), working with the association of wet disk milling and ozonolysis, found that this combination favored sugar release after enzymatic hydrolysis. These high values were probably achieved thanks to the small particle size caused by wet disk milling, very different from the milling method used in this work.

The only remarkable interaction found when analyzing the experimental results appears between the parameters of moisture (A) and ozone/oxygen flow (C) (Fig. 1d), corresponding to 25.72% of the total ANOVA sum of squares of significant factors for glucose (Table 4). Whereas the increment of moisture content improves glucose yields at 30 L/h flow, it decreases glucose release at 90 L/h. In the first case, thanks to increasing moisture content, the ozone accessibility to sugarcane bagasse increases too. Nevertheless, with a 90 L/h flow, the positive effect of solubilization is surpassed by hydrodynamic problems, and lower results were obtained with higher moisture contents. This could be due to the formation of preferential pathways within the porous structure of the fixed bed, resulting in smaller unreacted ozone escape times (Table 3) and non-homogeneous reaction. This problem

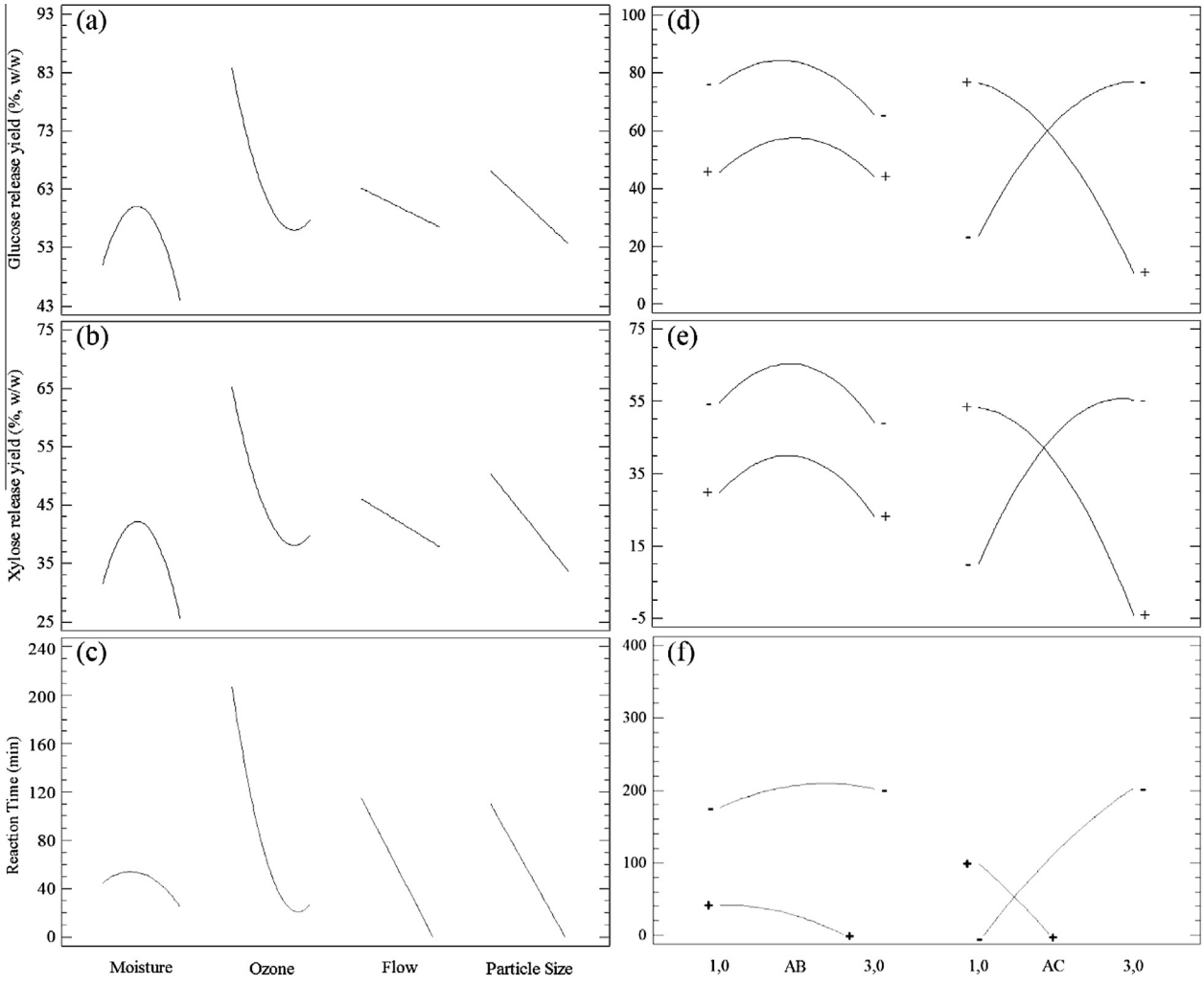

Fig. 1. Individual plots of the process parameters' effect on glucose (a) and xylose (b) release yields, and reaction time (c). Interaction plots of process parameters (moisture content vs ozone concentration, called AB, and moisture content vs ozone/oxygen flow, called AC), for glucose (d) and xylose (e) release yields, and reaction time (f). Graphics provided by STATGRAPHICS® Centurion XVI Version 16.2.04 (64-bit) $L_9(3)^4$ orthogonal array analysis. In figures d, e and f, X-axis represents the level of the factor A, and minus and plus signs over the curves indicate the level of the second factor (B or C) +: level 3; −: level 1. The factors of ozone/oxygen flow and particle size are maintained at level 2 for interaction AB, and the factors of ozone concentration and particle size at level 2 for interaction AC.

was also pointed out by experiment number 7, and other previous experiments (not shown here) with high moisture and ozone/oxygen flow. As described before, when the assay was conducted at 90 L/h flow with 70% moisture content, the ozone escaped from the reactor from Time = zero onwards. Even when working with the final conditions in experiment 7, with 72 L/h flow and 70% moisture, the smallest reaction time and glucose and xylose release yields were obtained, presumably due to hydrodynamic problems inside the fixed bed reactor. Analogous interactions were found for xylose and time (Fig. 1d and e), with the interaction AC being the only significant one, with 19.61% and 15.55% of the total ANOVA sum of squares (Table 4).

3.2. Process parameters optimization for sugars release

The values of sugars release yields obtained in the OA experiments (Table 3) were fitted by STATGRAPHICS® as described in Section 2.6, and the equations with regression coefficients for tested parameters and interactions were obtained. The regression model equations for glucose and xylose release yields are shown in Eqs. (3) and (4), respectively, fitted as sugar yield in% (w/w, sugar release divided by sugar content in raw SCB):

$$\text{Glucose yield}\%(\text{w/w}) = -6.80 + 103.31A - 61.12B + 56.36C - 6.23D - 12.85A^2 + 2.33AB - 29.83AC + 10.85B^2 \quad (3)$$

$$\text{Xylose yield}\%(\text{w/w}) = -17.43 + 102.71A - 54.29B + 47.34C - 8.31D - 13.43A^2 - 0.28AB - 25.69AC + 10.53B^2 \quad (4)$$

Equations were solved using the OA values matrix, and then the standard deviation between the predicted values (data not shown) and the experimental sugar yields (Table 3) was calculated in order to validate the accuracy of the equations. The maximum deviation found for glucose and xylose release yields was 2.09 and 2.10% (w/w), respectively, corresponding to percentages of relative deviation of 3.43 and 4.54%, which indicates a good fitting between the model and the experimental results. This accuracy indicates also that the uncertainty introduced by changing the factor's C level for experiment 7 (Section 3.1 OA analysis) did not interfere in the OA analysis.

The equations were processed to obtain the optimal level combination for maximum sugars release in each case. For glucose, the

maximum predicted yield reached 100%, meaning that no physical hindrance exists if optimal conditions are used. However, for xylose this value was 86.43%. The impossibility of achieving a 100% yield can be related to a slight xylose degradation during pretreatment, or to a non-accessible hemicellulose fraction in our SCB.

The optimization values of factors levels provided by the software, and the respective approximation of real parameters values are shown in Table 5. For moisture content, the optimum values were very different for glucose and xylose. As is thoroughly mentioned in literature, xylose is more sensitive to degradation than glucose, and water has a protective effect during the oxidative pretreatment (Travaini et al., 2015). The low ozone concentration improves the sugar release, finding the optimum at the lowest tested value 2% (mol/mol) for both sugars. In most of the published ozonolysis literature, researchers usually worked at an ozone concentration higher than 2%. Only Schultz-Jensen et al. (2011a) and Kádár et al. (2015) pretreated wheat straw using ozone concentrations of around 1% (mol/mol), but they obtained glucose release yields after enzymatic hydrolysis similar or lower than those obtained by other authors using higher ozone concentration (Travaini et al., 2016). For ozone/oxygen flow the results of optimization are not clear, with different results for glucose and xylose, probably due to interactions between moisture and flow. The increase on particle size favors the sugar release yields, in spite of the small influence detected for this parameter.

The optimization results, compared to the experimental runs, showed that experiment 4 is very close to the optimal parameters combination for both sugars (OA experiment 4, Table 5): Parameters A and C are at level 2, an intermediate value between optimums for both sugars; B is at level 1, being the optimum; and only the particle size differs, with a level 3 instead of the optimum level 1. Nevertheless, particle size had the lowest influence compared to the other individual factors and interactions, as mentioned before. Experiment 4 provided the highest glucose and xylose yields 77.55% and 56.94%, respectively.

3.3. Analysis of sugars produced in relation to ozone expended

In order to analyze the pretreatment economy, and therefore the process viability, the yield in terms of grams of sugars released per gram of expended ozone is a key process parameter. As noted before, the sugar release increased with ozone reacted in a near linear correlation, however, a more in-depth analysis of the results showed a non-linear correlation between the amounts of released sugars per g of expended ozone vs g of expended ozone (R^2 of the linear fit 0.7965; experiment 7 results were not considered due to the related hydrodynamic problems). For OA experiment 4 (where the highest sugars yields were obtained), 2.28 g of sugars were released per g of ozone expended, whereas for OA experiment 2 (the lowest sugar release, excluding OA experiment 7) 2.98 g of sugars were released per g of ozone.

The statistical analysis of the results in terms of total sugars release per g of expended ozone, applying ANOVA, showed the order of effect of the parameters as follows: A ≫ B, with the moisture content being the most important process parameter, with 32.32% of influence. The individual plot for this factor (not shown) passes through a minimum near to level 2, and then grows quickly until level 3, where the highest sugar release per g of expended ozone can be achieved. The influence of ozone concentration, the most important parameter for sugars release, was only 6.82%, when the goal was to achieve maximum sugar production per gram of expended ozone. Ozone/oxygen flow and particle size had no influence ($P > 0.05$) on the process, corroborating their low impact on sugars release yields. The regression coefficients for this optimization are shown in Eq. (5):

$$\text{g of sugars/g of ozone expended} = 5.42 - 4.78A + 3.66B - 3.23C + 0.36D + 0.81A^2 - 0.70AB + 1.69AC - 0.43B^2 \quad (5)$$

Although the optimization of sugars release yields can provide hydrolysates with high sugars contents (desirable for fermentation and product separation processes), the increase in sugar release implies higher expended ozone per g of released sugar. Further economic studies, out of the scope of this work, are necessary in order to optimize the global process, taking into account the expenditure on ozone production, but also on downstream processes.

3.4. Alcoholic fermentation of hydrolysates for ethanol production

With the objective of studying the fermentability of ozonated SCB hydrolysates and evaluating the inhibitory effect of degradation compounds produced during the pretreatment, two different yeasts were tested. Fermentation experiments were carried out using a bakery strain of *S. cerevisiae* (the most widely spread ethanol fermentative microorganism) and the diauxic yeast *P. stipitis* DSM 3651, which is able to ferment hexoses and pentoses. Hydrolysates with glucose concentration of ⩾15 g/L were selected for the fermentation assays.

The results obtained with *S. cerevisiae* are shown in terms of ethanol concentration (g/L) and glucose-to-ethanol conversion yield (%) in Fig. 2. The one-way ANOVA of glucose-to-ethanol conversion yields (%) showed a non-significant statistical difference between the tested hydrolysates ($F = 2.29$ and p-value = 0.1192), indicating that combinations of parameters levels did not affected fermentation by *S. cerevisiae*. Differences on ethanol production are related to the sugar concentrations on hydrolysates of the pretreated samples, but not to the fermentation yields. For OA experiment 4, the highest ethanol production was found, obtaining 7.19 ± 0.09 g/L of ethanol corresponding to 76% glucose-to-ethanol conversion; for OA experiment 1, the one with the lowest ethanol production, 6.20 ± 0.16 g/L ethanol was reached, an 80% glucose-to-ethanol conversion.

A model fermentation containing 15 g/L of glucose was also conducted, to evaluate the possible inhibitory effect of degradation compounds on ethanol production. It provided 5.46 ± 0.04 g/L of ethanol, and a glucose-to-ethanol conversion yield of 71.22 ± 0.51%. The one-way ANOVA of glucose-to-ethanol conversion yields (%) between actual values obtained from hydrolysates and model fermentation indicated a significant statistical

Table 5
Optimization of ozonolysis process parameters for sugarcane bagasse sugars release yields and comparison with orthogonal array experiment 4 parameters.

Parameter optimized	Level of optimized factors				Real values of optimized parameters			
	A	B	C	D	Moisture content (%)	Ozone concentration (mol/mol)	Flow (L/h)	Particle Size (mm)
Glucose	1.51	1.01	2.97	1.04	40	2	90	Ø < 4.76
Xylose	2.86	1.03	1.00	1.00	68	2	30	Ø < 4.76
OAE4*	2	1	2	3	50	2	60	Ø < 1.25

* Process parameters conditions for orthogonal array experiment number 4.

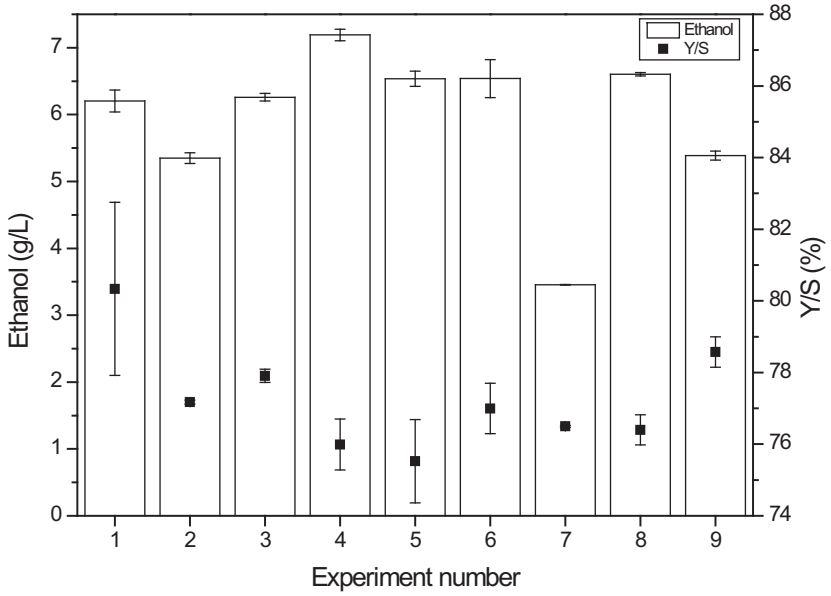

Fig. 2. Ethanol production by *Saccharomyces cerevisiae* bakery's strain by whole slurry fermentation hydrolysates from the orthogonal array experiments. Results are shown in ethanol production g/L (bars) and Y/S (points): glucose-to-ethanol conversion in fermentations divided by the maximum theoretical, multiplied by 100.

difference ($F = 22.66$ and p-value = 0.0002). These values not only revealed a non-inhibitory trend, but also demonstrated a positive effect increasing the ethanol production by hydrolysates. Glucose was not detected after fermentation by *S. cerevisiae* bakery strain, both for model and hydrolysates, and the xylose content did not change by the fermentation step.

No microorganism growth was observed when hydrolysates were used as the carbon source for fermentation by *P. stipitis* DSM 3651, although an 80% of sugars-to-ethanol conversion was achieved for model fermentation. After fermentation, no glucose was detected, and 93% of the initial xylose content was consumed. Degradation compounds produced by the ozonolysis pretreatment showed a clear inhibitory effect of this yeast, and hence no ethanol production.

Previous tests have proven the efficiency of water washing as a detoxifying step, removing degradation compounds which inhibit *P. stipitis* DSM 3651 (Toquero and Bolado, 2014). So, further studies are necessary to explore alternatives on how to make the most of the sugar content of this lignocellulosic waste, producing ethanol from both pentoses and hexoses.

From an energetic point of view, a preliminary evaluation, considering only the pretreatment step and a sugars-to-ethanol conversion of 80%, was done to evaluate the feasibility of the process. Nowadays, the production of 100 g of ozone requires 1.65 MJ of energy, while 100 g of ethanol releases 2.67 MJ of energy (Travaini et al., 2016). From our experimental results, at the best conditions for sugars release, (OA experiment number 4), producing ethanol only from glucose fermentation can yield 3.85 MJ of energy per kg of raw SCB; whereas when fermenting both glucose and xylose, the energy production can reach 5.52 MJ per kg of raw SCB. Since only the ozone production at the experimental conditions requires 3.66 MJ per kg of SCB, a viable process to valorize SCB for ethanol production demands the fermentation of all the sugar content from the biomass. Considering the experiment with the lowest sugar release yield but also the lowest ozone consumption (experiment 2), more favorable energetic assessments were obtained. 2.49 MJ of energy per kg of raw SCB can be produced by fermenting only glucose, and 3.42 MJ through glucose and xylose fermentation, with an energetic consumption for ozone production of 1.73 MJ.

Beyond these figures, a drastic reduction in energy requirements for ozone generation should be considered. Five years ago, the energetic consumption needed to produce 100 g of ozone was estimated at 2.38 MJ (Schultz-Jensen et al., 2011b), whereas in 2015 it was around 1.65 MJ, giving good future perspectives.

4. Conclusions

The SCB ozonolysis analysis revealed ozone concentration as the most important parameter for sugars release, with an optimal value of 2% mol/mol. The moisture showed a great influence on the sugars production per gram of expended ozone, with a positive effect. The increase in sugar release results in higher ozone consumption. Selected optimal operation conditions would provide 137 kg of ethanol by ton of SCB by *S. cerevisiae* fermentation; nevertheless, *P. stipitis* was unable to grow. The energetic balance between ethanol combustion potential and ozone production was positive, but currently the global process economy would require glucose and xylose fermentation.

Acknowledgments

This work was supported by the UIC 071 research unit of the regional government "*Junta de Castilla y León – JCyL*", Spain. Rodolfo Travaini is also grateful to "*Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq*", Brazil (238059/2012-0) for providing him with a Doctorate Scholarship.

References

APHA-AWWA-WEF, 2005. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, D.C.

Barros, R.D.R.O.De., Paredes, R.D.S., Endo, T., Bon, E.P.D.S., Lee, S.H., 2013. Association of wet disk milling and ozonolysis as pretreatment for enzymatic saccharification of sugarcane bagasse and straw. Bioresour. Technol. 136, 288–294.

Bhattarai, S., Bottenus, D., Ivory, C.F., Gao, A.H., Bule, M., Garcia-Perez, M., Chen, S., 2015. Simulation of the ozone pretreatment of wheat straw. Bioresour. Technol. 196, 78–87.

de Cassia Pereira, J., Travaini, R., Paganini Marques, N., Bolado-Rodríguez, S., Bocchini Martins, D.A., 2016. Saccharification of ozonated sugarcane bagasse using enzymes from *Myceliophthora thermophila* JCP 1–4 for sugars release and ethanol production. Bioresour. Technol. 204, 122–129.

Garcia-Cubero, M.T., Coca, M., Bolado, S., Gonzalez-Benito, G., 2010a. Chemical oxidation with ozone as pre-treatment of lignocellulosic materials for bioethanol production. Chem. Eng. Trans. 21, 1273–1278.

García-Cubero, M.T., González-Benito, G., Indacoechea, I., Coca, M., Bolado, S., 2009. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresour. Technol. 100, 1608–1613.

Garcia-Cubero, M.T., Marcos, M., Bolado, S., Coca, M., Gonzalez-Benito, G., 2010b. Optimization of the operating conditions in enzymatic hydrolysis of pretreated lignocellulosic materials. Chem. Eng. Trans. 21, 1285–1290.

Garcia-Cubero, M.T., Palacin, L.G., Gonzalez-Benito, G., Bolado, S., Lucas, S., Coca, M., 2012. An analysis of lignin removal in a fixed bed reactor by reaction of cereal straws with ozone. Bioresour. Technol. 107, 229–234.

