UNIVERSIDAD MICHOACANA

DE SAN NICOLÁS DE HIDALGO

DIVISIÓN DE ESTUDIOS DE POSGRADO

FACULTAD DE INGENIERÍA QUÍMICA

APROVECHAMIENTO DE BAGAZO DE AGAVE PRETRATADO PARA LA PRODUCCIÓN DE BIOETANOL

TESIS PRESENTADA POR:

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PARA OBTENER EL GRADO DE:

DOCTOR EN CIENCIAS EN INGENIERÍA QUÍMICA

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MORELIA, MICHOACÁN AGOSTO 2018

Dedicatoria

Dedico esta tesis a Lupita que es mi compañera y apoyo incondicional, a Carlitos que es el motor que me impulsa a dar todo cada día para llevar a cabo esta aventura que culmina.

A los doctores Castro y Rutiaga ser la guía en esta travesía.

A mis sinodales por sus buenos consejos y paciencia.

A mis padres, hermanos y amigos.

A todos los que me apoyaron para escribir y concluir esta tesis.

Para ellos es esta dedicatoria de tesis, pues es a ellos a quienes se las debo por su apoyo incondicional.

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Asunto: Impresión de Tesis.

Oficio número 327/2018/2018.

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U.M.S.N.H.
Presente.-**

Por este medio nos dirigimos a usted de la manera más atenta para notificarle que después de haberle dado seguimiento al desarrollo de la tesis: “APROVECHAMIENTO DE BAGAZO DE AGAVE PRETRATADO PARA LA PRODUCCIÓN DE BIOTANOL” Después de haber revisado el manuscrito que presentó del alumno **Juan Carlos Farías Sánchez con matrícula 1232235B**, concluimos que cumple con los requisitos académicos y con lo establecido en el Reglamento General de Estudios de Posgrado para el desarrollo de tesis.

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RESUMEN

Los materiales lignocelulósicos constituyen una fuente de materia prima importante para la obtención de productos de amplia utilización. Dentro de estos materiales se encuentran el bagazo de agave, desecho de la industria del tequila. En este trabajo se probaron tres diferentes pretratamientos: alcalino-oxidativo, explosión de vapor con ácido y organosolv con etanolamina, seguidos de sacarificación enzimática como parte del proceso global de producción de bioetanol de segunda generación. Para cada uno de ellos se plantearon diseños experimentales que permitieron estudiar el efecto de las diferentes variables sobre la conversión de los materiales lignocelulósicos hacia azúcares fermentables. En la sacarificación o hidrólisis enzimática se utilizaron enzimas de última generación como son: Cellic Ctec3 y Cellic Htec3, las cuales se obtuvieron mejores rendimientos a una dosis sustancialmente menor en comparación con otras enzimas. Los resultados generados son sumamente atractivos ya que se lograron concentraciones altas de azúcares tales como: 165,67 g/l (59,29% glucosa, 29,05% de xilosa, 11,66% de fructosa) con el pretratamiento alcalino-oxidativo con 6% NaOH (% p/v), 6% de H_2O_2 (%p/v) y relación L/S de 5:1; 71,1 g/ con el ptetratamieto de explosión de vapor con ácido a 15.85 kg/cm^2 y 0.5% de ácido sulfúrico; 122.2 g/L con el pretratamiento organosolv con 50% de etanolamina, relación de L/S de 3:1, 160°C y 30 minutos. Finalmente por cada tonelada de bagazo se obtuvieron 286.21 kg de azúcares fermentables para organosolv, a la cual le sigue la explosión de vapor con 285.53 kg y por último el alcalino-oxidativo
con 265.81 kg.

Con estos resultados, se puede concluir que es viable la producción de bioetanol de segunda generación a partir del aprovechamiento de bagazo de agave. Además de que estos residuos no están siendo aprovechados e incluso generan contaminación al ambiente.

Palabras clave

Etanol, Lignocelulosico, pretratamiento, azúcares y fermentables.