Kádár, Z., Schultz-Jensen, N., Jensen, J.S., Hansen, M.a.T, Leipold, F., Bjerre, A.-B., 2015. Enhanced ethanol production by removal of cutin and epicuticular waxes of wheat straw by plasma assisted pretreatment. Biomass Bioenergy 81, 26–30.

Kojima, Y., Yoon, S.-L., 2008. Improved enzymatic hydrolysis of waste paper by ozone pretreatment. J. Mater. Cycles Waste Manag. 10, 134–139.

Kovacs, K., Macrelli, S., Szakacs, G., Zacchi, G., 2009. Enzymatic hydrolysis of steam-pretreated lignocellulosic materials with *Trichoderma atroviride* enzymes produced in-house. Biotechnol. Biofuels 2, 14.

Li, C., Wang, L., Chen, Z., Li, Y., Wang, R., Luo, X., Cai, G., Li, Y., Yu, Q., Lu, J., 2015. Ozonolysis pretreatment of maize stover: the interactive effect of sample particle size and moisture on ozonolysis process. Bioresour. Technol. 183, 240–247.

Mamleeva, N.A., Autlov, S.A., Bazarnova, N.G., Lunin, V.V., 2009. Delignification of softwood by ozonation. Pure Appl. Chem. 81, 2081–2091.

Neely, W.C., 1984. Factors affecting the pretreatment of biomass with gaseous ozone. Biotechnol. Bioeng. 26, 59–65.

Panneerselvam, A., Sharma-Shivappa, R.R., Kolar, P., Ranney, T., Peretti, S., 2013. Potential of ozonolysis as a pretreatment for energy grasses. Bioresour. Technol. 148, 242–248.

Schultz-Jensen, N., Kádár, Z., Thomsen, A.B., Bindslev, H., Leipold, F., 2011a. Plasma-assisted pretreatment of wheat straw for ethanol production. Appl. Biochem. Biotechnol. 165, 1010–1023.

Schultz-Jensen, N., Leipold, F., Bindslev, H., Thomsen, A., 2011b. Plasma-assisted pretreatment of wheat straw. Appl. Biochem. Biotechnol. 163, 558–572.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Determination of ash in biomass: Laboratory Analytical Procedure (LAP), NREL/TP-510-42622. National Renewable Energy Laboratory (NREL), Golden, Colorado, USA.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2012. Determination of structural carbohydrates and lignin in biomass: Laboratory Analytical Procedure (LAP), NREL/TP-510-42618. National Renewable Energy Laboratory (NREL), Golden, Colorado, USA.

Soccol, C.R., Vandenberghe, L.P. de S., Medeiros, A.B.P., Karp, S.G., Buckeridge, M., Ramos, L.P., Pitarelo, A.P., Ferreira-Leitão, V., Gottschalk, L.M.F., Ferrara, M.A., 2010. Bioethanol from lignocelluloses: status and perspectives in Brazil. Bioresour. Technol. 101, 4820–4825.

Souza-Correa, J.A., Oliveira, C., Nascimento, V.M., Wolf, L.D., Gómez, E.O., Rocha, G.J. M., Amorim, J., 2014. Atmospheric pressure plasma pretreatment of sugarcane bagasse: the influence of biomass particle size in the ozonation process. Appl. Biochem. Biotechnol. 172, 1663–1672.

Souza-Correa, J.A., Oliveira, C., Wolf, L.D., Nascimento, V.M., Rocha, J.G.M., Amorim, J., 2013. Atmospheric pressure plasma pretreatment of sugarcane bagasse: the influence of moisture in the ozonation process. Appl. Biochem. Biotechnol. 171, 104–116.

Thangavelu, S.K., Ahmed, A.S., Ani, F.N., 2016. Review on bioethanol as alternative fuel for spark ignition engines. Renew. Sustain. Energy Rev. 56, 820–835.

Toquero, C., Bolado, S., 2014. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour. Technol. 157, 68–76.

Travaini, R., Marangon-Jardim, C., Colodette, J.L., Morales-Otero, M.D., Bolado-Rodríguez, S., 2015. Ozonolysis. In: Pandey, A., Negi, S., Binod, P., Larroche, C. (Eds.), Pretreatment of Biomass. Elsevier, USA, pp. 105–135.

Travaini, R., Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodríguez, S., 2016. Ozonolysis: an advantageous pretreatment for lignocellulosic biomass revisited. Bioresour. Technol. 199, 2–12.

Travaini, R., Otero, M.D.M., Coca, M., Da-Silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment: effect on enzymatic digestibility and inhibitory compound formation. Bioresour. Technol. 133, 332–339.

UNICA, 2015. Relatório final da safra de 2014/2015, região centro-sul, UNICADATA, avaliable at http://www.unicadata.com.br/, last accessed sep. 10, 2015. São Paulo, Brasil.

Wanderley, M.C.D.A., Martín, C., Rocha, G.J.D.M., Gouveia, E.R., 2013. Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. Bioresour. Technol. 128, 448–453.

Wang, Z., Cheng, J.J., 2011. Lime pretreatment of coastal bermudagrass for bioethanol production. Energy Fuels 25, 1830–1836.

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Travaini, R., Barrado, E., Bolado-Rodríguez, S., 2016. Bioresour. Technol. In press.

CAPITULO 6

Effect of ozonolysis parameters on the inhibitory compound generation and on the production of ethanol by *Pichia Stipitis* and Acetone-Butanol-Ethanol by *Clostridium* from ozonated and water washed sugarcane bagasse

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PII: S0960-8524(16)30992-0
DOI: http://dx.doi.org/10.1016/j.biortech.2016.07.028
Reference: BITE 16790

To appear in: *Bioresource Technology*

Received Date: 8 June 2016
Revised Date: 7 July 2016
Accepted Date: 8 July 2016

Please cite this article as: Travaini, R., Barrado, E., Bolado-Rodríguez, S., Effect of ozonolysis parameters on the inhibitory compound generation and on the production of ethanol by *Pichia Stipitis* and Acetone-Butanol-Ethanol by *Clostridium* from ozonated and water washed sugarcane bagasse, *Bioresource Technology* (2016), doi: http://dx.doi.org/10.1016/j.biortech.2016.07.028

Effect of ozonolysis parameters on the inhibitory compound generation and on the production of ethanol by *Pichia Stipitis* and Acetone-Butanol-Ethanol by *Clostridium* from ozonated and water washed sugarcane bagasse

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ABSTRACT

Sugarcane bagasse (SCB) was ozone pretreated and detoxified by water washing, applying a $L_9(3)^4$ orthogonal array (OA) design of experiments to study the effect of pretreatment parameters (moisture content, ozone concentration, ozone/oxygen flow and particle size) on the generation of inhibitory compounds and on the composition of hydrolysates of ozonated-washed samples. Ozone concentration resulted the highest influence process parameter on delignification and sugar release after washing; while, for inhibitory compound formation, moisture content also had an important role. Ozone expended in pretreatment related directly with sugar release and inhibitory compound formation. Washing detoxification was effective, providing non-inhibitory hydrolysates. Maximum glucose and xylose release yields obtained were 84% and 67%, respectively, for ozonated-washed SCB. Sugar concentration resulted in the decisive factor for biofuels yields. Ethanol production achieved an 88% yield by *Pichia stipitis*, whereas *Clostridium acetobutylicum* produced 0.072 $g_{BUTANOL}/g_{SUGAR}$ and 0.188 g_{ABE}/g_{SUGAR}, and, *Clostridium beijerinckii* 0.165 $g_{BUTANOL}/g_{SUGAR}$ and 0.257 g_{ABE}/g_{SUGAR}.

Keywords: ozonolysis pretreatment; sugarcane bagasse; hydrolysates detoxification; ethanol; butanol.

1. Introduction

Despite great advances driven by fossil fuels use, it is also responsible for many environmental, political and economic problems and recently complete oil depletion has been forecasted. For these reasons, there has been a growing necessity to replace fossil fuels by environmentally friendly and renewable energy sources and many alternatives have been proposed. Within the alternatives, the biorefinery approach has appeared to

be one of the most promising, using lignocellulosic residues for integrated bioenergy and value-added compound production. Due to the recalcitrant nature of lignocellulosic materials, a pretreatment stage is required to open biomass structures and liberate lignin. After pretreatment, enzymatic hydrolysis is responsible for depolymerize carbohydrates into their monomeric sugar forms (mainly glucose and xylose), able to be converted into biofuels by microorganisms (Alvira et al., 2010).

Sugarcane is a crop extensively cultivated in many countries, such as Brazil, India and Colombia, where is used for ethanol and aliments production. The SCB is the residue generated in factories after sugarcane juice extraction (Souza Dias et al., 2016), with around 280 kg of SCB generated by ton of sugarcane processed (UNICA, 2015). It is a fibrous lignocellulosic material rich in polymeric sugars with low ash content, making it a promising raw material for biofuel production.

Many pretreatments have been studied for SCB conversion into biofuels, such as acid hydrolysis, wet oxidation, thermal alkali, steam explosion, etc (Chandel et al., 2013; Jonglertjunya et al., 2014; Pang et al., 2016; Su et al., 2015). However, most of them still had technological barriers for application, like low sugar yields, inhibitory compound formation which prevents fermentation, and unfeasible energetic balances. In the last decade, ozonolysis has gained visibility by its many advantages over other pretreatments (Travaini et al., 2015). Two advantages that stand out are its high sugar yields and its selectivity for lignin degradation preserving carbohydrates polymers with no generation of furfural and 5-hydroxymethylfurfural, reported for other pretreatments (Bellido et al., 2014). The most studied ozonolysis configuration uses plug flow reactors and had been applied extensively to wheat straw with systematical studies available about how process parameters influence the compositional content of biomass, sugar

release yields and fermentation (Garcia-Cubero et al., 2012; García-Cubero et al., 2009; Schultz-Jensen et al., 2011b). For SCB ozonolysis, there are published works studying how it affects the structural composition and sugar release yield (Travaini et al., 2013); how the combination with other pretreatments can improve yields (Barros et al., 2013); and, how lignin is decomposed during pretreatment (Souza-Correa et al., 2013b).

The main inhibitory compounds generated by the ozonolysis pretreatment are organic acids and low molecular weight phenolic compounds (Ben'ko et al., 2013; Mamleeva et al., 2009; Schultz-Jensen et al., 2011a). These organic acids were reported as strongly inhibitory, especially for diauxic microorganisms, making the utilization of the xylose fraction of ozonated biomass impossible (Bellido et al., 2011; Toquero and Bolado, 2014). Recently, a systematical study about SCB ozonolysis has been published, highlighting how process parameters affect sugar release yields, time of reaction, ozone consumption and ethanol production by *Saccharomyces cerevisiae* (Travaini et al., 2016a). This previous work also revealed a strong inhibitory effect on *P. stipitis*, that was unable to grow in SCB ozonated hydrolysates. The same study concluded that xylose utilization is necessary to achieve an energetically feasible process. Some published works studied the effect of process parameters on chemical composition of ozonated SCB and on sugar release, using one factor at a time methodology. In these studies, an alkali washing was applied before enzymatic hydrolysis; nevertheless, the alkali washing step's goal was to remove the residual lignin and the study did not measure its detoxification effect (Souza-Correa et al., 2014, 2013a). Other work is available about the fermentation of detoxified SCB ozonated hydrolysates, but only glucose fraction was used for ethanol conversion by *Saccharomyces cerevisiae* (de Cassia Pereira et al., 2016).

The literature states the necessity for a detoxification step, between pretreatment and hydrolysis, to produce hydrolysates able to be fermented by diauxic microorganisms. Nevertheless, no systematic study is available in literature evaluating the influence of ozonolysis pretreatment process parameters on inhibitory compound formation nor the efficiency of detoxification steps. With this objective, in this work, a previously developed OA $L_9(3)^4$ experimental design (Travaini et al., 2016a) was applied to study how ozonolysis process parameters and water washing affect inhibitory compound generation and sugar release yields, taking into account the mass losses during the washing step. Four process parameters were analyzed: moisture content, ozone concentration, ozone/oxygen flow and particle size. Their influence on compositional content (lignin and carbohydrates polymers), sugar release yield (glucose and xylose), and inhibitory compound formation (formic, acetic and oxalic acids and phenolic compounds) were measured by the OA ANOVA sum of squares. In each case, results obtained were model-fitted to predict the combination of parameters for maximal sugar release or inhibitory compound formation. Inhibitory compound removal by the washing detoxification step was measured. Selected hydrolysates of washed-ozonated SCB were fermented by the diauxic yeast *P. stipitis*, for alcoholic fermentation and by the bacteria *C. acetobutylicum* and *C. beijerinckii* for ABE fermentation. A preliminary energetic balance between the energy expended in pretreatment and the combustion of produced biofuels is also presented.

2. Material and methods

2.1. Raw material

The SCB utilized in this study was kindly donated by Usina Virgolino de

Oliveira S/A Açúcar e Álcool, José Bonifácio, São Paulo State, Brazil. The SCB was recovered from piles at the factory, washed with distilled water to remove residual soluble sugars and particulate material and dried in a ventilated oven at 37 °C. The washed and dried SCB was sieved manually, using nº 4 mesh (4.76 mm) and nº 120 mesh (0.125 mm) sieves, to obtain three particle size ranges: Ø<4.76, 4.76>Ø>1.25 mm and Ø<1.25 mm. The averages of the SCB compositional analysis in terms of acid insoluble lignin (AIL), acid soluble lignin (ASL), cellulose and xylose are at Table 1.

2.2. Ozonolysis pretreatment

Ozonolysis pretreatment experiments were conducted in a fixed bed reactor (glass column, 50 cm in height and 2.7 cm in diameter) connected to an ozone generator, Sander 301, fed with industrial oxygen. The inlet oxygen flow was set before each experiment using a rotameter and the outlet flow was saturated with water using a scrubber bottle. Before each test, the reactor was filled with 24±1 g (dry basis) of SCB previously moistened to the required water content in % (w/w, water:SCB). Ozone concentration in the flow was set by energy supply, and measured iodometrically (APHA-AWWA-WEF, 2005). The outlet reactor gas flow was passed through 2% KI solution, and the absorbance was periodically determined each 30 seconds, after color appearance, by spectrophotometry at 464 nm. Ozonolysis pretreatment experiments were stopped 15 minutes after KI solution reached an absorbance of 0.800 (corresponding to 0.028 g of O_3 escaped). The ozone concentration and ozone/oxygen flow in each experiment were used for calculating the ozone expended in pretreatment.

The resulting ozone pretreated SCB in the reactor was divided into four parts within the length of the reactor and the section just before the gas outlet was discharged

in order to avoid non-homogeneous pretreated samples. The other three quarters were gently mixed, the moisture content measured by gravimetry, and stored hermetically in a freezer until use (Travaini et al., 2016). All experiments were conducted by duplicate, according to the experimental design described in section 2.7. A fraction of the solid was used for control enzymatic hydrolysis in order to determine the inhibition compound production. Another fraction was washed with water for compositional analysis, enzymatic hydrolysis assays and fermentations.

2.3. Washing detoxification

Since most of the inhibitory compounds formed in ozonolysis pretreatment are low chain carboxylic acids (Travaini et al., 2016b), the detoxification method selected was a water washing. Ozone pretreated SCB, resulting from each experimental run, was shaken in distillated water (6% w/w, dry basis) for 1 h, at 25 ºC and 300 rpm. Then, the material was filtered with a vacuum pump and the solid fraction was washed with distilled water three times. The final water expended was 100 mL by g of ozone pretreated SCB (dry basis) (Travaini et al., 2013). The procedure was made by duplicate for mass loss measurement and compositional analysis. The procedure was repeated by duplicate for enzymatic hydrolysis assays and for each fermentation test.

For assays of enzymatic hydrolysis, the resulting wet solid from washing detoxification was transferred to Erlenmeyer flasks. The mass lost during the washing steps was not taken into account, in order to refer the sugar release and fermentation results to the mass of initial SCB in all experiments.

2.4. Enzymatic hydrolysis

The ozonated and ozonated-washed SCB samples were enzymatically hydrolyzed in Erlenmeyer flasks (6% w/w, dry basis). The enzyme cocktails Celluclast 1.5L and Novozym 188 were used to provide 10 FPU and 30 CBU per gram of cellulose (dry basis), according to the cellulose content in the raw material. Hydrolysis were buffered with citrate buffer at pH 4.80 (0.05 M). Acetate buffer was used in hydrolysis experiments destined to ABE fermentation because citric acid provided an inhibitory effect on *Clostridium* microorganisms (data not shown). No significant differences were found in hydrolysates sugar yields using acetate or citrate buffer (data not shown). Hydrolysis were performed by duplicate at 50 ºC, 300 rpm for 48 h. The hydrolysates were centrifuged at 20000 xg for 5 min and the supernatant was filtered through 0.22 µm filters and stored for sugars and inhibitory compounds quantification. The mass of released sugars from ozonated-washed SCB samples was divided by the corresponding sugar polymer in raw SCB multiplied by 100 to obtain the sugar release yield (%). For selected experiments, the whole slurries from hydrolysis were fermented.

2.5. Fermentations

2.5.1. Ethanol production

Pichia stipitis DSM 3651 was obtained from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Germany. It was maintained in YEPX agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose, and 20 g/L agar) at 4 ºC. For the pre-inoculum, the yeast was grown aerobically in Erlenmeyer flasks with YEPX liquid medium (10 g/L yeast extract and 20 g/L peptone previously autoclaved at 121ºC, 20 min; and 20 g/L of sterile filtered xylose – 0.22 µm filters - added later at room temperature to avoid sugar degradation) at 30 ºC, 175 rpm, 24h.

For ethanol fermentations, selected hydrolysates whole slurries or sugar model solution (14.00 g/L glucose and 3.50 g/L xylose) were transferred to penicillin flasks and supplemented with 20 g/L peptone, 10 g/L yeast extract, 12.8 g/L KH_2PO_4, 0.51 g/L Na_2HPO_4, 0.47 g/L $(NH_4)_2SO_4$ and 0.47 g/L $MgSO_4 \cdot 7H_2O$ (Toquero and Bolado, 2014). Penicillin flasks were inoculated with 10% (v/v) of pre-inoculum, and fermentations occurred semi-anaerobically (with the oxygen present in the empty flask space), incubated at 30 ºC under 175 rpm for 168 hours. Fermentations were centrifuged at 20000 xg for 5 min, supernatants were filtered through 0.22 µm and analyzed by chromatography for sugar and ethanol content. Sugar-to-ethanol conversion yield was calculated with respect to the maximum theoretical value, according to Eq. 1:

Sugar-to-ethanol conversion yield (%) = [(g/L of ethanol obtained)/((g/L of initial glucose+xylose)*0.511)]*100 (Eq. 1).

For energetic balance, the energy spent for ozone production was considered at 16.5 MJ by kg of ozone (Travaini et al., 2016a) and 26.7 MJ/kg was used as the heating value for ethanol (Schultz-Jensen et al. 2011). The amount of energy, in MJ, produced by kg of SCB pretreated, hydrolyzed and alcoholic fermented, was calculated by Eq. 2:

Energetic balance (MJ/kg_{SCB})= [(kgO_3 expended by kg of SCB)*(16.5 MJ/kgO_3 produced)]-[(kg of ethanol produced)*(26.7 MJ/kg of ethanol)] (Eq. 2)

2.5.2 ABE fermentation

Clostridium acetobutylicum DSM 792 and *Clostridium beijerinckii* DSM 6422 were obtained from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Germany. The strains were submitted to a heat shock at 80 ºC for 3.5 min (to stimulate spores' germination) in Hungate tubes (18 x 150 mm) with nitrogen

flushed Reinforced Clostridial Medium (Fluka, Sigma-Aldrich, Spain). They were grown anaerobically for 120 h for spore formation at 37 and 35 ºC, respectively for *C. acetobutylicum* and *C. beijerinckii*. The spore stock cultures were conserved at -20 ºC.

For pre-inoculums preparation, the spore stock cultures were submitted to a heat shock as described before, and 10% (v/v) of the heat-activate stock were transferred to the growth medium. It was composed of four solutions; the values of g/L or mg/L are the concentration of compounds in the total final volume. (I) sugar solution: 30 g/L glucose and 1 g/L yeast extract; (II) phosphate-acetate buffer: 0.50 g/L K_2HPO_4, 0.50 g/L KH_2PO_4 and 2.20 g/L CH_3COONH_4; (III) salts solution: 0.20 g/L of $MgSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$; 0.01 g/L NaCl and 0.01 g/L $FeSO_4 \cdot 7H_2O$; (IV) vitamins solution: 1 mg/L of p-aminobenzoic acid, 1 mg/L mg thiamine and 0.01 mg/L of biotin (Bellido et al., 2015). Solution I was autoclaved for 20 min at 121 ºC, while solutions II, III and IV were passed through sterile filters (0.22 µm). Pre-inoculums were grown for 24 h statically in duplicate at the temperature required for each *Clostridium*.

For ABE fermentations, selected hydrolysates whole slurry or model solution (14.00 g/L glucose and 3.50 g/L xylose) were transferred to penicillin flasks and supplemented with solutions II, III and IV in the same concentrations of pre-inoculum and flushed with nitrogen for oxygen removal. The penicillin flasks were inoculated with 10% (v/v) of pre-inoculum and maintained at the temperature required for each *Clostridium* as aforementioned, at 120 rpm during 96h (Bellido et al., 2015). Fermentations were centrifuged at 20000 xg for 5 min, supernatants were filtered through 0.22 µm and analyzed by chromatography for sugar, butyric acid, acetone, butanol and ethanol content. Yields in terms of g of butanol produced by g of sugar in the tested media, was calculated according to Eq. 3 and g of ABE by g of sugar in the

tested media according to Eq. 4:

$g_{BUTANOL}/g_{SUGAR}$= (g/L of butanol obtained)/(g/L of sugars in the tested media) (Eq. 3).

g_{ABE}/g_{SUGAR}= (g/L of acetone + g/L of butanol + g/L of ethanol after 96h fermentation)/ (g/L of sugars in the tested media) (Eq. 4).

For energetic balance, the energy spent for ozone production and the heating value for ethanol were used as aforementioned, as well as the heating values for butanol (33.1 MJ/kg) and acetone (28.6 MJ/kg) from Jin et al. (2011). The amount of energy, in MJ, produced by kg of SCB pretreated, hydrolyzed and ABE fermented, was calculated according to Eq. 5:

Energetic balance (MJ/kg_{SCB})= [(kgO_3 expended by kg of SCB)*(16.5 MJ/kgO_3 produced)]-{ [(kg of acetone produced)*(28.6 MJ/kg of acetone)]+ [(kg of butanol produced)*(33.1 MJ/kg of butanol)]+[(kg of ethanol produced)*(26.7 MJ/kg of ethanol)]} (Eq. 5).

2.6. Analytical methods

The chemical compositional analysis of raw SCB fractions and ozone-washed pretreated samples were performed in terms of AIL, ASL, cellulose (as glucose), xylan (as xylose) and ashes, following the NREL (*National Renewable Energy Laboratory*, USA) laboratory analytical procedures: *Determination of structural carbohydrates and lignin in biomass* and *Determination of ash in biomass* (Sluiter et al., 2012, 2008). Percentage of mass loss during the washing step was determined gravimetrically by the difference between the dry mass of ozonated SCB before and after the washing detoxification step, divided by the initial dry mass and multiplied by 100.

Sugars, organic acids and alcohols were measured by HPLC in a Waters

Alliance e2695 separation module equipped with a Waters 2998 photodiode array detector and a Waters 2414 refractive index detector, all controlled and processed by Empower 3 (build 3471 from 2010) software (Waters). Sugars from compositional analysis procedures, hydrolysates and ethanol from fermentation were measured using the refraction index with a Phenomenex® HPLC column Rezex™ RPM-Monosaccharide Pb+2 (8%), 300 x 7.8 mm, at 80 ºC, with MilliQ water as the eluent, at 0.6 mL/min (Travaini et al., 2016a). Inhibitory compounds (oxalic, acetic and formic acids) in hydrolysates were measured by UV at 210 nm with a Bio-Rad Aminex® HPLC HPX-87H column, 300 x 7.8 mm, at 50 ºC, with 25 mM H_2SO_4 as the eluent, at 0.6 mL/min. For sugars and products of ABE fermentation, the cited Bio-Rad column was used with the photodiode array and refractive index detector, at 30 ºC, with 5 mM H_2SO_4 at 0.6 mL/min as eluent (Bellido et al., 2015).

Total phenolic compounds (TPC) in hydrolysates were determined by the Folin-Ciocalteu method (Toquero and Bolado, 2014), using galic acid as calibration standard.

2.7. Design of experiments using orthogonal array

In order to study how SCB ozonolysis process parameters affect sugar release yields and inhibitory compound formation, a previously developed OA experimental design $L_9(3)^4$ was used (Travaini et al., 2016a). The effect of parameters A (SCB moisture content, % (w/w)), B (Ozone concentration (%, mol/mol in ozone/oxygen), C (Ozone/oxygen flow (L/h)) and D (Particle size (mm)) were analyzed. The combinations of factors for each experimental run is available in Table 1. The percentages of influence of these parameters on SCB composition, sugar release yields and inhibitory compound production were calculated using the OA ANOVA sum of

squares. Only values with a 95.00% confidence level were taken into account; therefore, sum of squares with a P-value lower than 0.05 were not used for influence calculation. The OA was optimized to calculate the best factor levels in each case. Experiments were made by duplicate, and their order randomized. The software STATGRAPHICS® Centurion XVI Version 16.2.04 was used to statically treat the results.

The process parameters levels were selected according to our previous work with SCB ozonolysis (Travaini et al., 2013). Moisture content was between 30% and 70% (w/w), ozone concentration between 2% and 4% (mol/mol in ozone/oxygen), ozone/oxygen flow between 30 and 90 L/h and particle size between 1.25 and 4.76 mm. The OA $L_9(3)^4$ matrix provided a process parameters combination for experiment 7 that generated hydrodynamic problems. For this reason, ozone/oxygen flow was diminished from 90 L/h to 72 L/h, keeping the rest of the parameters as designated by the matrix (Travaini et al,2016). The analysis of results from alcoholic fermentations included a one-way ANOVA followed by Tukey´s test with a confidence level of 95.00%.

All experiments, analytical procedures, enzymatic hydrolysis and fermentations were conducted by duplicate.

3. Results and discussion

3.1. Effect of ozonolysis on mass losses and composition of SCB after washing

The ozonolysis pretreatment followed by the washing detoxification had a high effect on biomass solubilization, the percentages of mass lost for each experimental run are provided in Table 1. Mass losses tended to increase with ozone spent augmentation, with experiments grouped in two ranges. For ozone spent lower than 0.11 g of ozone by g of pretreated SCB (experiments 2, 7 and 9), mass losses ranged between 13.8% and

16.7%. For the rest of the experiments, with ozone spent higher than 0.11 g by g of SCB, the mass lost after washing varied between 24.2% and 30.3% with the highest loss corresponding to the highest ozone spent (experiment 4). These values of solubilization are similar to those found for other ozonated substrates, as aspen wood chips with 35% (Ben'ko et al., 2013) and 20.4% for corn stalks (Quesada et al., 1999).

The compositional analysis of the ozonated-washed SCB for all experimental runs are shown in Table 1. All biomass component percentages diminished in the resulting solid after pretreatment and washing compared to raw SCB, except cellulose that increased. The component with highest diminution was xylan, with the highest reduction of 49.97% for experiment 8. This reduction is high, when compared to the 36% reduction found by Souza-Correa et al. (2013), with SCB ozonated for 6 h and washed with 1% NaOH 1:10 (v/w NaOH solution:SCB). Nevertheless, xylan reduction varies in a wide range for ozonated samples depending on the substrate used and ozonization process parameters, e.g., 49.5% for corn stalks, 43% for rye straw or 16% for wheat straw (Garcia-Cubero et al., 2012; Quesada et al., 1999). Ozonolysis pretreatment has a known effect on xylan solubilization (Miura et al., 2012), but also on its degradation into organic acids (Schultz-Jensen et al., 2011b). In this way, its high diminution can be related to its leaching as oligomers, monomers or degradation compounds into the washing water. For AIL, the highest diminution was found in experiment 4, where it decreased by 42.06%. Similar AIL reductions were also found in experiments 1, 6 and 8. Souza-Correa et al. (2014), in their work with SCB ozonated and washed with NaOH, found a maximal lignin reduction of 80%, which can be attributed to the long ozonolysis reaction time applied (6 h) and to the alkaline washing. In literature, AIL diminution is related to the oxidation of lignin producing organic

acids and low molecular weight phenolic compounds (Garcia-Cubero et al., 2012; García-Cubero et al., 2009). AIL reduction was not associated with ASL increase, as described in previous works without the detoxification step (Garcia-Cubero et al., 2012; Travaini et al., 2013), probably due to ASL leaching on the washing water. Cellulose removal was found as depreciable in many cases for ozonated SCB (Souza-Correa et al., 2014, 2013a; Travaini et al., 2013); nevertheless, in our work an average of 13% augmentation was found in the pretreated and washed solid for all experimental runs, indicating cellulose losses lower than total biomass solubilization.

The amount lost from each component, calculated from the biomass loss and the composition of ozonated-washed samples in each experiment, are represented in the function of the ozone spent in Fig. 1a. The amount of lost ASL remains almost constant for all the experiments, probably by the aforementioned leaching of this component during the washing step. The xylan losses increased with the ozone spent, but reaching a constant value for consumptions higher than 0.160 g of ozone by g of pretreated SCB. Cellulose and AIL losses show a relative dependency on the ozone expended, even though other factors seem to influence these results.

The OA analysis by ANOVA sum of squares for AIL losses after ozonolysis and washing (data not shown), showed ozone concentration as the most important process parameter with 18.80% of influence, followed by moisture content (13.87%), ozone/oxygen flow (9.87%) and particle size (1.36%). The quadratic terms of moisture content and ozone concentration were 14.40% and 17.83%, respectively. The interactions reinforce these results, with 19.57% for moisture and ozone concentration, and only 4.30% for moisture and flow. The OA optimization showed that AIL losses go through maxima values for moisture content (45% (w/w)), and for ozone concentration

(2.53% (mol/mol)). In contrast, AIL losses increased linearly when the ozone/oxygen flow and the particle size decrease.

For cellulose losses after ozonolysis and washing, the OA analysis by ANOVA sum of squares (data not shown) also showed moisture content as the pretreatment parameter with most influence (25.18%) followed by ozone concentration (9.61%), but no influence of ozone/oxygen flow and particle size was found. The quadratic term of moisture content and the interaction between moisture content and ozone concentration were also relevant, with 52.21% and 13.01% of influence, respectively. The OA optimization showed minimal cellulose losses for high moisture contents, achieving the maximum loss at 53% (w/w), and diminishing moderately for lower moisture contents. Conversely, cellulose losses increase linearly with increasing ozone concentration.

3.2. Sugar release yields

Table 2 shows the glucose and xylose release yields by enzymatic hydrolysis of ozonated-washed SCB, calculated in base on the cellulose and xylan content of the ozonated-washed SCB. The sugar release yields ranged in wide intervals, from 34% to 84% for glucose and 19% to 67% for xylose. The sugar release yields increased with ozone spent, the lineal correlation providing coefficients of determination R^2 of 0.9632 and 0.8632, for glucose and xylose respectively. This behavior was reported before for wheat straw, corn straw and unwashed SCB (García-Cubero et al., 2009; Shi et al., 2015; Travaini et al., 2013). Despite the higher SCB delignification obtained by Souza-Correa et al., 2014, as previous mentioned, just 78.8% glucose release was found, proving the importance of the optimization of process parameters.

The sugar release yields of this study are slightly higher than those obtained by

Travaini et al. (2016a) from no washed ozone pretreated SCB, applying the same OA experimental design $L_9(3)^4$. In that study, yields from 32% to 76% were reported for glucose, and from 19% to 57% for xylose. The higher differences of sugar release yields between these two works were found for experiments 3, 4 and 5, with around 10 percentage points for glucose and 20 percentage points for xylose. The OA analysis by ANOVA sum of squares in terms of total sugar release yields resulted in a coefficient of determination R^2 that explains 98.26% of the variability. The sum of squares, P-values and percentage of estimated effects provided by ANOVA are presented in Table 3. When only individual effects are taken into account, ozone is the most influential parameter, with 22.24%. The order of influence is as follows: B>>A>D, with no influence from the C factor, ozone/oxygen flow. Nevertheless, an important percentage of influence, with 32.94% of the total sum of squares, was found for the interaction between flow and moisture content, AC. A possible explanation of this interaction could be the drying effect of the gas flow through the bed (Mamleeva et al., 2009). Travaini et al., (2016) reported very similar operation parameters effects for sugar released from non-washed ozonated SCB, but some influence from ozone/oxygen flow, similar to the particle size effect, was found for those samples. The optimization of the ozonolysis parameters for maximum sugars release after enzymatic hydrolysis provided very similar values to those of experiment 4 (Table 1), with 57% (w/w) of moisture content, 2% (mol/mol) of ozone, 30 L/h of flow and particle size 4.76mm<Ø<1.25mm.

Fig. 1b compares the glucose and xylose concentrations in the hydrolysates of washed-ozonated samples obtained in this work with those previously published from non-washed ozonated SCB samples (Travaini et al., 2016a). Identical ozonolysis experiments and initial amounts of raw material were used in both works. Despite the

higher percentages of sugar release yields obtained by hydrolysis from washed samples, the global process provided lower sugar concentrations due to the washing step. The increase of sugar release yields did not counteract the losses of sugar by washing. As shown in Fig. 1b glucose and xylose concentrations increase with ozone expended, with parallel results for washed and unwashed hydrolysates, although this augmentation is reduced for ozone consumption higher than 0.170 g by g of SCB. Maybe, the difficulty to hydrolyze the remaining sugar polymers is more related to their crystallinity, than to the pretreatment efficiency (Alvira et al., 2010).

3.3. Effect of process parameters on inhibitory compounds formation and washing detoxification efficiency

The concentration of degradation compounds in hydrolysates from washed-ozonated samples and control non-washed ozonated samples are shown in Table 2. For control samples, the concentrations ranged between 0.64-2.44 g/L for formic acid, 0.47-1.87 g/L for acetic acid and 1.50-4.02 g/L for oxalic acid; and, for phenolic compounds, as TPC, from 259.80-585.63 mg/L. The lineal fitting of the organic acid concentrations vs. ozone expended assays provided R^2 of 0.8394, 0.7955 and 0.8082 for formic, acetic and oxalic acids, respectively. Low lineal dependence of phenolic compounds vs. ozone consumption was found, with R^2 of 0.7601, which could be attributed to their oxidation to other compounds (Schultz-Jensen et al., 2011b; Souza-Correa et al., 2013b).

The OA ANOVA sum of squares analysis for process parameters influence on inhibitory compounds generated in control unwashed ozonated SCB hydrolysis are provided in Table 3. Moisture and ozone concentration are the individual parameters with the highest effect on the formation of degradation compounds, with similar

percentages around 10%, except for TPC where 37% of moisture influence was found. Despite the closer percentages of influence for moisture content and ozone concentration, the quadratic terms confirm the importance of moisture influence on inhibitory compound generation, as described by other works (Mamleeva et al., 2009; Schultz-Jensen et al., 2011a; Travaini et al., 2013). For all the degradation compounds, ozone/oxygen flow and particle size had minor effects (~5%), with particle size being completely non-influent for oxalic acid generation. For the three acids, the lowest generation was found for the highest moisture content, reaching their maximum concentration at 50%, decreasing gently for lower moisture contents. Meanwhile, phenolic compound concentrations increased with the moisture content decrease, with this effect being very important for elevated moisture values. Concentrations of all the inhibitory compounds increased when decreasing the ozone concentration, with the highest productions at 2% (mol/mol). Again, an appreciable influence of the interaction between moisture content and flow was found, which could be related to the drying effect of the gas flow through the bed mentioned at section 3.2.

The OA optimization for acid formation provided the highest acid concentrations for moisture 50% (w/w), ozone concentration 2% (mol/mol), ozone/oxygen flow 30 L/h and particle size <4.76mm. The highest amount of phenolic compounds was obtained for moisture 30% (w/w), ozone concentration 2% (mol/mol), ozone/oxygen flow 85 L/h and particle size <4.76mm. These results allow us to infer that using low moisture contents, reactions go through the phenolic compounds formation, while, higher moisture content carry reactions to organic acids formation. This information, can be useful in process parameters selection for fermentations, taking into account the main inhibitory compounds of the selected microorganisms.

The process parameters combination producing the highest inhibitory compounds is very close to that reported for the maximum glucose release yield from non-washed SCB; nevertheless, very different from the combination for maximum xylose release yield (Travaini et al, 2016a). Despite the xylose fermentability, glucose rich hydrolysates are required for fermentation due to its higher biological conversion yields (Bellido et al., 2013; Raganati et al., 2015). These conclusions, associated with the total inhibition of diauxic microorganisms in hydrolysates of non-washed ozonated SCB (Travaini et al., 2016a), reinforce the need of a detoxification step.

The applied washing detoxification step was very effective in removing all the studied inhibitory compounds, providing hydrolysates with inhibitory compound concentrations in a narrow range (Table 2). No formic acid was detected in hydrolysates of washed samples, and oxalic acid removals from 90% to 97% were achieved. Some lower removal efficiencies (60-82%) were obtained for acetic acid, with maximum concentration of 0.52 g/L, for experiment 3. Previous studies with *Pichia stipitis* (Toquero and Bolado, 2014) provided only 60% ethanol production yield with 0,5 g/L acetic acid model solutions. TPC removal ranged from 52 to 77%.

3.4. Hydrolysates fermentation and energetic balances

In order to study the fermentability of the hydrolysates from ozonated-washed SCB, four samples were selected: runs 4 and 8, because of their high sugars concentrations, and, runs 2 and 9, due to the high ratio of sugar released by ozone expended. Experimental run 3 was dismissed due to its high inhibitory compounds content. Aside from hydrolysates fermentation, model fermentations were done with the three tested microorganisms using commercial glucose and xylose as the carbon source.

The sugar concentrations used in model fermentations were an average of the selected hydrolysates concentrations (14.00 g/L of glucose and 3.50 g/L of xylose).

3.4.1. Ethanol production by P. stipitis

Pichia stipitis was able to grow in all selected experiments with high fermentation yields, in spite of the acetic acid concentrations of the samples. The results of fermentations in terms of sugar conversion, ethanol production (g/L) and ethanol yield are shown in Table 4. In all the experiments, about 90% of the initial sugar content was converted, with only a xylose fraction not consumed. Model fermentation resulted in an 80% fermentation yield, while hydrolysates provided yields between 75% and 88%. These values are high when compared to those obtained from other SCB pretreatments fermented with *P. stipitis spp.*, like the 59% ethanol yield for detoxified hydrolysates of diluted sulfuric acid (Canilha et al., 2010) and the 57% for thermal ammonia hydrolysates (Chandel et al., 2013); and, very close to the 80% obtained with ozonated washed wheat straw hydrolysates (Bellido et al., 2013).

No correlation between hydrolysates inhibitory compounds concentration and ethanol yields was found. A one-way ANOVA between model and hydrolysates fermentation yields resulted in a F-ratio of 2.19 and a P-value of 0.1843, indicating no significant differences between them. This result is corroborated by the lineal fit between initial sugar concentration in fermentations and ethanol productions, resulting in a coefficient of determination R^2 of 0.9644. So, the operation parameters maximizing sugar production are also optimal for ethanol production.

The results of preliminary energy balances are shown in Table 4. The balance between energy expended for ozone generation and energy liberated by combustion of

the produced ethanol (calculated by Eq. 2) is positive in all experiments. The highest results were found for experiments 9 (low ozone consumption) and 8 (high sugar concentration), both with 1.03 MJ generated per kg of SCB pretreated and fermented. This value is higher than those obtained from unwashed hydrolysates, where only glucose was fermented by *Saccharomyces cerevisiae* bakery strain (Travaini et al., 2016a). In this case, the best results obtained were 0.19 MJ per kg of SCB pretreated and fermented from experiment 4, and 0.76 MJ from experiment 2.

3.4.2. ABE fermentation by Clostridium spp.

The two selected bacteria, *C. acetobutylicum* and *C. beijerinckii* were able to grow and produce solvents in all the selected hydrolysates.

For *C. acetobutylicum*, sugar conversions increased from 76% for sample 2 (the one with the highest acetic and oxalic acids concentration), to 94-96% for the other samples, with both glucose and xylose remaining after fermentation. Sugar conversion was 96% for model fermentation, with complete glucose conversion and only xylose remaining. Raganati et al. 2015 demonstrated the wide capacity of this microorganism to use hexoses and pentoses as carbon sources and its preference of sugar consumption varying with initial sugar concentration and ratio, with no clear relation.