ABSTRACT

Lignocellulosic materials are a source of important raw material for important products. Within these materials they find agave bagasse, a waste of the tequila industry.

In this work three different pretreatments were tested: alkaline-oxidative, steam explosion with acid and organosolv with ethanolamine, followed by enzymatic saccharification as part of the global second-generation bioethanol production process. For each of them, experimental designs were proposed that allowed study the effect of different variables on the conversion of materials lignocellulosics towards fermentable sugars. In saccharification or hydrolysis, enzymes were used, such as: Cellic Ctec3 and Cellic Htec3, which obtained better yields at a dose substantially lower compared to other enzymes. The results generated are extremely attractive since high concentrations of sugars such as: 165.67 g / l (59.29% glucose, 29.05% xylose, 11.66% fructose) with the alkaline-oxidative pretreatment with 6% NaOH (% w / v), 6% H2O2 (% p / v) and L / S ratio of 5: 1; 71.1 g / with the steam explosion ptetratamieto with acid at 15.85 kg / cm2 and 0.5% sulfuric acid; 122.2 g / L with pretreatment organosolv with 50% ethanolamine, L / S ratio of 3: 1, 160 ° C and 30 minutes.

Finally, for each ton of bagasse, 286.21 kg of fermentables sugar were obtained for organosolv, which is followed by steam explosion with 285.53 kg and finally the alkaline-oxidative with 265.81 kg. With these results, it can be concluded that the production of bioethanol from second generation from the use of agave bagasse. In addition to that these residues are not being used and even generate pollution to the environment.

Keywords

Ethanol, Lignocellulosic, pretreatment, sugars and fermentables.

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CAPITULO 1. INTRODUCCIÓN

El alcohol etílico, etanol o bioetanol es un producto químico obtenido a partir de la fermentación de los azúcares que se encuentran en los productos vegetales, tales como cereales, remolacha, caña de azúcar, sorgo o biomasa. Estos azúcares están combinados en forma de sacarosa, almidón, hemicelulosa y celulosa. Las plantas crecen gracias al proceso de fotosíntesis, en el que la luz del sol, el dióxido de carbono de la atmósfera, el agua y los nutrientes de la tierra forman moléculas orgánicas complejas como el azúcar, los hidratos de carbono y la celulosa, que se concentra en la parte fibrosa la planta. El bioetanol se produce por la fermentación de los azúcares contenidos en la materia orgánica de las plantas. En este proceso se obtiene el alcohol hidratado, con un contenido aproximado del 5% de agua, que tras ser deshidratado se puede utilizar como combustible.

Los materiales lignocelulósicos son los que ofrecen un mayor potencial para la producción de bioetanol, el uso de residuos de procesos agrícolas, forestales o industriales, con alto contenido en biomasa son ideales. Estos residuos pueden ir desde la paja de cereal a las "limpias" forestales, pasando por los Residuos Sólidos Urbanos (RSU) o las cáscaras de cereal o de arroz. Los residuos tienen la ventaja de su bajo coste, los cuales son la parte no necesaria de otros productos o procesos, salvo cuando son utilizados en la alimentación del ganado.

1.1 Bioetanol como combustible alternativo

A nivel mundial, aproximadamente 90% de la energía consumida proviene de fuentes no renovables, por lo que estos recursos fósiles se están agotando aceleradamente y su tasa de disminución es cada vez mayor. Por lo anterior, desde hace algunos años, distintas naciones han incursionado en la búsqueda de fuentes alternas de energía. Aproximadamente, la mitad de los ingenios del país cuentan con destilerías, unas más, otras menos modernas, pero pueden producir bioetanol (96° GL). (Velasco, 2009)

A pesar de que el bioetanol se ha utilizado mayoritariamente como base en la producción de bebidas alcohólicas, tales como cervezas, vinos, licores, etc., tiene además una serie de aplicaciones en la industria química, farmacéutica, y más recientemente como combustible para automóviles. El bioetanol es producido por la fermentación de granos tales como: trigo, cebada, maíz, madera y caña de azúcar, o por residuos forestales y DSM (papel, alimento y basura de patio, plásticos, maderas, llantas). El bioetanol es un alcohol combustible con potencial de llegar a ser ampliamente usado como combustible de automóvil.