High concentrations of butyric acid were obtained for all the samples. The best hydrolysate fermentation was found with experiment 8, with yields of 0.072 $g_{BUTANOL}/g_{SUGAR}$ and 0.188 g_{ABE}/g_{SUGAR}. Studies about *C. acetobutylicum* ABE fermentation using SCB lignocellulosic hydrolysates as the carbon source are scant in the literature. The results obtained in this work were 38 times higher than the 0.0019 $g_{BUTANOL}/g_{SUGAR}$ reported using the liquid fraction from SCB pretreated with diluted

sulfuric acid, but they worked with a ratio glucose:xylose of 1:5.6 and just 60 h of fermentation time (Jonglertjunya et al., 2014). Pang et al. (2016) reported higher yields than those found in this work, using SCB alkali-pretreated (0.15 $g_{BUTANOL}/g_{SUGAR}$ and 0.24 $g_{ABE}/g_{SUGAR)}$. However, they worked with an initial sugar concentration of 38.48 g/L. Their study also concluded that initial sugar concentration is decisive in the final solvents production. The lineal fittings of our butanol or ABE concentration in g/L results vs. initial sugar concentration, provided R^2 of 0.8720 and 0.9044 respectively, supports the same conclusion.

For *C. beijerinckii*, the solvents production was higher than those found for *C. acetobutylicum*, in the experiments 8 and 4, and, in the model fermentation. The sugar conversion in model fermentation was 94%, and, for hydrolysates varied between low values around 60% for experiments 2 and 9, and values close to 100% for hydrolysates 8 and 4. All the glucose was consumed for hydrolysates 8 and 4, and model fermentation, the three assays where butyric acid was found, but in concentrations always lower than in assays using *C. acetobutylicum*. For *C. beijerinckii*, the highest butanol and ABE fermentation yields were obtained from experiment 4, with 0.165 $g_{BUTANOL}/g_{SUGAR}$ and 0.257 g_{ABE}/g_{SUGAR}. Su et al. (2015) using sequential SCB milling-liquid hot water-microwave pretreatments achieved a maximum of 0.07 $g_{BUTANOL}/g_{SUGAR}$ and 0.15 g_{ABE}/g_{SUGAR} for separated hydrolysis and fermentation as done in our work. For washed ozonated wheat straw, the fermentation reached 0.18 $g_{BUTANOL}/g_{SUGAR}$ and 0.32 g_{ABE}/g_{SUGAR}, but working with an initial sugar concentration 50% higher than this work. As found for *C. acetobutylicum*, the lineal fit of butanol or ABE solvents in g/L vs. initial sugar content for *C. beijerinckii* provided R^2 of 0.9276 and 0.9282 respectively, proving the dependence of yields on sugar concentrations.

The preliminary energetic balances calculated by Eq. 5, considering the energy generated by the combustion of ethanol, butanol and acetone, and the energy expended for ozone generation are shown in Table 4. Even with the appreciable solvents production by ABE fermentation using washed-ozonated SCB hydrolysates, energetic balances were always negative for both *Clostridia* tested. The less negative energetic balance was achieved with the hydrolysate from experiment 8 for *C. beijerinckii*. Many other possibilities should be analyzed in order to achieve economic feasibility: the value of generated butyric acids for industrial processes; the increase of hydrolysates concentration to increase production of solvents, as predicted by aforementioned studies; and, the valorization of sugar leached in washing water and fermentation residues for biogas production. The development of new technologies for ozone production could also be emphasized which could considerably decrease the energy spent for ozone generation, as occurred in the last five years, with a 144% of reduction (Schultz-Jensen et al., 2011b; Travaini et al., 2016b).

Conclusions

SCB ozonolysis optimization revealed a process parameters combination for maximum inhibitory compounds formation very similar to those found for maximum sugar release yield, reinforcing the need for a detoxification step. Ozonated SCB water-washed provided hydrolysates with low inhibitory contents, able to be fermented by the diauxic microorganisms tested. Optimal process parameters combination can provide 154 kg of ethanol by ton of SCB with *P. stipitis*, or, 87 kg of ABE solvents by *C. beijerinckii*. Preliminary energetic balances between fermentation products and ozone production were positive only for ethanol production, while for ABE production,

improvements are needed.

Acknowledgments

This work was supported by the UIC 071 research unit of the regional government "*Junta de Castilla y León – JCyL*", Spain. Rodolfo Travaini is also grateful to "*Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq*", Brazil (238059/2012-0) for providing him with a Doctorate Scholarship.

References

1. Alvira, P., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis : A review. Bioresour. Technol. 101, 4851–4861.

2. APHA-AWWA-WEF, 2005. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, D.C.

3. Barros, R.D.R.O. De, Paredes, R.D.S., Endo, T., Bon, E.P.D.S., Lee, S.H., 2013. Association of wet disk milling and ozonolysis as pretreatment for enzymatic saccharification of sugarcane bagasse and straw. Bioresour. Technol. 136, 288–294.

4. Bellido, C., Bolado, S., Coca, M., Lucas, S., González-Benito, G., García-Cubero, M.T., 2011. Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by Pichia stipitis. Bioresour. Technol. 102, 10868–10874.

5. Bellido, C., González-Benito, G., Coca, M., Lucas, S., García-Cubero, M.T., 2013. Influence of aeration on bioethanol production from ozonized wheat straw hydrolysates using Pichia stipitis. Bioresour. Technol. 133, 51–8.

6. Bellido, C., Infante, C., Coca, M., González-Benito, G., Lucas, S., García-Cubero,

M.T., 2015. Efficient acetone-butanol-ethanol production by Clostridium beijerinckii from sugar beet pulp. Bioresour. Technol. 190, 332–8.

7. Bellido, C., Loureiro Pinto, M., Coca, M., González-Benito, G., García-Cubero, M.T., 2014. Acetone–butanol–ethanol (ABE) production by Clostridium beijerinckii from wheat straw hydrolysates: Efficient use of penta and hexa carbohydrates. Bioresour. Technol. 167, 198–205.

8. Ben'ko, E.M., Manisova, O.R., Lunin, V. V., 2013. Effect of ozonation on the reactivity of lignocellulose substrates in enzymatic hydrolyses to sugars. Russ. J. Phys. Chem. A 87, 1108–1113.

9. Canilha, L., Carvalho, W., de Almeida Felipe, M. das G., de Almeida e Silva, J.B., Giulietti, M., 2010. Ethanol Production from Sugarcane Bagasse Hydrolysate Using Pichia stipitis. Appl. Biochem. Biotechnol. 161, 84–92.

10. Chandel, A.K., Antunes, F.A.F., Silva, M.B., da Silva, S.S., 2013. Unraveling the structure of sugarcane bagasse after soaking in concentrated aqueous ammonia (SCAA) and ethanol production by Scheffersomyces(Pichia)stipitis. Biotechnol. Biofuels 6, 102.

11. de Cassia Pereira, J., Travaini, R., Paganini Marques, N., Bolado-Rodríguez, S., Bocchini Martins, D.A., 2016. Saccharification of ozonated sugarcane bagasse using enzymes from Myceliophthora thermophila JCP 1-4 for sugars release and ethanol production. Bioresour. Technol. 204, 122–129.

12. García-Cubero, M.T., González-Benito, G., Indacoechea, I., Coca, M., Bolado, S., 2009. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresour. Technol. 100, 1608–1613.

13. Garcia-Cubero, M.T., Palacin, L.G., Gonzalez-Benito, G., Bolado, S., Lucas, S., Coca, M., 2012. An analysis of lignin removal in a fixed bed reactor by reaction of

cereal straws with ozone. Bioresour. Technol. 107, 229–34.

14. Jin, C., Yao, M., Liu, H., Lee, C.F., Ji, J., 2011. Progress in the production and application of n-butanol as a biofuel. Renew. Sustain. Energy Rev. 15, 4080–4106.

15. Jonglertjunya, W., Makkhanon, W., Siwanta, T., Prayoonyong, P., 2014. Dilute acid hydrolysis of sugarcane bagasse for butanol fermentation. Chiang Mai J. Sci. 41, 60–70.

16. Mamleeva, N. a., Autlov, S. a., Bazarnova, N.G., Lunin, V. V., 2009. Delignification of softwood by ozonation. Pure Appl. Chem. 81, 2081–2091.

17. Miura, T., Lee, S.-H., Inoue, S., Endo, T., 2012. Combined pretreatment using ozonolysis and wet-disk milling to improve enzymatic saccharification of Japanese cedar. Bioresour. Technol. 126, 182–6.

18. Pang, Z.-W., Lu, W., Zhang, H., Liang, Z.-W., Liang, J.-J., Du, L.-W., Duan, C.-J., Feng, J.-X., 2016. Butanol production employing fed-batch fermentation by Clostridium acetobutylicum GX01 using alkali-pretreated sugarcane bagasse hydrolysed by enzymes from Thermoascus aurantiacus QS 7-2-4. Bioresour. Technol. 212, 82–91.

19. Quesada, J., Rubio, M., Gómez, D., 1999. Ozonation of Lignin Rich Solid Fractions from Corn Stalks. J. Wood Chem. Technol. 19, 115–137.

20. Raganati, F., Olivieri, G., Götz, P., Marzocchella, A., Salatino, P., 2015. Butanol production from hexoses and pentoses by fermentation of Clostridium acetobutylicum. Anaerobe 34, 146–55.

21. Schultz-Jensen, N., Kádár, Z., Thomsen, A.B., Bindslev, H., Leipold, F., 2011a. Plasma-assisted pretreatment of wheat straw for ethanol production. Appl. Biochem. Biotechnol. 165, 1010–1023.

22. Schultz-Jensen, N., Leipold, F., Bindslev, H., Thomsen, A., 2011b. Plasma-

Assisted Pretreatment of Wheat Straw. Appl. Biochem. Biotechnol. 163, 558–572.

23. Shi, F., Xiang, H., Li, Y., 2015. Combined pretreatment using ozonolysis and ball milling to improve enzymatic saccharification of corn straw. Bioresour. Technol. 179, 444–51.

24. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Determination of ash in biomass: Laboratory Analytical Procedure (LAP), NREL/TP-510-42622. National Renewable Energy Laboratory (NREL) - Golden, Colorado, USA.

25. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2012. Determination of structural carbohydrates and lignin in biomass: Laboratory Analytical Procedure (LAP), NREL/TP-510-42618. National Renewable Energy Laboratory (NREL) - Golden, Colorado, USA.

26. Souza Dias, M.O. de, Cavalett, O., Filho, R.M., Bonomi, A., 2016. Integrated first- and second-generation processes for bioethanol production from sugarcane, in: Sugarcane-Based Biofuels and Bioproducts. John Wiley & Sons, Inc, Hoboken, NJ, USA, pp. 311–332.

27. Souza-Correa, J.A., Oliveira, C., Nascimento, V.M., Wolf, L.D., Gómez, E.O., Rocha, G.J.M., Amorim, J., 2014. Atmospheric pressure plasma pretreatment of sugarcane bagasse: the influence of biomass particle size in the ozonation process. Appl. Biochem. Biotechnol. 172, 1663–72.

28. Souza-Correa, J.A., Oliveira, C., Wolf, L.D., Nascimento, V.M., Rocha, J.G.M., Amorim, J., 2013a. Atmospheric pressure plasma pretreatment of sugarcane bagasse: the influence of moisture in the ozonation process. Appl. Biochem. Biotechnol. 171, 104–16.

29. Souza-Correa, J.A., Ridenti, M.A., Oliveira, C., Araújo, S.R., Amorim, J., 2013b.

Decomposition of lignin from sugar cane bagasse during ozonation process monitored by optical and mass spectrometries. J. Phys. Chem. B 117, 3110–3119.

30. Su, H., Liu, G., He, M., Tan, F., 2015. A biorefining process: Sequential, combinational lignocellulose pretreatment procedure for improving biobutanol production from sugarcane bagasse. Bioresour. Technol. 187, 149–60.

31. Toquero, C., Bolado, S., 2014. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour. Technol. 157, 68–76.

32. Travaini, R., Barrado, E., Bolado-Rodríguez, S., 2016a. Effect of ozonolysis pretreatment parameters on the sugar release, ozone consumption and ethanol production from sugarcane bagasse. Bioresour. Technol. 214, 150–158.

33. Travaini, R., Marangon-Jardim, C., Colodette, J.L., Morales-Otero, M.D., Bolado-Rodríguez, S., 2015. Ozonolysis, in: Pandey, A., Negi, S., Binod, P., Larroche, C. (Eds.), Pretreatment of Biomass. Elsevier, USA, pp. 105–135.

34. Travaini, R., Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodríguez, S., 2016b. Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited. Bioresour. Technol. 199, 2–12.

35. Travaini, R., Otero, M.D.M., Coca, M., Da-Silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation. Bioresour. Technol. 133, 332–339.

36. UNICA, 2015. Relatório final safra 2014/2015, região centro-sul, UNICADATA, at http://www.unicadata.com.br/, last accessed may, 2016. São Paulo, Brasil.

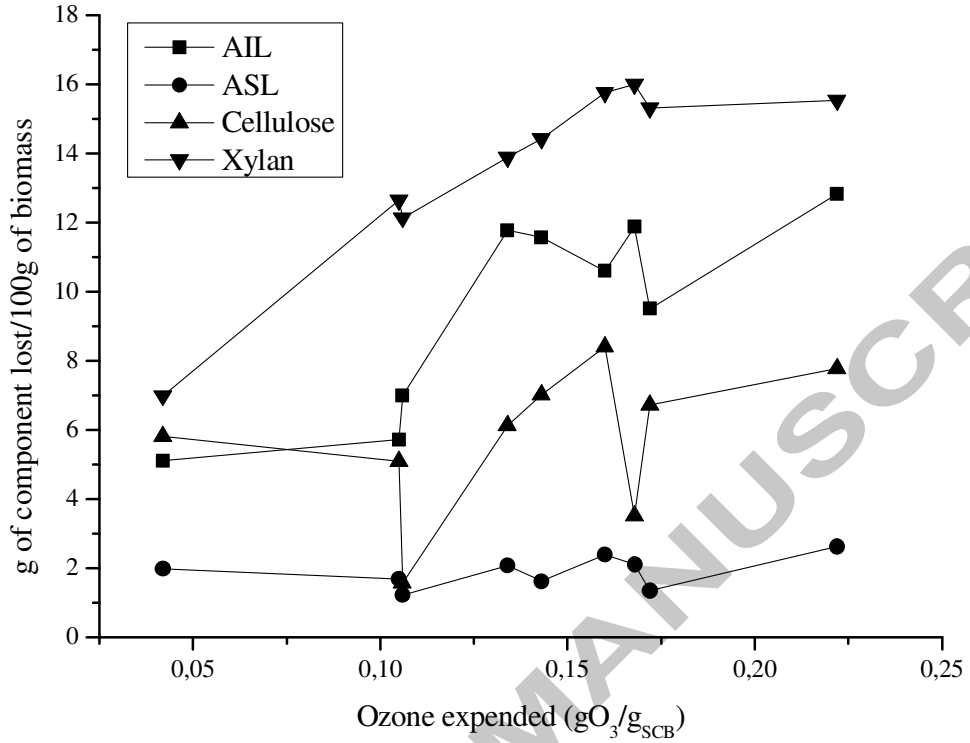
Figures captions

Figure 1: Effect of ozone expended in the experimental runs on sugarcane bagasse components reduction and on sugars release: (a) losses of acid insoluble lignin, acid soluble lignin, cellulose and xylan after ozonolysis and washing (g of component lost by

100 g of biomass); (b) sugars release (g/L) by hydrolysates of ozonated-washed sugarcane bagasse (W), and, from ozonated-unwashed (U) (from Travaini et al. 2016a).

Figure 1

(a)



(b)

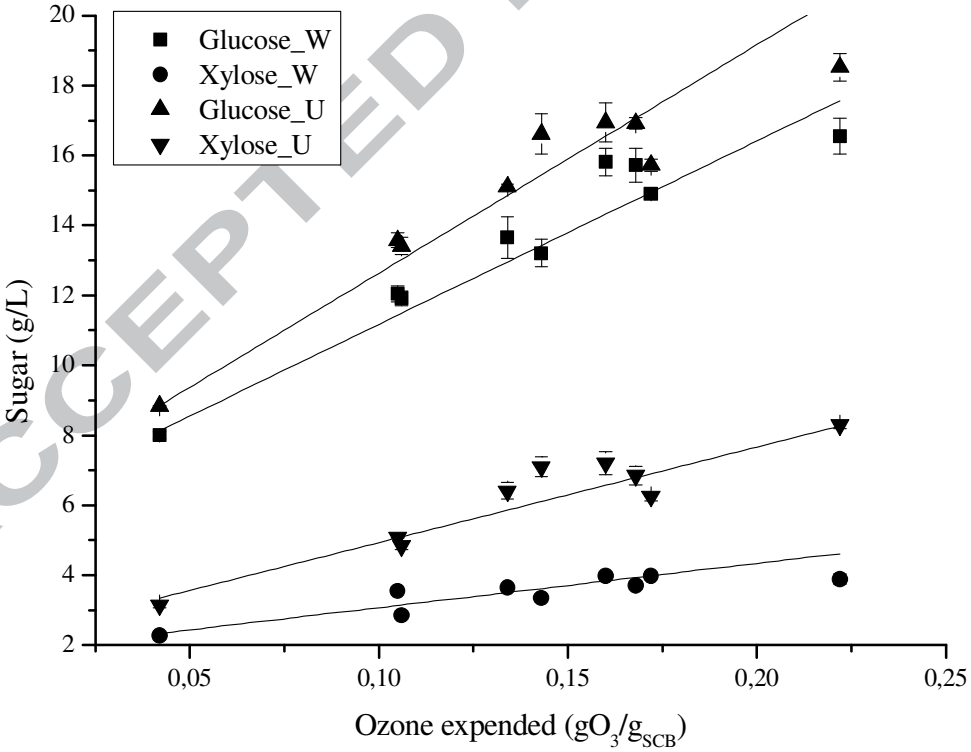


Table 1: Orthogonal array experimental design $L_9(3)^4$ matrix; ozone expended in pretreatments experimental runs; mass loss in washing detoxification; and, compositional analysis of raw and ozonated-washed sugarcane bagasse, in percentage of acid insoluble lignin, acid soluble lignin, cellulose and xylan.

Orthogonal array matrix[a]					Ozone expended gO_3/g_{SCB}[f]	Mass loss in % (w/w)	Compositional analysis in % (w/w)			
Exp. No.	Process parameters levels values									
	A[b]	B[c]	C[d]	D[e]			AIL[g]	ASL[h]	Cellulose	Xylan
1	30	2	30	Ø<4.76	0.134	24.16±0.32	12.86±0.49	3.27±0.01	50.89±1.11	15.63±0.10
2	30	3	60	4.76>Ø>1.25	0.105	16.74±0.23	19.00±0.77	3.45±0.09	47.62±0.61	15.72±0.22
3	30	4	90	Ø<1.25	0.172	23.80±0.02	15.79±0.30	4.22±0.03	49.88±0.50	13.68±0.38
4	50	2	60	Ø<1.25	0.222	30.27±0.60	12.48±0.43	2.78±0.09	53.00±0.39	14.62±0.46
5	50	3	90	Ø<4.76	0.160	29.94±0.56	15.61±0.23	3.09±0.08	51.85±1.70	14.23±0.30
6	50	4	30	4.76>Ø>1.25	0.143	27.04±0.32	13.66±0.26	4.02±0.09	51.70±0.30	15.50±0.24
7	70	2	72*	4.76>Ø>1.25	0.042	13.84±0.59	19.07±0.37	2.99±0.14	45.16±0.32	21.76±0.33
8	70	3	30	Ø<1.25	0.168	24.37±0.32	12.77±0.40	3.25±0.05	54.50±1.51	12.88±0.14
9	70	4	60	Ø<4.76	0.106	13.79±0.11	16.86±0.12	3.87±0.02	50.07±1.21	15.77±0.41
Raw**	--	--	--	--	--	--	21.54±0.44	4.56±0.12	44.74±0.47	25.74±0.26

[a]Orthogonal array matrix used in the experimental design $L_9(3)^4$

[b]Sugarcane bagasse moisture content, % (w/w).

[c]Ozone concentration (%, mol/mol in ozone/oxygen).

[d]Ozone/oxygen flow (L/h).

[e]Sugarcane bagasse particle size (mm).

[f]g of ozone expendend by g of sugarcane bagasse pretreated in each experiment (Travaini et al., 2016a).

[g]Acid insoluble lignin.

[h]Acid soluble lignin.

*For experiment number 7, factors and levels of the OA provided a combination where unreacted ozone escaped from reactor since time zero making impossible its use for statistical analysis. To complete the OA matrix, the assay was conducted changing the flow from 90 L/h (level 3) to 72 L/h (Travaini et al., 2016a).

**Compositional analysis of raw sugarcane bagasse used. Ash content: 5.55±0.01% (w/w).

Table 2: Sugar release yields from ozonated and washed experiments and inhibitory compounds concentration in hydrolysates of washed and unwashed ozonated sugarcane bagasse.

	Hydrolysates from washed ozonated sugarcane bagasse						Hydrolysates from ozonated sugarcane bagasse			
Exp.	Sugars yields % (w/w)[a]		Inhibitory compounds concentration				Inhibitory compounds concentration			
No.	Glucose	Xylose	Formic (g/L)	Acetic (g/L)	Oxalic (g/L)	TPC[b] (mg/L)	Formic (g/L)	Acetic (g/L)	Oxalic (g/L)	TPC[b] (mg/L)
1	58.81±1.99	50.89±0.44	ND	0.40±0.00	0.17±0.00	146.12±5.52	1.83±0.13	1.45±0.03	2.93±0.05	541.42±22.00
2	49.93±0.94	43.36±0.21	ND	0.43±0.01	0.19±0.00	171.58±1.86	1.40±0.04	1.14±0.06	2.43±0.02	424.23±1.33
3	73.71±0.10	67.55±0.01	ND	0.52±0.03	0.13±0.01	251.42±6.72	1.72±0.02	1.43±0.02	2.55±0.00	524.74±11.40
4	84.18±2.11	67.41±0.98	ND	0.35±0.02	0.12±0.00	133.49±4.11	2.44±0.07	1.87±0.01	4.02±0.06	585.63±22.73
5	72.28±1.50	65.96±0.52	ND	0.42±0.03	0.13±0.01	154.98±2.21	2.21±0.07	1.83±0.15	3.29±0.22	488.66±5.56
6	59.06±2.76	47.00±0.06	ND	0.37±0.01	0.14±0.01	188.29±3.35	2.10±0.14	1.71±0.03	3.12±0.04	510.83±47.27
7	33.78±0.36	19.28±0.03	ND	0.27±0.01	0.11±0.00	137.04±3.43	0.64±0.04	0.47±0.04	1.35±0.01	259.80±12.25
8	71.52±1.90	67.35±0.81	ND	0.41±0.01	0.16±0.01	179.95±10.03	1.96±0.08	1.50±0.09	2.99±0.00	445.75±5.39
9	46.51±0.20	35.23±0.36	ND	0.34±0.02	0.16±0.01	123.75±3.58	1.16±0.02	0.85±0.03	1.75±0.03	332.05±2.86

[a]Calculated in base of the cellulose and xylan content of the ozonated-washed SCB.
[b]Total phenolic compounds.

Table 3: Orthogonal array ANOVA analysis for percentage of influence or process parameters on sugars release yields and inhibitory compounds formation. Sum of squares, P-value and percentage of influence or process parameter.

	Sugars release yield			Inhibitory compounds formation[b]											
	G+X[a]			Formic acid			Acetic acid			Oxalic acid			TPC		
Parameter	SS*	P-value	I (%)	SS	P-value	I (%)	SS	P-value	I (%)	SS	P-value	I (%)	SS	P-value	I (%)
A: Moisture	91.14	0.0003	8.59	0.480	0.0002	9.66	0.472	0.0000	13.00	1.11	0.0000	13.35	68349	0.0000	37.19
B: Ozone	257.1	0.0000	24.22	0.565	0.0001	11.38	0.360	0.0001	9.92	0.930	0.0000	11.19	19208	0.0008	10.45
C: Flow	11.99	0.0673	--	0.270	0.0012	5.44	0.211	0.0009	5.80	0.232	0.0021	2.80	8119	0.0110	4.42
D: Particle Size	51.4	0.0020	4.84	0.198	0.0031	3.98	0.181	0.0015	4.99	0.066	0.0504	--	5404	0.0285	2.94
AA	201.9	0.0000	19.02	2.57	0.0000	51.76	1.75	0.0000	48.22	5.22	0.0000	62.80	45835	0.0000	24.94
AB	0.123	0.8378	--	0.025	0.1920		0.049	0.0437	1.35	0.004	0.6088	--	0.105	0.3292	--
AC	349.7	0.0000	32.94	0.688	0.0000	13.86	0.483	0.0000	13.31	0.696	0.0000	8.37	21073	0.0006	11.47
BB	110.3	0.0001	10.39	0.195	0.0032	3.93	0.124	0.0047	3.42	0.124	0.0125	1.49	15799	0.0016	8.60
ANOVA total sum of squares:	1061	--	--	4.97	--	--	3.63			8.31			183786		

[a]Sum of squares for glucose plus xylose release yields (%, w/w) in hydrolysates using washed-ozonated sugarcane bagasse.
[b]Sum of squares for inhibitory compounds concentration (g/L) in control hydrolysates using ozonated sugarcane bagasse.
*Abbreviations: SS, ANOVA sum of squares; I, percentage of influence (calculated by the sum of squares of each individual factor, quadratic terms or interactions, divided by the total sum of squares multiplied by 100).

Table 4: Fermentations sugars conversion, products and yields, and, preliminary energetic balances, for experiments 2, 4, 8, 9 and model.

Pichia stipitis DSM 3651

Exp.	Reimaging sugars (g/L)			Etanol production		EB
No.	Glucose	Xylose	SC (%)[a]	(g/L)	Y/S (%)[b]	MJ/kgSCB[c]
2	ND	1.25±0.07	92	5.97±0.11	75	0.92
9	ND	1.84±0.23	91	6.25±0.10	83	1.03
8	ND	2.14±0.12	89	8.55±0.06	86	1.03
4	ND	1.18±0.04	92	9.23±0.34	88	0.44
model	ND	1.23±0.13	93	7.12±0.22	80	--

Clostridium acetobutylicum DSM 792

Exp.	Reimaging sugars (g/L)			Fermentation products (g/L)				Yields (g/g_{SUGAR})		EB
No.	Glucose	Xylose	SC (%)	Butyric	Acetone	Butanol	Ethanol	Butanol	ABE	MJ/kgSCB[c]
2	2.90±0.06	0.85±0.02	76	3.26±0.25	1.06±0.16	0.27±0.00	ND	0.017	0.085	-1.08
9	0.54±0.01	0.13±0.02	96	3.57±0.08	0.91±0.02	0.43±0.02	ND	0.029	0.091	-1.08
8	0.96±0.00	0.23±0.00	94	3.24±0.03	1.60±0.04	1.40±0.08	0.65±0.01	0.072	0.188	-0.95
4	0.96±0.00	0.22±0.09	94	3.42±0.18	1.59±0.10	1.36±0.11	0.62±0.01	0.066	0.175	-1.88
model	ND	0.78±0.02	96	2.50±0.16	0.74±0.05	1.10±0.01	0.32±0.00	0.063	0.123	--

Clostridium beijerinckii DSM 6422

Exp.	Reimaging sugars (g/L)			Fermentation products (g/L)				Yields (g/g_{SUGAR})		EB
No.	Glucose	Xylose	SC (%)	Butyric	Acetone	Butanol	Ethanol	Butanol	ABE	MJ/kgSCB[c]
2	5.10±0.06	1.50±0.02	58	ND	0.19±0.00	0.29±0.02	ND	0.019	0.031	-1.48
9	4.08±0.01	0.98±0.02	66	ND	0.26±0.02	0.33±0.03	ND	0.022	0.040	-1.45
8	ND	0.78±0.00	96	2.40±0.17	2.06±0.03	2.62±0.09	ND	0.135	0.241	-0.35
4	ND	ND	100	2.09±0.24	1.86±0.16	3.38±0.12	ND	0.165	0.257	-0.91
model	ND	1.08±0.03	94	1.46±0.33	1.30±0.11	2.36±0.11	ND	0.135	0.209	--

[a]Sugar conversion during fermentation. Initial sugars concentration minus final, divided by initial sugars multiplied by 100.
[b]Sugars-to-ethanol conversion yield respect to the maximum theoretical.
[c]Energetic balance between the energy produced by the combustion of fermentation products minus the energy expended to ozone generation for sugarcane bagasse pretreatment.

Around 30% of mass was lost during washing, due especially to xylan removal

Washing decreased the global sugar release compared to unwashed samples

Intermediate moisture contents favor organic acids formation and sugars release

Lower moisture contents generate mainly phenolic compounds than organic acids

Washing detoxification allowed xylose fraction utilization by diauxic microorganisms

Saccharification of ozonated sugarcane bagasse using enzymes from Myceliophthora thermophila JCP 1-4 for sugars release and ethanol production.

de Cassia Pereira, J., Travaini, R., Paganini Marques, N., Bolado-Rodríguez, S., Bocchini Martins, D.A., 2016. Bioresour. Technol. 204, 122–129.

CAPITULO 7

Bioresource Technology 204 (2016) 122–129

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Saccharification of ozonated sugarcane bagasse using enzymes from *Myceliophthora thermophila* JCP 1-4 for sugars release and ethanol production

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HIGHLIGHTS

- *Myceliophthora thermophila* JCP 1-4 is a new enzymes source for biomass hydrolysis.
- A fast 8 h hydrolysis was enough for ozonated sugarcane bagasse saccharification.
- The fungus was highlighted as a glucose isomerase producer, releasing fructose.
- Ozonated sugarcane bagasse acted as enzymes activities activator during hydrolysis.
- Hydrolysate from ozonated bagasse was greatly fermented for bioethanol production.

ARTICLE INFO

Article history:
Received 13 October 2015
Received in revised form 16 December 2015
Accepted 17 December 2015
Available online 23 December 2015

Keywords:
Sugarcane bagasse
Enzymatic saccharification
Ozonolysis
Fungal enzymes
Ethanol

ABSTRACT

The saccharification of ozonated sugarcane bagasse (SCB) by enzymes from *Myceliophthora thermophila* JCP 1-4 was studied. Fungal enzymes provided slightly higher sugar release than commercial enzymes, working at 50 °C. Sugar release increased with temperature increase. Kinetic studies showed remarkable glucose release (4.99 g/L, 3% w/w dry matter) at 60 °C, 8 h of hydrolysis, using an enzyme load of 10 FPU (filter paper unit). FPase and β-glucosidase activities increased during saccharification (284% and 270%, respectively). No further significant improvement on glucose release was observed increasing the enzyme load above 7.5 FPU per g of cellulose. Higher dry matter contents increased sugars release, but not yields. The fermentation of hydrolysates by *Saccharomyces cerevisiae* provided glucose-to-ethanol conversions around to 63%.

1. Introduction

The use of lignocellulosic residues – such as SCB – as raw material for second generation fuels production is a promissory research field. Lignocellulosic wastes stand out as a renewable bioenergy resource to reduce environmental, economic and political problems related to the use of the traditional fossil fuels. These residues can be used as substrates for microbial enzymes depolymerization to produce monomeric sugars that can be converted into second generation and other biobased products.

Brazilian government established, in 1975, the *PRO-ÁLCOOL* program, a policy to reduce the dependence from another countries fuel. The program was based in the increase of SCB ethanol production to be used as gasoline substitute as well as to blend it in a proportion up to 24%. In Brazil, SCB is generated in large quantities as a residue of sugar production and first generation ethanol factories (more than 800 million tons of sugarcane processed in the 2014/2015′ crop) (UNICA, 2015). Considering this scenario and taking into account the environmental issues, scientific researches for new energy sources in Brazil have been focused on the use of SCB to obtain second generation ethanol and other value-add products.

For the production of second-generation ethanol from SCB, a step of pretreatment is necessary to remove or disrupt lignin and

Abbreviations: AIL, acid insoluble lignin; ASL, acid soluble lignin; CBU, cellobiohydrolase unit; FPU, filter paper unit; SCB, sugarcane bagasse; HPLC, high pressure liquid chromatography; SSF, solid state fermentation; TL, total lignin.

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http://dx.doi.org/10.1016/j.biortech.2015.12.064
0960-8524/

to decrease cellulose crystallinity. Subsequently, carbohydrates polymers have to be chemical or enzymatically saccharified into glucose, which finally will be converted to ethanol by fermentative microorganisms (Tomás-Pejó et al., 2011).

Ozonolysis was studied decades ago as a chemical oxidative pretreatment of lignocellulosic materials (Neely, 1984) and has been rediscovered recently (García-Cubero et al., 2010, 2012; Li et al., 2015; Travaini et al., 2013). Ozonolysis pretreatment promotes kinetic-selective degradation of lignin, because its high quantity of electron-rich centers prone to ozone attach. Ozone converts acid insoluble lignin (AIL) into acid soluble lignin (ASL) and low molecular weight compounds, mainly the carboxylic acids oxalic, formic and acetic. Lignin removal increases enzymes accessibility to polysaccharides and sugar release (Travaini et al., 2015). Among the many advantages of ozonolysis on other pretreatments, these can be highlighted: high saccharification yields; low inhibitory compounds formation; the process occurs at ambient pressure and temperature; a single solid phase is generated, avoiding problems related to products dilution; ozone can be generated *in situ* and residual ozone can be easily destroyed, preventing environmental problems (Travaini et al., 2016).

Enzymatic saccharification of lignocellulosic materials to obtain glucose requires the use of microbial enzymes cocktails comprising at least three hydrolases: endoglucanase, exoglucanase and β-glucosidase (Arantes and Saddler, 2010). These enzymes are produced by many microorganisms, especially filamentous fungi (Maeda et al., 2013). Among them, thermophilic fungi stand out, since they can use lignocellulosic wastes as substrates to produce enzymes by solid state fermentation (SSF). In general, they better support temperature rise, common in SSF processes, and produce more active and stable enzymes under high temperatures, compared to mesophilic fungi (Gomes et al., 2007).

The search for new microbial cellulases that can be produced by SSF, using lignocellulosic residues as substrates, is interesting to reduce the costs of second-generation ethanol production, since enzymatic saccharification is one of the most expensive steps in the global process. These enzymes must have some characteristics, such as: high hydrolysis rate; activity and stability at high temperatures; and low inhibition by end-product and by secondary compounds that could be generated in the process (Mielenz, 2001). All the works available in scientific literature related to enzymatic saccharification of lignocellulosic material pretreated with ozone use commercial cellulases (García-Cubero et al., 2009; Li et al., 2015; Travaini et al., 2013).

In this work, the enzymatic extract produced by SSF from the new isolated thermophilic fungus *Myceliophthora thermophila* JCP 1-4 was used to saccharify ozone pretreated SCB. This fungus shows significant production of cellulases when growing in a variety of lignocellulosic substrates, including avicelase, the enzyme responsible for hydrolysis of crystalline cellulose (Cassia Pereira et al., 2015). Glucose isomerase activity was also detected in the enzymatic extract, which is not usual in fungi. Commercial enzymes were also used for comparison purposes. The influence of time, temperature, enzyme and bagasse load in saccharification were evaluated. Enzyme activities were monitored along with saccharification, and the endurance of enzymes subjected to concentration using a rotary evaporator was also studied. Hydrolysates were fermented using a bakery strain of *Saccharomyces cerevisiae* to obtain ethanol.

2. Methods

2.1. Sugarcane bagasse

SCB was donated by Usina Vale, city of Onda Verde, São Paulo State, Brazil. It was washed with distilled water to remove sugar residues and particulate material, dried in a ventilated oven at 37 °C and ground in an agricultural crusher (Trapp Model TRF-400) to a size of 3–5 mm. *In natura* SCB chemical composition (%) was: 3.13 ± 0.04 of ASL, 19.54 ± 0.03 of AIL, 22.67 ± 0.07 of total lignin (TL), 20.86 ± 0.05 of xylan and 46.21 ± 0.10 of cellulose (Travaini et al., 2013).

2.2. Ozonolysis pretreatment

Pretreatment was carried out in a fixed bed reactor (glass column of 50 cm in height and 2.7 cm in diameter) provided with a porous glass diffuser, as described by Travaini et al. (2013). The optimal operation parameters found in the previously mentioned work were applied: moisture content of 80%, ozone/oxygen concentration of 3.44% (mol/mol) at a flow of 60 L/h, for 45 min. After pretreatment, SCB was washed with distilled water. Ozonated SCB chemical composition (%) was: 6.22 ± 0.07 of ASL, 12.99 ± 0.02 of AIL, 19.20 ± 0.09 of TL, 20.74 ± 0.12 of xylan and 44.86 ± 0.75 of cellulose (Travaini et al., 2013).

2.3. Microorganism

The fungus *M. thermophila* JCP 1-4 was isolated from SCB silage piles in Potirendaba, São Paulo State, Brazil. It was chosen for the present study, among 26 thermophilic fungi, as the best producer of cellulases and β-glucosidase, when cultivated by SSF, at 45 °C, using a variety of lignocellulosic materials as substrates (Cassia Pereira et al., 2015). β-glucosidase from *M. thermophila* JCP 1-4 is resistant to glucose inhibition (Cassia Pereira et al., 2015), an important characteristic for saccharification experiments. Stock cultures are maintained in cryo tubes, in 20% glycerol solution at −80 °C.

2.4. Enzymes production by solid state fermentation

To obtain enzymatic extract, *M. thermophila* JCP 1-4 was precultivated on Sabouraud agar plates for 72 h at 45 °C. Five mycelial disks (1 cm diameter) from plates were used as inoculum for each 250 mL Erlenmeyer flask, containing 2.5 g of *in natura* SCB and 2.5 g of soybean meal (both washed and oven dried at 37 °C). Soybean meal was donated by Trouw Nutrition, Mirassol, São Paulo State, Brazil. Each Erlenmeyer flask was moisturized with 11 mL of saline solution as described by Toyama and Ogawa (1978), providing an initial substrate moisture close to 70%. Erlenmeyer flasks with substrates and saline solution were autoclaved at 121 °C, 1 atm, during 20 min before inoculation. Erlenmeyer flasks were inoculated and incubated at 45 °C, for 96 h, time for a satisfactory cellulolytic enzymes production (Cassia Pereira et al., 2015). After this period, 50 mL of distilled water were added to each flask, the mixture was homogenized with a glass bar, stirred in an orbital shaker at 100 rpm for 1 h. Then, it was filtered through nylon cloth disks and centrifuged at 10,000*g*, for 15 min, at 5 °C. Supernatants were collected, lyophilized and stored. For use, the enzymes were resuspended in water in the same water ratio of the original extract, for reproduce the same conditions when crude enzymes can be used. The enzymatic activities in the reconstituted extract were 0.33 FPU/mL and 1.00 CBU/mL (1:3 FPU/CBU ratio, the same found in crude extract).

2.5. Enzyme concentration

Fungal enzymatic extract had to be concentrated for some of the saccharification experiments (those in which the influence of dry matter content was evaluated), in order to achieve the desired FPU per g of cellulose. Concentration was performed by rotaevaporation, the extract was concentrated by 5-fold (initial volume

divided by final volume) on a rotatory evaporator at 60 °C under 100 rpm in vacuum. Each concentration procedure took approximately 2 h.

2.6. Enzyme activities

FPase activity was determined as described by Ghose (1987) and expressed as PFU. β-glucosidase activity was assayed according to Leite et al. (2008) and expressed as CBU. Endoglucanase and exoglucanase (avicelase) activities were determined as described by Cassia Pereira et al. (2015).

Glucose isomerase activity was determined according to Zhang et al. (2013), with modifications. Reaction tubes were composed of: 0.5 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.2 mL of 1 M D-glucose, 0.1 mL of 0.1 M $MgSO_4 \cdot 7H_2O$, 0.1 mL of 0.01 M $CoCl_2 \cdot 6H_2O$, 0.9 mL of distilled water and 0.2 mL of crude enzymatic extract. Reaction tubes were incubated at 40, 50 or 60 °C, for 1 h, and then reaction was stopped by addition of 2 mL 0.5 M perchloric acid. One unit of glucose isomerase activity was defined as the amount of enzyme needed to produce 1 μmol of fructose per minute, under the assay conditions.

2.7. Sugarcane bagasse enzymatic saccharification

Saccharification assays were performed in 100 mL Erlenmeyer flasks containing *in natura* or ozone pretreated SCB (3–10%, w/w dry basis) in sodium citrate buffer 0.05 M, pH 5.0 and the desired load of FPU per g of cellulose, provided by the enzymatic extract of *M. thermophila* JCP 1-4 or commercial enzymes, with 25 mL of final volume. The commercial enzymes used were Celluclast 1.5 L and Novozym 188 (Novozymes, Denmark). The first one was used as FPU source and the second as β-glucosidase source, to achieve the same FPU/CBU of 1:3 ratio found in the fungal enzymatic extract. Flasks were incubated on an orbital shaker, at different temperatures and 300 rpm, for up to 48 h. Substrate blank experiments were performed without enzyme addition, using only bagasse and buffer solution. Enzyme blank experiments were performed without bagasse, only with enzyme and buffer solution. At each time interval, a flask was taken, cooled in an ice bath, its content was filtered using 0.22 μm filters and sugars in the liquid fraction were determined and quantified by HPLC (high pressure liquid chromatography). The values of sugars (g/L) in blank experiments were subtracted from the values obtained in saccharification media. Yields were expressed as g of glucose divided per g of cellulose in the raw material, multiplied by 100 (expressed as a percentage). Experiments were performed as duplicates.