El uso del bioetanol en la gasolina tiene varios beneficios ambientales; entre los que destacan (Castillo-Hernández y col., 2012, Torres y col., 2002)

- Reducción de CO: el uso de bioetanol causa un 25 a 30% de reducción en las emisiones de monóxido de carbono (CO) en una mezcla del 10% en volumen con gasolina.

- Reducción de CO_2: aunque el dióxido de carbono es liberado cuando se quema el bioetanol, éste es reciclado dentro de tejido orgánico durante el desarrollo de la planta; de hecho, el uso del bioetanol en la gasolina puede resultar en una reducción neta de los niveles de dióxido de carbono atmosférico.

- Fuente renovable: el bioetanol es un derivado de una fuente biológica renovable.

- Reducción de emisiones de ozono: el uso de 10% de bioetanol en la mezcla gasolina puede resultar en una reducción neta del ozono - que forma emisiones como monóxido de carbono e hidrocarburos.

El uso intensivo del bioetanol puede ser motivado por su habilidad para sustituir a la gasolina o utilizarlo como componente oxigenante de la gasolina y antidetonante.

- En vehículos de gasolina (90% gasolina y 10% bioetanol en volumen) gasoil, esto se practica sin ninguna modificación al motor.

- Bioetanol como sustituto de la gasolina. Una mezcla de 85 % bioetanol y 15% gasolina (E85) es un combustible viable para vehículos ligeros, éstos pueden operar con cualquier proporción de bioetanol mezclado con gasolina, teniendo como límite 85%. Algunos autobuses y camiones con la adecuada modificación a sus motores diesel, pueden operar con bioetanol casi puro.

- Como un componente de la gasolina. El bioetanol puede ser usado para manufacturar ETBE; (etil terciario butil éter) elemento para la gasolina reformulada. Al ser mezclado con la gasolina aumenta el octanaje de la gasolina y por lo tanto previene el golpeteo.

Existe también la opción de utilizar vehículos de combustible flexible, los cuales están diseñados para usar una gran variedad de combustibles, siempre y cuando estén en el mismo estado físico (es decir líquido).

El uso de un combustible en el autotransporte, requiere que sus características físicas y químicas sean las adecuadas para poder lograr una buena autonomía, buen desempeño y confiabilidad (Castillo-Hernández y col., 2012, Velasco, 2009).

1.2 Bioetanol en la actualidad

Dada la disminución de las reservas de materias orgánicas fósiles, en los últimos años en todo el mundo se dedica mucha atención al tratamiento químico y biotecnológico de la biomasa vegetal de madera y plantas agrícolas. La biomasa vegetal (fitomasa) se forma como resultado de la actividad fotosintética de las plantas. A diferencia de las fuentes fósiles, las reservas de fitomasa se renuevan como resultado de la actividad de las plantas superiores. Cada año en el planeta se forman cerca de 200 millones de toneladas de biomasa vegetal celulósica. La biosíntesis de la celulosa es la mayor de las síntesis en el pasado, presente y futuro. En este siglo los principales tipos de materiales orgánicos serán los productos lignocelulósicos y el carbón de piedra que a su vez es de origen vegetal. Por una valoración aproximada, las reservas conocidas de petróleo son prácticamente iguales, sin embargo, los recursos petroleros se agotan, al tiempo que los agrícolas, como resultado del crecimiento natural, se elevan (Jolkin, 1989)

En un futuro no lejano, nos espera el paso de la producción petroquímica a la elaboración química y bioquímica de bioetanol a partir del bagazo de agave y otros materiales vegetales. Por otro lado, entre los problemas más acuciantes que enfrenta la humanidad en el momento actual, ocupan un lugar prominente la explosión demográfica, con su incidencia cada vez mayor en la producción alimentaria y la crisis energética mundial, con la perspectiva sombría del agotamiento no lejano del combustible fósil.