2.8. Statistical analysis

The effects of temperature, time, enzyme load and dry matter in ozonated SCB saccharification by *M. thermophila* JCP 1-4 enzymes were evaluated, using a one-factor-at-a-time (OFAT) design of experiments.

Statistical analysis of the data from saccharification experiments included a one-way ANOVA followed by Tukeýs test with a 5% significance level. All analyses were run in the STATGRAPHICS® Centurion XVI Version 16.2.04 (64-bit).

2.9. Alcoholic fermentation

For preculture production, a fresh bakery strain of *S. cerevisiae* acquired in a grocery shop was grown on commercial YEPD (Yeast Extract Peptone Dextrose) (1% yeast extract, 2% peptone and 2% glucose), aerobically, on a rotatory shaker at 30 °C, 175 rpm, for 24 h.

Hydrolysate slurries were transferred to 100 mL penicillin flasks, supplemented with 4 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4 and 0.75 g/L $MgSO_4 \cdot 7H_2O$ and inoculated with 5.5% (v/v) of the preculture (Wanderley et al., 2013). Flasks were sealed and fermentation occurred with the oxygen present in the empty flask space, at 30 °C, under 175 rpm, for 24 h. The supernatants were filtrated and analyzed by HPLC.

Ethanol yield was calculated with respect to the maximum theoretical production. Ethanol content (g/L) was divided by the glucose content (g/L), multiplied by the stoichiometric factor 0.511, and then the value was multiplied by 100 to give the percentage.

2.10. Analytical methods

Ethanol analysis was performed using the same HPLC setup previously described by Toquero and Bolado (2014), equipped with a Biorad Aminex® HPX-87H column, with 0.6 mL/min sulfuric acid 5 mM as eluent, at 50 °C. For sugars analysis, the system was equipped with a Phenomenex® HPLC column Rezex™ RPM-Monosaccharide Pb^{+2} (8%), 300 × 7.8 mm, with 0.6 mL/min MilliQ water as eluent, at 80 °C.

3. Results and discussion

3.1. Saccharification of sugarcane bagasse using fungal or commercial enzymes

In order to compare the efficiency of *M. thermophila* JCP 1-4 enzymatic extract in relation to the commercial enzymes mixture, preliminary saccharification assays were performed using *in natura* or ozonated SCB (3% w/w – dry basis, at 50 °C, for 24 h). The enzyme load used was 10 FPU and 30 CBU per g of cellulose content in raw material, for both fungal and commercial enzymes. These conditions are commonly cited in studies about sugarcane enzymatic saccharification (Cassia Pereira et al., 2015; Travaini et al., 2013). Besides, 50 °C is the optimum temperature for the commercial enzymes activities, and the enzymatic extract from *M. thermophila* JCP 1-4 shows good activities of endoglucanase, exoglucanase, β-glucosidase and FPase under this temperature (55.16, 2.01, 1.02 U/mL and 0.51 FPU, respectively).

As shown in Table 1, for *in natura* SCB, fungal enzymatic extract provided a glucose release of approximately 1.4 folds higher than commercial enzymes. Xylose release from *in natura* SCB was also higher (3.9 folds) when using *M. thermophila* JCP 1-4 enzymes. Regarding cellobiose release from *in natura* bagasse, very close values were obtained when using fungal or commercial enzymes. A standard one-way ANOVA analysis indicated significant differences in glucose release when using MT or commercial enzymes to saccharify in natura SCB ($F = 213.2$ and p-value = 0.003).

Table 1
Sugars released from *in natura* and ozonated bagasse, in saccharification assays (3% – w/w dry basis, 10 FPU and 30 CBU per g of cellulose, 50 °C, 24 h) using *Myceliophthora thermophila* JCP 1-4 enzymatic extract (MT) or commercial enzymes*.

Enzyme source	SCB	Sugar (g/L)**		
		Glucose***	Xylose	Cellobiose
MT	*In natura*	1.40 ± 0.02	0.50 ± 0.02	0.15 ± 0.04
Commercial		0.95 ± 0.20	0.13 ± 0.16	0.18 ± 0.01
MT	Ozonated	4.19 ± 0.02	1.94 ± 0.02	0.23 ± 0.05
Commercial		4.13 ± 0.20	1.08 ± 0.44	0.04 ± 0.00

* Mixture of Celluclast 1.5 L and Novozym 188.
** No sugars were detected in the substrate blank and enzyme blank under the HPLC method limit of detection (>0.01 g/L).
*** No significant difference found on sugar released applying one-way ANOVA analysis ($p < 0.005$), using ozonated SCB saccharified with MT or commercial enzymes.

However, there was no significant difference in glucose release from ozonated SCB when using MT or commercial enzymes ($F = 0.106$ and p-value = 0.8).

Regarding ozonated SCB, the use of fungal enzymes provided a glucose release very close to that observed when using the commercial ones, and release of xylose and cellobiose 44% and 83% higher, respectively. These data clearly show the efficiency of fungal enzymes to saccharify ozonated SCB. Furthermore, ozone pretreatment improved the releasing of reducing sugars in saccharification for both enzymes.

From these results, further ozone pretreated SCB saccharification experiments were performed using the enzymatic extract from *M. thermophila* JCP 1-4. Works dealing with the use of non-commercial microbial enzymes to saccharify ozonated SCB or other pretreated lignocellulosic materials are scarce in literature. Besides, the use of new microbial enzymes with appropriate characteristics for lignocellulosic material saccharification can greatly cheapen biorefineries processes.

3.2. Influence of temperature and time on saccharification of ozonated bagasse

Saccharification assays were performed at 40, 50 and 60 °C (temperatures around the optimal for commercial enzymes), using 3% (w/w, dry basis) ozonated SCB. *M. thermophila* JCP 1-4 enzymatic extract was loaded for 10 FPU and 30 CBU per g of cellulose content in the raw material and samples were taken at different time intervals.

Glucose release increased with the increasing of temperature (Fig. 1a). The higher release of this sugar (5.38 g/L; 39% yield) was observed at 48 h and 60 °C. Additionally, at this temperature, a glucose release of 4.99 g/L (36% yield) was obtained at only 8 h of saccharification, which can be considered a satisfactory sugar release in a short hydrolysis time, indicating that it is not advantageous to extend the experiment. The profiles of glucose releasing at 40 and 50 °C were similar, and the amount of glucose release tends to stabilize after 16 h of saccharification (around 4.0 g/L). A very close glucose yield (41.79%) was obtained by Travaini et al. (2013) with ozonated SCB produced in identical conditions of this work, using the commercial enzymes NS50013 and NS50010 (from Novozymes) during 24 h saccharification at 50 °C. Souza-Correa et al. (2013, 2014), working with ozonated SCB achieved cellulose-to-glucose conversions between 65% and 78.8%; however they used different ozonolysis conditions, 20 FPU per g of substrate and worked with just 1% of substrate concentration, during 24 h at 50 °C.

Surprisingly, fructose was also detected in hydrolysates (Fig. 1b) but not in the fungal enzymatic extract and even in substrate chemical composition, which was considered an indicative that *M. thermophila* JCP 1-4 produced a glucose isomerase. To confirm this hypothesis, glucose isomerase activity in the enzymatic extract was determined at 40, 50 and 60 °C. It is a rare characteristic, since glucose isomerase (responsible for glucose isomerization into fructose) is hardly found among fungi. The traditional glucose isomerase source are bacteria, and its production was also reported for some basidiomycetes (Horwath and Irbe, 1984), but there is no report in literature about this enzyme production by other fungi.

Relationship between temperature of saccharification and fructose release was opposite to that observed for glucose (Fig. 1b). The highest amount of fructose (1.40 g/L) was obtained after 48 h, at 40 °C. Again, from 8 h on very close amounts were obtained. Glucose isomerase activity was measured in fungal enzymatic extract. From these data it can be inferred that, during the saccharification, part of the glucose content was converted to fructose by the action of glucose isomerase, avoiding β-glucosidase inhibition and, consequently, increasing the conversion of cellobiose to glucose (Fig. 1).

Enzyme activities at 40, 50 and 60 °C were 0.36, 0.31 and 0.23 U/mL, respectively. Thus, fructose production is directly related to glucose isomerase enzymatic activity, since both decreased with the increasing of temperature. It is worth to note that in the chemical characterization of SCB it was not observed the presence of fructose, which can only have arisen from glucose conversion.

Cellobiose concentration initially increased, increasing the kinetic with temperature, but decreased after around 16 h saccharification time (Fig. 1c).

After defining the most suitable condition for saccharification, based on glucose release (8 h and 60 °C), new experiments were performed, in order to evaluate the efficiency of commercial enzymes after 8 h saccharification at 60 °C and at the optimum temperature of the commercial enzymes (50 °C). At 60 °C, *M. thermophila* JCP 1-4 enzymes provided a glucose release around 30% higher when compared to commercial enzymes mixture (4.99 and 3.50 g/L, respectively). It is interesting to observe that the saccharification efficiency of commercial enzymes at 50 and 60 °C was very close (glucose release of 3.62 and 3.50 g/L, respectively), which indicates that at 60 °C their activity is not being considerably reduced.

3.3. Monitoring fungal enzymes activities during enzymatic saccharification

FPase and β-glucosidase activities of fungal extract were monitored during saccharification experiments. Results discussed below are presented as percentage relative to the enzymatic activity measured at zero time in the saccharification media. Enzymatic extract was loaded at saccharification media to achieve 0.14 FPU and 0.42 CBU/mL in the final volume (corresponding to 10 FPU and 30 CBU by g of cellulose content in raw SCB). Identical or very similar enzymatic activities were obtained from the measurements at time zero in the saccharification media (ANOVA analysis showed no-significant differences, and so, these data are not shown).

Initially, an experiment using *in natura* SCB at 3% (w/w, dry basis) as substrate and an enzyme blank experiment containing only buffer and enzymatic extract were conducted, at 50 °C (enzyme load as described in Section 3.2). In the enzyme blank experiment, FPase activity decreased clearly with time, retaining around 70% of its original activity between 16 and 24 h (Fig. 2a). Regarding β-glucosidase activity (Fig. 2b), a slight increase was observed during the first 8 h (around 5%) and the enzyme retained 100% of its original activity until 16 h. At 24 h, β-glucosidase activity decreased only 7.5%, indicating that this enzyme is more stable than FPase. Activities of FPase and β-glucosidase were improved slightly in the experiment with *in natura* bagasse (11% and 10% higher when compared to their original activities, at 8 and 24 h of saccharification, respectively) (Fig. 2a and b). Bagasse seems to weakly stimulate the enzymatic activity of both enzymes from the fungal extract.

FPase and β-glucosidase activities were also monitored in the saccharification experiment using ozonated bagasse (described in Section 3.2). A remarkable increase of enzymatic activities was observed during the saccharification course. The most outstanding increases in FPase activity were observed in 48 h at 50 and 60 °C (284% and 274%, respectively) (Fig. 2c). Concerning β-glucosidase, it can be highlighted a maximum increase of 269.93% in 48 h, at 50 °C (Fig. 2d). This considerable increase in enzyme activities was not related to an increase in glucose release. Glucose release increased very slightly after 8 h of saccharification, probably because there were no more polysaccharides suitable for conversion or due to some complex enzymatic inhibition process. From these results, lignocellulose structure breakdown by pretreatment seems to be the limiting step of the global process. Nevertheless,

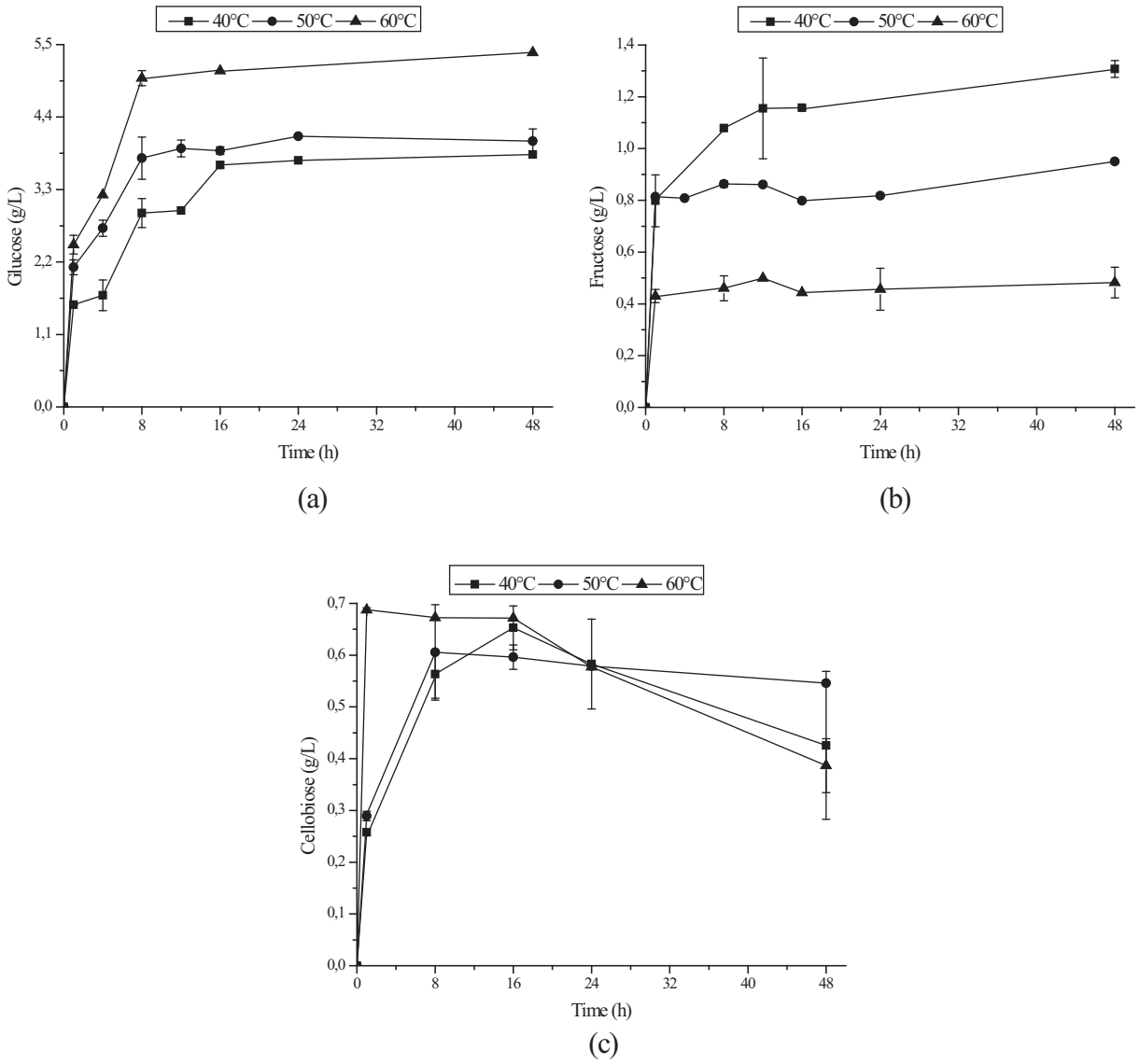

Fig. 1. Glucose (a), fructose (b) and cellobiose (c) release during saccharification of ozonated sugarcane bagasse (3% w/w, dry basis, 10 FPU and 30 CBU per g of cellulose) at different temperatures. Enzymes provided from *Myceliophthora thermophile* JCP 1-4.

the enzymatic activity increases could be very interesting when using other pretreatments.

Fructose has been described as a stabilizer of β-glucosidase activity (Weijers and Van't Riet, 1992). Since fructose was produced during saccharification by the action of glucose isomerase, the activities of FPase β-glucosidase in the enzymatic extract from *M. thermophila* JCP 1-4 were measured, at 50 °C, in the presence of this sugar. Fructose was added to the assay tubes at 0.95 g/L (the concentration found when the highest FPase and β-glucosidase activities increases were observed). In this assays, practically no increase was found on FPase activity. On the other hand, β-glucosidase activity was increased by 180% in the presence of fructose. These results suggest that fructose exerts specific activation effect on β-glucosidase activity. However, the increase in β-glucosidase activity may improve FPase action in a global view, since it removes cellobiose from media, reducing its inhibitory effect on cellulases.

The fructose effect is not enough to explain the observed increase on FPase and β-glucosidase activities during ozonated bagasse saccharification experiments. Increase on enzymatic activities are higher for ozonated SCB than for in natura SCB in all the experiments. Enzymatic activities decreased since time zero, as expected, for enzyme blank experiments without substrate. The lower increase in enzyme activation and activity retention in time found for in natura SCB can be attributed to enzymes active site protection and protein rearrangement (Kokkinidis et al., 2012). Since the increase on these enzymes activities was low during *in natura* bagasse saccharification experiments, we can infer that some compound(s) produced or released after ozonolysis pretreatment could have a more expressive effect on their activities. Apart from fructose, other factors may contribute to enzymatic activation, such as ions, which can act as cofactors helping to maintain the structure of polymeric proteins and also stabilizing active sites (Hernández-Salas et al., 2009; Iyer and Ananthanarayan, 2008).

3.4. Influence of enzyme load in ozonated SCB saccharification

Working with the best conditions of time and temperature (8 h at 60 °C) for enzymatic ozonated SCB (3% w/w, dry basis) saccharification found in Section 3.2, the influence of enzyme load was

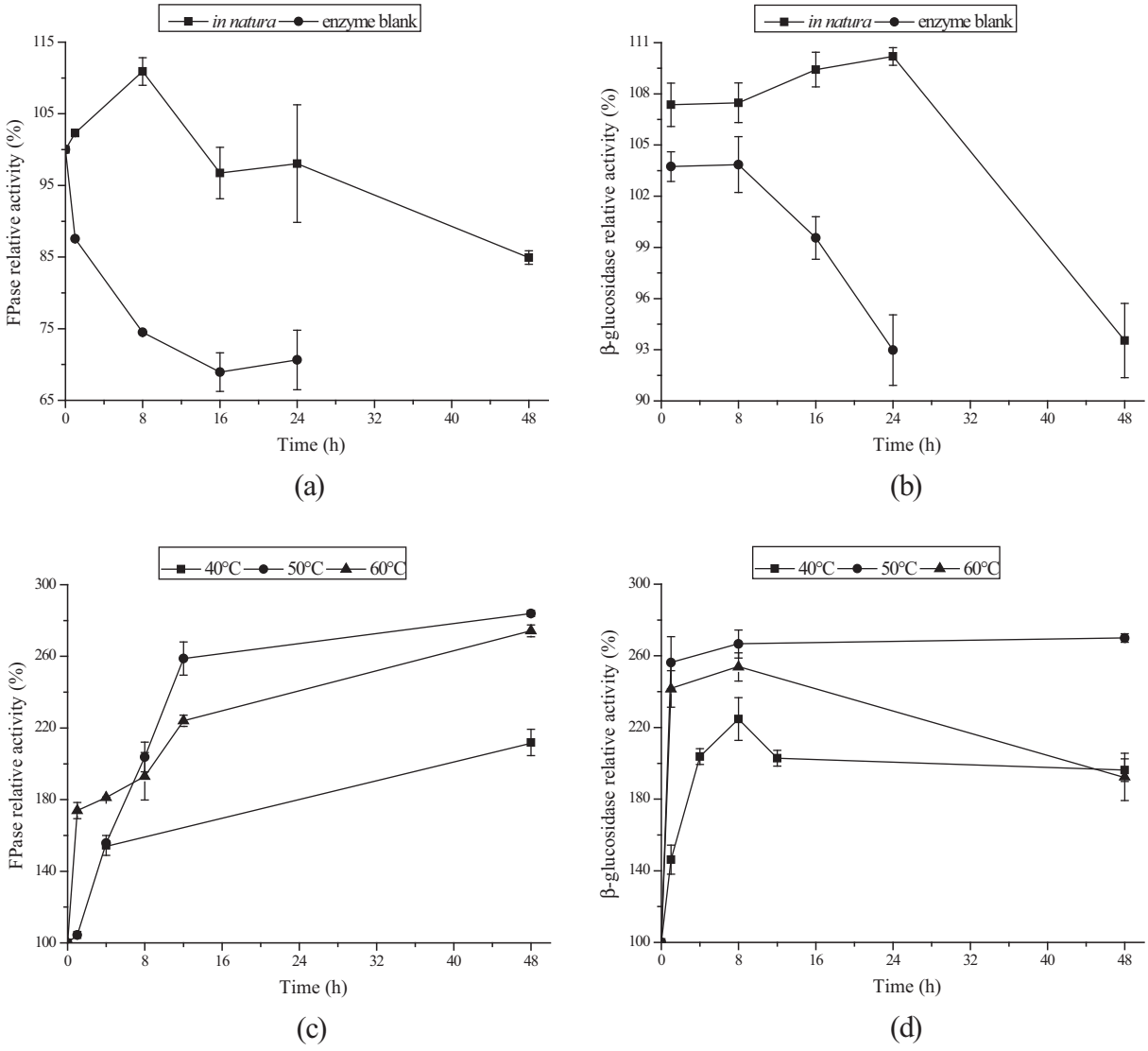

Fig. 2. *Myceliophthora thermophila* JCP 1-4 enzymes relative activities during saccharification experiments. FPase (a) and β-glucosidase (b) activities for control *in natura* bagasse (3% w/w dry basis, 50 °C, 10 FPU and 30 CBU per g of cellulose) and enzyme blank experiments (50 °C); FPase (c) and β-glucosidase (d) activities during ozonated sugarcane bagasse saccharification at different temperatures (3% – w/w dry basis, 10 FPU and 30 CBU per g of cellulose). Relative enzymatic activities compared to the activities at zero time in saccharification media (0.14 FPU and 0.42 CBU/mL, in the final volume to achieve 10 FPU and 30 CBU per g of cellulose content in SCB).

evaluated using the enzymatic extract from *M. thermophila* JCP 1-4 with basis on FPU. Enzyme loads in the range from 2.5 to 15 FPU - per g of cellulose were used. Glucose release markedly increased with enzyme loads up to 7.5 FPU per g of cellulose (4.86 g/L) (Fig. 3). Higher enzyme loads provided very slight increases on glucose release (5.21 g/L using 15 FPU per g of glucose). This enzyme load (7.5 FPU per g of cellulose) is lower than other cited by scientific literature for SCB saccharification: 10 FPU per g of cellulose (Travaini et al., 2013; Wanderley et al., 2013); 15 FPU per g of cellulose (Aguiar Souza et al., 2013; Mesa et al., 2011); 20 FPU per g of cellulose (Hongdan et al., 2013); and even values notably higher, such as 60 and 65 FPU per g of cellulose (Benjamin et al., 2013; Jiang et al., 2013).

It is well known that high enzyme loads increase glucose release during saccharification, however this is one of the most expensive steps in second-generation fuels production and in biorefineries factories. Thus, studies involving new microbial enzymes that present characteristics suitable for lignocellulosic material saccharification (high saccharification efficiency with low enzyme load, low product inhibition and stability during saccharification) are necessary.

3.5. Influence of dry matter content in ozonated SCB enzymatic saccharification and hydrolysates fermentation

The amount of dry matter is an important parameter in the study of enzymatic saccharification, since it refers to the amount of substrate to be converted by enzymes which, in turns, is related to the final hydrolysate sugar concentration, including fermentable sugars.

To achieve the desired FPU per g of cellulose in experiments with high dry matter concentration, a previous fungal enzymatic extract concentration was required. For this purpose, the fungal extract was concentrated 5 times by rotaevaporation (60 °C and 100 rpm, in vacuum, for 2 h). FPase and β-glucosidase activities were measured after concentration. Enzymes retained 90% and 64% of their original activities, respectively, providing a FBU/CBU ratio of 1:2.15 in the concentrated extract.

Saccharification experiments were performed at 60 °C, 8 h and 7.5 FPU per g of cellulose (the best conditions found in Sections 3.2 and 3.4), varying the amount of ozonated SCB in the range from 3% to 10% (w/w, dry basis). Results, in terms of glucose release and yield, are shown in Fig. 4. As expected, 10% of bagasse provided the

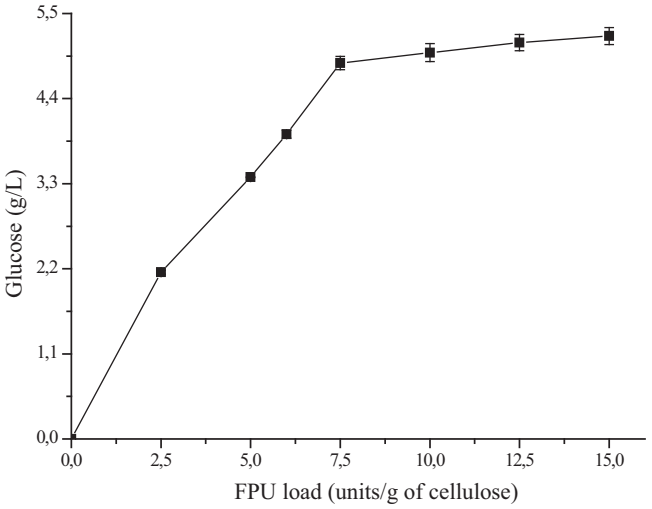

Fig. 3. Influence of enzyme load (FPU per g of cellulose) on glucose release from ozonated sugarcane bagasse in saccharification at 60 °C, 3% (w/w dry basis) during 8 h. Enzymes provided from *Myceliophthora thermophila* JCP 1-4, with a ratio FPU: CBU of 1:3.

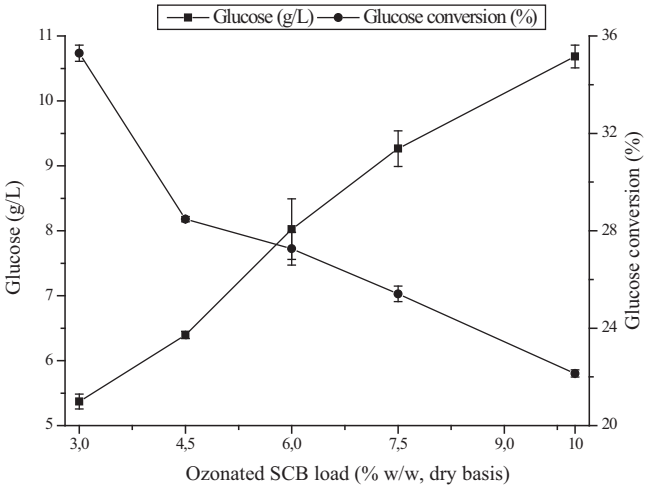

Fig. 4. Influence of dry matter content (% w/w dry basis) on glucose release (g/L) and glucose conversion from ozonated sugarcane bagasse in saccharification at 60 °C, 7.5 FPU and 15.1 CBU per g of cellulose, at 8 h. Enzymes provided from *Myceliophthora thermophila* JCP 1-4.

highest glucose release (10.19 g/L). Nevertheless, in terms of saccharification yield, the best result (30.11%) was obtained with 3% of ozonated SCB. Maitan-Alfenas et al. (2015) worked with 8% (w/v, dry basis) acid and basic pretreated SCB for saccharification with 5-fold concentrated fungal extract of *Chrysophorte cubensis*. They achieved 5.32 g/L (12.5% yield) and 2.94 g/L (7.7% yield) of glucose, respectively, for each pretreatment. However, they used 10 FPU per g of dry pretreated SCB and 72 h of saccharification at 50 °C.

High solid loads in saccharification may confer advantages and disadvantages and require an accurate analysis. A higher solid load provides higher glucose concentration after hydrolysis, increasing fermentation yield and reducing the costs related to the hydrolysate concentration. However, high solid loads usually bring lower enzymatic hydrolysis yields, due to some main factors: (1) increase on the dry matter concentration hinders the mass transfer and enzymes accessibility to biomass, being necessary higher stirring power and (2) high dry matter concentration leads to liquid absorption, resulting in a decrease of free water, reducing medium viscosity. Water is essential for hydrolysis reactions and necessary for solubilization. So, the increase on dry matter concentration can increase the process energy requirements and the production costs (Modenbach and Nokes, 2013).

3.6. Alcoholic fermentation

There is no previously published data about alcoholic fermentation of hydrolysates obtained from the saccharification of ozonated SCB. In order to test its fermentability, hydrolysates produced with fungal enzymes were fermented. Whole slurry hydrolysates from saccharification assays performed with 7.5 and 10% (w/w – dry basis) ozonated SCB were fermented with *S. cerevisiae* bakery strain to obtain ethanol. The 7.5% (w/w) dry matter hydrolysate provided 2.81 g/L of ethanol from 8.77 g/L of glucose (63% of the maximum theoretical conversion), whereas the 10% (w/w) dry matter hydrolysate gave 3.15 g/L of ethanol from 10.19 g/L (61% of the maximum theoretical conversion). Both fermentation yields are really close, indicating the absence of inhibitory compounds that could have been accumulated by high solids content. Wanderley et al. (2013) obtained 46.47% (5.33 g/L) of ethanol efficiency using enzymatic hydrolysates in the same conditions of biomass load, but using commercial enzymes.

4. Conclusions

M. thermophila JCP 1-4 is a promising fungus for bioethanol production. Its enzymes efficiency to saccharify ozonated SCB was higher than that of the commercial enzymes used and increased with temperature. Fructose was found in hydrolysates, indicating glucose isomerase production (rare among fungi). Ozonated bagasse increased FPase and β-glucosidase activities during saccharification, suggesting that pretreatment is the limiting step. The evaluated saccharification parameters influenced sugars release: optimal results were obtained at 60 °C, with low enzyme load (7.5 FPU), in only 8 h. Glucose released was converted into ethanol with a satisfactory yield, indicating no fermentation inhibition.

Acknowledgements

This work was supported by the "*Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP*", *Brazil* (2011/19554-0) and by the research unit UIC 071 of the regional government "*Junta de Castilla y León – JCyL*", *Spain*. Josiani de Cassia Pereira thanks to "*Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP*", *Brazil* for her Master Scholarship (2011/12794-6) and for the internship scholarship "*Bolsa Estágio de Pesquisa no Exterior – BEPE*" (2012/20403), which allowed her to develop this work at the University of Valladolid. Rodolfo Travaini is also grateful to "*Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq*", *Brazil* (238059/2012-0) for providing his Doctorate Scholarship.

References

Aguiar Souza, R., Silveira, M.H.L., Pitarelo, A.P., Corazza, M.L., Ramos, L.P., 2013. Kinetics of enzyme-catalyzed hydrolysis of steam-exploded sugarcane bagasse. Bioresour. Technol. 147, 416–423.

Arantes, V., Saddler, J.N., 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol. Biofuels 3, 4.

Benjamin, Y., Cheng, H., Görgens, J.F., 2013. Evaluation of bagasse from different varieties of sugarcane by dilute acid pretreatment and enzymatic hydrolysis. Ind. Crops Prod. 51, 7–18.

Cassia Pereira, J., Paganini Marques, N., Rodrigues, A., Brito de Oliveira, T., Boscolo, M., Da-Silva, R., Gomes, E., Bocchini Martin, D., 2015. Thermophilic fungi as new

sources for production of cellulases and xylanases with potential. J. Appl. Microbiol. 118, 928–939.
García-Cubero, M., Coca, M., Bolado, S., González-Benito, G., 2010. Chemical oxidation with ozone as pre-treatment of lignocellulosic materials for bioethanol production. Chem. Eng. Trans. 21, 1273–1278.
García-Cubero, M.T., González-Benito, G., Indacoechea, I., Coca, M., Bolado, S., 2009. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresour. Technol. 100, 1608–1613.
García-Cubero, M.T., Palacín, L.G., González-Benito, G., Bolado, S., Lucas, S., Coca, M., 2012. An analysis of lignin removal in a fixed bed reactor by reaction of cereal straws with ozone. Bioresour. Technol. 107, 229–234.
Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268.
Gomes, E., Guez, M.A.U., Martin, N., da Silva, R., 2007. Enzimas termoestáveis: fontes, produção e aplicação industrial. Quim. Nova 30, 136–145.
Hernández-Salas, J.M., Villa-Ramírez, M.S., Veloz-Rendón, J.S., Rivera-Hernández, K. N., González-César, R.A., Plascencia-Espinosa, M.A., Trejo-Estrada, S.R., 2009. Comparative hydrolysis and fermentation of sugarcane and agave bagasse. Bioresour. Technol. 100, 1238–1245.
Hongdan, Z., Shaohua, X., Shubin, W., 2013. Enhancement of enzymatic saccharification of sugarcane bagasse by liquid hot water pretreatment. Bioresour. Technol. 143, 391–396.
Horwath, R., Irbe, R., 1984. Glucose isomerase from fungi of the basidiomycetes class. Patent Number US4447531A.
Iyer, P.V., Ananthanarayan, L., 2008. Enzyme stability and stabilization—aqueous and non-aqueous environment. Process Biochem. 43, 1019–1032.
Jiang, L.Q., Fang, Z., Li, X.K., Luo, J., Fan, S.P., 2013. Combination of dilute acid and ionic liquid pretreatments of sugarcane bagasse for glucose by enzymatic hydrolysis. Process Biochem. 48, 1942–1946.
Kokkinidis, M., Glykos, N.M., Fadouloglou, V.E., 2012. Protein Flexibility and Enzymatic Catalysis. In: Donev, R. (Ed.), Advances in protein chemistry and structural biology. Elsevier, USA, pp. 181–218.
Leite, R.S.R., Alves-Prado, H.F., Cabral, H., Pagnocca, F.C., Gomes, E., Da-Silva, R., 2008. Production and characteristics comparison of crude β-glucosidases produced by microorganisms *Thermoascus aurantiacus* e *Aureobasidium pullulans* in agricultural wastes. Enzyme Microb. Technol. 43, 391–395.
Li, C., Wang, L., Chen, Z., Li, Y., Wang, R., Luo, X., Cai, G., Li, Y., Yu, Q., Lu, J., 2015. Ozonolysis pretreatment of maize stover: the interactive effect of sample particle size and moisture on ozonolysis process. Bioresour. Technol. 183, 240–247.
Maeda, R.N., Barcelos, C.A., Anna, L.M.M.S., Pereira, N., 2013. Cellulase production by *Penicillium funiculosum* and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. J. Biotechnol. 163, 38–44.
Maitan-Alfenas, G.P., Visser, E.M., Alfenas, R.F., Nogueira, B.R.G., de Campos, G.G., Milagres, A.F., de Vries, R.P., Guimarães, V.M., 2015. The influence of pretreatment methods on saccharification of sugarcane bagasse by an enzyme extract from *Chrysoporthe cubensis* and commercial cocktails: a comparative study. Bioresour. Technol. 192, 670–676.
Mesa, L., González, E., Romero, I., Ruiz, E., Cara, C., Castro, E., 2011. Comparison of process configurations for ethanol production from two-step pretreated sugarcane bagasse. Chem. Eng. J. 175, 185–191.
Mielenz, J.R., 2001. Ethanol production from biomass: technology and commercialization status. Curr. Opin. Microbiol. 4, 324–329.
Modenbach, A., Nokes, S.E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings – a review. Biomass Bioenergy 56, 526–544.
Neely, W.C., 1984. Factors affecting the pretreatment of biomass with gaseous ozone. Biotechnol. Bioeng. 26, 59–65.
Souza-Correa, J.A., Oliveira, C., Wolf, L.D., Nascimento, V.M., Rocha, J.G.M., Amorim, J., 2013. Atmospheric pressure plasma pretreatment of sugarcane bagasse: the influence of moisture in the ozonation process. Appl. Biochem. Biotechnol. 171, 104–116.
Souza-Correa, J.A., Oliveira, C., Nascimento, V.M., Wolf, L.D., Gómez, E.O., Rocha, G.J. M., Amorim, J., 2014. Atmospheric pressure plasma pretreatment of sugarcane bagasse: the influence of biomass particle size in the ozonation process. Appl. Biochem. Biotechnol. 172, 1663–1672.
Tomás-Pejó, E., Alvira, P., Ballesteros, M., Negro, M.J., 2011. Chapter 7 – Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion. In: Pandey, A., Larroche, C., Ricke, S.C., Dussap, C.-G., Gnansounou, E. (Eds.), Biofuels. Elsevier, USA, pp. 149–176.
Toquero, C., Bolado, S., 2014. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour. Technol. 157, 68–76.
Toyama, N., Ogawa, K., 1978. Cellulase production of *trichoderma viride* in solid and submerged culture methods. In: Ghose, T. (Ed.), International Symposium on Bioconversion of Cellulosic Substances into Energy Chemicals and Microbial Proteins, pp. 305–312, New Delhi, India.
Travaini, R., Marangon-Jardim, C., Colodette, J.L., Morales-Otero, M.D., Bolado-Rodríguez, S., 2015. Ozonolysis. In: Pandey, A., Negi, S., Binod, P., Larroche, C. (Eds.), Pretreatment of Biomass. Elsevier, USA, pp. 105–135.
Travaini, R., Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodríguez, S., 2016. Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited. Bioresour. Technol. 199, 2–12.
Travaini, R., Otero, M.D.M., Coca, M., Da-Silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment: effect on enzymatic digestibility and inhibitory compound formation. Bioresour. Technol. 133, 332–339.
UNICA, 2015. Relatório final da safra de 2014/2015, região centro-sul, UNICADATA, available at <http://www.unicadata.com.br/>, last accessed September 10, 2015. São Paulo, Brasil.
Wanderley, M.C.D.A., Martín, C., Rocha, G.J.D.M., Gouveia, E.R., 2013. Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. Bioresour. Technol. 128, 448–453.
Weijers, S.R., Van't Riet, K., 1992. Enzyme stability in downstream processing part 1: enzyme inactivation, stability and stabilization. Biotechnol. Adv. 10, 237–249.
Zhang, F., Duan, X., Chen, S., Wu, D., Chen, J., Wu, J., 2013. The addition of Co^{2+} enhances the catalytic efficiency and thermostability of recombinant glucose isomerase from *Thermobifida fusca*. Process Biochem. 48, 1502–1508.

CONCLUSIONES

CAPITULO 8

8.1 CONCLUSIONES ESPECÍFICAS

1. El estudio de las condiciones de operación sobre el pretratamiento por ozonólisis ha revelado que el tiempo de reacción disminuye con el aumento de la concentración de ozono. Se ha demostrado también que la humedad posee un papel protector sobre la biomasa: cuanta más alta, menos ataque a los polímeros de carbohidratos y menor la generación de ácidos inorgánicos, que podrían actuar como inhibidores de fermentación. La recuperación de carbohidratos post pretratamiento ha sido alta, con las menores recuperaciones en los ensayos con mayor delignificación.

No se detectaron los inhibidores furfural y 5-(hidroximetil)furfural en los hidrolizados de bagazo ozonizado, siendo encontrados mayoritariamente los ácidos fórmico, acético y oxálico y compuestos fenólicos.

El pretratamiento ha aumentado considerablemente la digestibilidad enzimática del bagazo, y la delignificación ha demostrado tener relación directa con los rendimientos de glucosa obtenidos. El análisis estadístico ha demostrado que el ozono y la humedad son los factores más importante en la delignificación, con sus óptimos en un 2% (mol/mol) y 45% (m/m), respectivamente. El ozono gastado en los pretratamientos ha presentado una relación directa con los rendimientos de azúcares, con la liberación aumentando paralelamente al gasto de ozono en el pretratamiento. La hidrólisis enzimática estándar aplicada se ha revelado eficiente en la sacarificación de los polímeros de azúcares, con conversiones de alrededor

de 80% para glucosa y entre 60-70% para xilosa. La sacarificación del bagazo ozonizado-lavado ha producido hidrolizados con más glucosa y menos xilosa, que los ensayos sin lavar, en términos de g/L producidos. La disminución en la liberación de la xilosa ha sido atribuida a su lixiviación durante el proceso de lavado. El aumento en el rendimiento de glucosa ha sido debido a la remoción de sustancias interferentes durante el lavado, lo que ha sido comprobado por los cambios morfológicos observados por microscopia electrónica de barrido.

2. El estudio con enzimas fúngicas ha demostrado que las enzimas producidas por el hongo *Myceliophtora thermophila* JCP 1-4 son más adecuadas para la hidrólisis del bagazo ozonizado que las enzimas comerciales testadas. Debido a las características de termoestabilidad presentadas por la celulasa fúngica producida, se han obtenido mayores rendimientos de azúcares trabajando a mayores temperaturas, con su mejor resultado cinético a 60 ºC y 8h de tiempo de hidrólisis. El monitoreo de las actividades enzimáticas durante la sacarificación ha demostrado un efecto inductor del bagazo, con la actividad aumentando con el tiempo de hidrólisis. El estudio de la cantidad de enzima utilizada, en FPU por gramo de celulosa, ha mostrado un óptimo en 7,5, valor por encima del cual un aumento en la cantidad de enzima no conlleva un aumento en los rendimientos. Para la carga de bagazo ozonizado en las hidrólisis se ha encontrado un efecto negativo, ya que al aumentar la concentración de materia seca se redujeron los rendimientos.

3. El estudio estadístico ha proporcionado información del efecto de cada parámetro estudiado (humedad, concentración de ozono, flujo de ozono/oxígeno y tamaño de partícula) sobre los rendimientos de azúcares, consumo de ozono y generación de inhibidores. Aunque la humedad ha sido reportada en la literatura de ozonólisis como el parámetro más importante, este estudio sistemático ha revelado que la concentración de ozono es el parámetro determinante en los rendimientos de azúcares, con su óptimo en 2% (mol/mol en ozono:oxígeno). Por otro lado, la humedad desempeña un papel fundamental en el consumo de ozono, y es el parámetro con mayor influencia cuando se busca producir la mayor cantidad de azúcares con el menor gasto de ozono. Cuando se ha tenido en cuenta la generación de inhibidores, la humedad ha sido el parámetro de mayor importancia, favoreciendo la generación de compuestos fenólicos cuanto más baja y la generación de ácidos orgánicos cuando intermedia (con su máximo en 45%, m/m).

4. La optimización de parámetros para la máxima producción de glucosa y xilosa ha resultado en una combinación de parámetros muy próxima a uno de los experimentos de la matriz ortogonal aplicada en el diseño experimental (50% (m/m) de humedad, 2% de ozono, 60 L/h de ozono/oxígeno y Ø<1,25mm). En este experimento, se han obtenido conversiones de un 77,6% de la celulosa y un 57,0% del xilano. Sin embargo, en estas mismas condiciones se ha producido la mayor cantidad de compuestos inhibidores, y el mayor consumo de ozono.

5. El proceso de detoxificación ha sido eficiente para todos los inhibidores cuantificados (ácidos fórmico, acético y oxálico, y compuestos fenólicos totales). Se han observado pérdidas de hasta un 30% (m/m) de materia durante el proceso de lavado, que se han atribuido mayoritariamente a xilano, ácidos orgánicos y fracciones solubilizadas de la lignina. Se ha observado que, con el aumento del consumo de ozono, el xilano en el sólido ozonizado-lavado disminuye hasta un máximo, a partir del cual su cantidad en el sólido permanece constante; para lignina ácida insoluble y celulosa otros factores parecen afectar su degradación/lixiviación.

6. Todos los hidrolizados obtenidos a partir de bagazo ozonizado fueron fermentados por la levadura *Saccharomyces cerevisiae* bakery, con rendimientos variando entre 80% y 88% respecto al máximo teórico con enzimas comerciales, y un 60% con enzimas fúngicas. La detoxificación por lavado ha permitido la fermentación de los hidrolizados por la levadura diaúxica *Pichia stipitis* DSM 3651 para producción de etanol, con rendimientos de alrededor de 80%, y por las bacterias anaerobias *Clostridium acetobutylicum* DSM792 y *Clostridium beijerinckii* DSM6422 para producción de butanol, obteniendo el máximo de disolventes por esta última con 0,165 $g_{BUTANOL}/g_{AZÚCAR}$ y 0,257 $g_{ABE}/g_{AZÚCAR}$. No se ha encontrado efecto inhibidor de los hidrolizados producidos con bagazo ozonizado-lavado, pero una relación directa ha sido encontrada entre la concentración inicial de azúcares en las fermentaciones y los productos obtenidos.

7. El estudio energético preliminar entre la energía gastada para la producción de ozono y la energía teórica generada por la combustión del

etanol obtenido por *Saccharomyces cerevisiae* bakery ha puesto de manifiesto la necesidad de utilizar de la fracción xilosa para conseguir balances energéticos viables. Esta conclusión ha sido confirmada por los mejores resultados obtenidos cuando los hidrolizados fueron fermentados por la levadura diaúxica *Pichia stipitis* DSM 3651. En cuanto a las fermentaciones para producción de butanol, el balance energético ha sido negativo para las dos bacterias testadas, habiendo mayor gasto de energía en la producción del ozono gastado que en la combustión teórica de los biocombustibles producidos. Sin embargo, posibilidades como la recuperación de los ácidos generados como producto, y la utilización de los azúcares lixiviados y residuos de fermentación para producción de biogás, podrían llevar a un proceso viable.

8.2 CONCLUSIONES GENERALES

Los resultados obtenidos en esta tesis doctoral han consolidado el gran potencial de la ozonólisis como pretratamiento del bagazo de caña de azúcar de caña para la producción de biocombustibles. Estos estudios han aportado información nunca antes publicada, cuantificando el efecto de los parámetros de la ozonólisis sobre los resultados obtenidos de rendimientos de azúcares, tiempo de reacción, consumo de ozono y generación de inhibidores. También se ha visto que la concentración de ozono tiene el papel principal en las modificaciones de composición y en los rendimientos de azúcares, debido a que está intrínsecamente relacionada con el poder de delignificación del pretratamiento. La humedad ha

presentado un papel de gran importancia, una vez que es factor controlante para la generación de inhibidores y para la máxima producción de azúcar por gramo de ozono gastado en el pretratamiento.

De los microorganismos testados, el único que no ha sufrido inhibición por los hidrolizados de bagazo ozonizado sin lavar ha sido la levadura *Saccharomyces cerevisiae* para la producción de etanol, pero proporciona un balance energético poco favorable. Todos los otros microorganismos testados fueron capaces de crecer en los hidrolizados de bagazo ozonizado-lavado, con los mejores balances energéticos para la producción de etanol por la levadura *Pichia stipitis*. A pesar del balance energético desfavorable en la producción de butanol por las dos *Clostridia* testadas, mejoras en las condiciones de fermentación y el aprovechamiento de los productos secundarios generados en las etapas intermedias podrían llevar a un proceso económicamente viable.

PERSPECTIVAS Y TRABAJOS FUTUROS

CAPITULO 9

A pesar de los grandes avances alcanzados en esta tesis doctoral, muchos aspectos quedan por resolver antes de la aplicación industrial de la ozonólisis al bagazo de caña de azúcar para producción de biocombustibles. La optimización de los parámetros de operación ha resultado en altos rendimientos de conversión de los polímeros de azúcares, por lo cual trabajos futuros deberían de ser enfocados a otras debilidades del proceso, de las cuales, se pueden destacar:

1. Optimizar la hidrólisis enzimática del bagazo ozonizado-lavado, buscando utilizar la menor cantidad de enzima con la mayor carga de bagazo, como forma de reducir los costes de esta etapa del proceso;
2. Concentrar los hidrolizados antes de los procesos de fermentación, ya que se ha visto que cuanto mayor la cantidad de azúcares iniciales, mejores los rendimientos;
3. Valorizar los azúcares lixiviados durante el proceso de lavado para la producción de compuestos de valor añadido;
4. Recuperar los ácidos producidos durante el pretratamiento, ya que la mayoría de ellos posee valor comercial;
5. Recuperar la lignina liberada y transfórmala en productos de valor añadido;
6. En el caso del butanol, recuperar el ácido butírico generado en las fermentaciones como un producto a más del proceso;
7. Utilizar los residuos de las fermentaciones como sustrato para producción de biogás, mejorando así el balance energético del proceso global.

Como continuación de este trabajo, será llevado a cabo un proyecto post doctoral dónde se estudiarán de forma integrada la producción de ácidos y azúcares empleándose la ozonólisis como pretratamiento. Se aprovechará el efecto observado

de degradación de la lignina por el ozono, para la producción de ácidos, manteniendo los hidratos de carbono para la producción de etanol y/o butanol.

El estudio será realizado de forma comparada para dos materias primas: el bagazo de caña de azúcar y la cascara de café. La primera materia prima se ha seleccionado debido a los resultados prometedores obtenidos en esta tesis doctoral, y la segunda debido a que es un residuo generado en grandes cantidades en muchos países y todavía muy poco explotado como materia prima lignocelulósica, con ningún trabajo publicado respecto a su ozonólisis.

Los experimentos serán desarrollados buscándose optimizar el pretratamiento para la producción de ácidos orgánicos a parir de la lignina, con el mínimo ataque a los carbohidratos. De esta manera, se espera producir un sólido pretratado que después de pasar por una etapa de lavado presentará alto contenido de polímeros de azúcares, mayoritariamente celulosa.

El sólido pretratado y lavado será sometido a hidrólisis y fermentación simultáneas para producción de etanol y butanol, intentando de esta manera trabajar con mayores concentraciones iniciales de sólido para obtención de mejores rendimientos. El líquido pretratado a su vez, será sometido a cuatro procesos distintos por separado, o de manera secuencial cuando convenga:

1. Electrodiálisis para separación de los ácidos orgánicos, y aprovechamiento secuencial del líquido resultante rico en xilosa para fermentación o producción de biogás (metano);
2. Recuperación de las fracciones de lignina presentes en el líquido de hidrolizado y su caracterización para buscar posibles aplicaciones de interés;

3. Utilización directa del líquido de lavado como sustrato para producción de biopolímeros polihidroxialcanoatos y polihidroxibutiratos.
4. Utilización directa del líquido de lavado como sustrato para producción de biogás (metano).

CURRICULUM VITAE

CAPITULO 10

PUBLICACIONES EN REVISTAS INTERNACIONALES

1. **Travaini, R.**, Barrado, E., Bolado-Rodríguez, S., 2016. Effect of ozonolysis parameters on generation of inhibitory compounds and on production of ethanol by *Pichia stipitis* and acetona-butanol-ethanol by *Clostridium* from ozonated and water washed sugarcane bagasse. Bioresour. Technol. In press.

2. **Travaini, R.**, Barrado, E., Bolado-Rodríguez, S., 2016. Effect of ozonolysis pretreatment parameters on the sugar release, ozone consumption and ethanol production from sugarcane bagasse. Bioresour. Technol. 214, 150–158.

3. **Travaini, R.**, Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodríguez, S., 2016. Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited. Bioresour. Technol. 199, 2–12.

4. Bolado-Rodríguez, S., Toquero, C., Martín-Juárez, J., **Travaini, R.**, García-Encina, P.A., 2016. Effect of thermal, acid, alkaline and alkaline-peroxide pretreatments on the biochemical methane potential and kinetics of the anaerobic digestion of wheat straw and sugarcane bagasse. Bioresour. Technol. 201, 182–190.

5. de Cassia Pereira, J., **Travaini, R.**, Paganini Marques, N., Bolado-Rodríguez, S., Bocchini Martins, D.A., 2016. Saccharification of ozonated sugarcane bagasse using enzymes from Myceliophthora thermophila JCP 1-4 for sugars release and ethanol production. Bioresour. Technol. 204, 122–129.

6. **Travaini, R.**, Otero, M.D.M., Coca, M., Da-Silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation. Bioresour. Technol. 133, 332–339.

CAPÍTULO DE LIBRO INTERNACIONAL

1. **Travaini, R.**, Marangon-Jardim, C., Colodette, J.L., Morales-Otero, M., Bolado-Rodríguez, S., 2015. Ozonolysis, in: Pandey, A., Negi, S., Binod, P., Larroche, C. (Eds.), Pretreatment of Biomass. Elsevier, USA, pp. 105–135.

CONTRIBUCIONES EN CONFERENCIAS

1. **Travaini, R.**, Barrado, E., Bolado, S. Sugarcane bagasse ozonolysis pretreatment – Hydrolysates fermentation by brewer's yeast Saccharomyces cerevisiae, Journal of Biotechnology, Volume 208, Supplement, 20 August 2015, Page S118. In: Eurobiotech 2015 – European Biotechnology Congress, Bucarest, Rumanía, 07-09 de mayo de 2015 (Presentación de póster).

2. **Travaini, R.**, Barrado, E., Bolado, S. Optimization of ozonolysis pretreatment for production of alcohols from sugarcane bagasse. In: 10th International Conference on Renewable Resources and Biorefineries, Valladolid, España, 4-6 de junio de 2014 (Presentación de póster).

3. **Travaini, R.**; Pereira, J. C.; Zavarizi, F.; Gomes, E., Bolado, S.; Bocchini Martins, D. A. Use of a cellulases cocktail from a new isolated thermophilic fungus for saccharification of sugarcane bagasse pretreated with ferric nitrate. In: 10th International Conference on Renewable Resources and Biorefineries, Valladolid, España, 4-6 de junio de 2014 (Presentación de póster).

4. Pereira, J. C, **Travaini, R.**, Gomes, E., Bocchini Martins, D. A., Bolado, S. Application of cellulase extract of the new isolated thermophilic fungus M*yceliophtora thermophila* JCP 1-4 on the saccharification of ozonated sugarcane bagasse. In: 10th International Conference on Renewable Resources and Biorefineries, Valladolid, España, 4-6 de junio de 2014 (Presentación de póster).

5. **Travaini, R.**; Pereira, J. C.; Zavarizi, F.; Bocchini Martins, D. A.; Gomes, E.; Bolado, S. R. Studies on the application of *Myceliophthora thermophila* JCP1-4 cellulases cocktail on sucargane bagasse pretreated by different methods. Journal of Biotechnology, Volume 185, Supplement, September 2014, Pages S122-S123. In: Eurobiotech 2014 – European Biotechnology Congress, Lecce, Itália, 14-18 de mayo de 2014 (Presentación de póster).

6. **Travaini, R.**, Leite; R. S. R., Da-Silva, R., Gomes, E., Bolado, S. *Penicillium viridicatum* RFC3, a new high β-glycosidase activity producer: a supplement of cellulases from *Trichoderma reesei* QM9414 for biomass saccharification, Current Opinion in Biotechnology, Volume 24, Supplement 1, July 2013, Page S139. In: Eurobiotech 2013 – European Biotechnology Congress, Bratislava, Eslováquia, 16-18 de mayo de 2013 (Presentación de póster).

7. Pereira, J. C., **Travaini, R.**, Gomes, E., Bolado, S., Bocchini-Martins, D. A. Enzymatic hydrolysis of ozone pretreated sugarcane bagasse with cellulases of a new isolated thermophilic fungus *Myceliophtora sp* JCP 1-4, Current Opinion in Biotechnology, Volume 24, Supplement 1, July 2013, Pages S140-S141. In: Eurobiotech 2013 – European Biotechnology Congress, Bratislava, Eslováquia, 16-18 de mayo de 2013 (Presentación de póster).

8. Zavarizi, F., Pereira, J. C., **Travaini, R.**, Bolado, S., Bocchini-Martins, D. A. Cellulolytic enzymes production by the newly isolated thermophilic filamentous fungus *Myceliophthora sp* DAMB 23-45, Current Opinion in Biotechnology, Volume 24, Supplement 1, July 2013, Page S141. In: Eurobiotech 2013 – European Biotechnology Congress, Bratislava, Eslováquia, 16-18 de mayo de 2013 (Presentación de póster).

9. **Travaini, R.**, Otero, M. D. M., Coca, M., Da-Silva, R., Gomes, E., Bolado, S. Evaluation of the ozone as a pretreatment for sugarcane bagasse enzymatic

hydrolysis In: 1st Brazilian BioEnergy Science and Technology Conference, Campos do Jordão, Brasil, 14-18 de agosto de 2011 (Presentación de póster).

10. **Travaini, R.**, Otero, M. D. M., Coca, M., Da-Silva, R., Gomes, E., Bolado, S. Chemical, structural and morphological changes on the bagasse pretreated with ozone In: 33st Symposium on Biotechnology for Fuels and Chemicals, Seattle, USA, 2011 (Presentación de póster).

11. **Travaini, R.**, Do-Amaral, L.A., Boscolo, M., Gomes, E., Da-Silva, R. Synergistic action of brute enzymatic extracts of *Thermoascus aurantiacus* CBMAI756 and *Thermomyces lanuginosus* on saccharification of sugarcane bagasse, Journal of Biotechnology, v. 150, p. 167, 2010. In: 14th International Biotechnology Symposium and Exhibition, Rimini, Italy, Rimini, Italia, 15-17 de noviembre de 2010 (Presentación de póster).

12. **Travaini, R.**, Boscolo, M., Gomes, E., Da-Silva, R. Synergistic action of *Trichoderma reesei* and *Penicillium viridicatum*'s enzymatic extracts on sugarcane bagasse for glucose production In: 32st Symposium on Biotechnology for Fuels and Chemicals, Clearwater Beach, USA, p. 121, 2010 (Presentación de póster).

13. **Travaini, R.**, Leite, R. S. R., Silva, D., Do-Amaral, L. R., Gomes, E., Da-Silva, R. Production of cellulases and hemicellulases by *Penicillium viridicatum* RFC3 on solid state fermentation In: 31st Symposium on Biotechnology for Fuels and Chemicals, San Francisco, USA, p. 133, 2009 (Presentación de póster).

14. Do-Amaral, L. R., **Travaini, R.**, Pinto, T. O. P., Gomes, E., Da-Silva, R. Comparative production and characterization of cellulolitic enzymes from thermophilic fungi *Thermoascus aurantiacus* CBMAI756 and *Thermomyces lanuginosus* In: 31st Symposium on Biotechnology for Fuels and Chemicals, San Francisco, USA, p.128, 2009 (Presentación de póster).

OTROS MÉRITOS

Estancia de Investigación:

1. En el Departamento de Ingeniería Química y Tecnología del Medio Ambiente, incorporado al Grupo de Investigación Tecnología de Procesos Químicos y Bioquímicos bajo la supervisión de la Prof. Dra. Silvia Bolado Rodríguez, Universidad de Valladolid - Valladolid, España, del 5 de septiembre al 5 de diciembre de 2010, con beca de movilidad académica internacional de la Asociación Universitaria Iberoamericana de Postgrado (AUIP, Salamanca, España), cuando estaba matriculado en el Máster en Química: Bioenergía, tratamiento y aprovechamiento de residuos, de la Universidade Estadual Paulista “Julio de Mesquita Filho”, campus de São José do Rio Preto, Brasil.

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1. Trabajo fin de grado. Alejandro Sánchez Calvete, 02/2016-07/2016. Producción integrada de etanol y metano a partir de bagazo de caña de azúcar pretratado mediante procesos térmicos. Grado en Ingeniería Química, Escuela de Ingenierías Industriales, Universidad de Valladolid.

2. Trabajo fin de máster. Edwar Tucto, 03/2014-07/2014. Producción de etanol a partir de bagazo de caña de azúcar por *Saccharomyces cerevisiae*: efecto de los inhibidores generados durante el proceso de pre-tratamiento. Máster en Ingeniería Ambiental, Escuela de Ingenierías Industriales, Universidad de Valladolid.

3. Trabajo fin de carrera en estancia de investigación. Franco Zavarizi, 04/2013-09/2013. Aplicación de celulasas producidas por un hongo filamentoso termófilo en la hidrólisis del bagazo de caña de azúcar in natura y sometido a pré-tratamiento térmico con nitrato férrico. Licenciatura en Química, Universidad Estadual Paulista "Julio de Mesquita Filho" – UNESP, Campus Araraquara, Brasil.

4. Trabajo fin de máster en estancia de investigación. Josiani de Cassia Pereira, 02/2013-07/2013. Evaluación de la influencia del pre-tratamiento por ozonólisis en la hidrólisis del bagazo de caña de azúcar con extracto enzimático producido por un hongo termófilo recién aislado. Máster en Biotecnología, Universidad Estadual Paulista "Julio de Mesquita Filho" – UNESP, Campus Araraquara, Brasil.

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   Trabajo fin de máster: Desarrollo de pre tratamientos y procesos bioquímicos para la producción de alcoholes de valor económico a partir de residuos lignocelulósicos. Tutora: Profa. Dra. Silvia Bolado Rodríguez.

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   Trabajo fin de máster: Utilización de los procesos de oxidación avanzada basados en ozono como pre tratamiento para el bagazo de caña, hidrólisis del bagazo tratado con enzimas combinadas de Penicillium viridicatum RFC3 y Trichoderma reesei QM9414. Tutor: Prof. Dr. Roberto da Silva.

3. 03/2005-12/2008: **Licenciatura en Química Ambiental** - Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, São Paulo, Brasil.
   Trabajo fin de carrera: Estudios sobre la producción y caracterización de enzimas celulolíticas y hemicelulolíticas producidas por Penicillium viridicatum RFC3 y Trichoderma reesei QM9414 y aplicación de los extractos enzimáticos en la hidrólisis del bagazo de caña. Tutor: Prof. Dr. Roberto da Silva.