1.3 Los residuos tequileros

Las tequileras generan residuos agroindustriales, los cuales pudieran ser aprovechados para obtener productos de interés industrial, entre estos desechos se encuentra el bagazo de Agave tequilana Weber. Estos esquilmos de los procesos de producción de tequila generando aproximadamente 1.05x10^8 kg de bagazo en México anualmente (Saucedo-Luna y col., 2010). Debido a que este material es rico en moléculas lignocelulósicas, puede ser fraccionado para posteriormente ser utilizado en la obtención de productos, tales como: alcoholes, ácidos orgánicos, compostas, biocombustibles, sustrato para la producción de hongos comestibles, alimento para ganado (ensilados) y en menor proporción, la extracción de otros metabolitos tales como: enzimas, compuestos fenólicos, biopolímeros y fibras solubles e insolubles (García y col., 2005)

1.4 Bagazo de Agave.

El bagazo es una fibra residual obtenida de la planta de agave utilizada para la producción de tequila y representa el 40% de la materia prima. De acuerdo a Saucedo-Luna y col., (2010), la producción anual de bagazo en México es aproximadamente 1.05x10^8 kg. De la cual, sólo una pequeña porción se utiliza para preparar abono y el resto es desechado. Por lo cual es evidente que esta situación en la actualidad está generando nuevas investigaciones para poder darle un mejor uso al bagazo de agave.

Hasta el momento se conoce que el material lignocelulósico que constituye al bagazo es considerado como fuente de energía renovable por su consumo en la producción biocombustibles y/o productos químicos, los cuales representan un gran potencial para suministrar energía (Saucedo-luna y col., 2010). Sin embargo la producción de estos es costosa ya que se requieren ataques enzimáticos específicos para su fermentación.

Tradicionalmente el bagazo de agave ha sido utilizado para la fabricación de ladrillos y colchones, pero para los grandes volúmenes que se generan este aprovechamiento no representa un impacto en la utilización de este material (Íñiguez y col., 2005).

1.5 Composición del Bagazo.

Uno de los principales componentes químicos que se ven implicados en el comportamiento mecánico y estructural del bagazo de agave, es el material lignocelulósico, que está constituido por un entramado de micro fibrillas de celulosa formando capas recubiertas de hemicelulosas y sobre las que se deposita la lignina

(Barroso Casillas, 2010). De acuerdo a Balam-Cocom y col., (2006), la fibra obtenida de diferentes tipos de agaves presenta una composición química distinta.

1.5.1. Celulosa

La celulosa es la macromolécula más abundante de la Tierra y su producción anual se ha estimado en 10^{11} toneladas (Brown, 2004). Este biopolímero es sintetizado por una gran cantidad de organismos vivos y, aunque la mayor parte de la celulosa procesada para la producción industrial proviene de plantas, algunas bacterias, cianobacterias, hongos y algas verdes también son capaces de sintetizarla (Nobles y col., 2001, Zogaj y col., 2001).

La celulosa es un polímero lineal de monómeros de D-glucosa, como se muestra en la Figura 1, que se unen por enlaces ß-(1→4) y cuyo peso molecular medio es de 100.000 (Zogaj y col., 2001). Se sintetiza en la naturaleza como moléculas individuales de D-glucosa que se van ensamblando en el mismo lugar en el que se producen. Las moléculas de celulosa se unen formando moléculas más largas conocidas como protofibrillas que se empaquetan en unidades mayores denominadas micro fibrillas. Las estructuras fibrilares están estabilizadas lateralmente por puentes de hidrógeno entre grupos hidroxilo intra e intermoleculares y todos los átomos de carbono están fijados en una posición concreta.

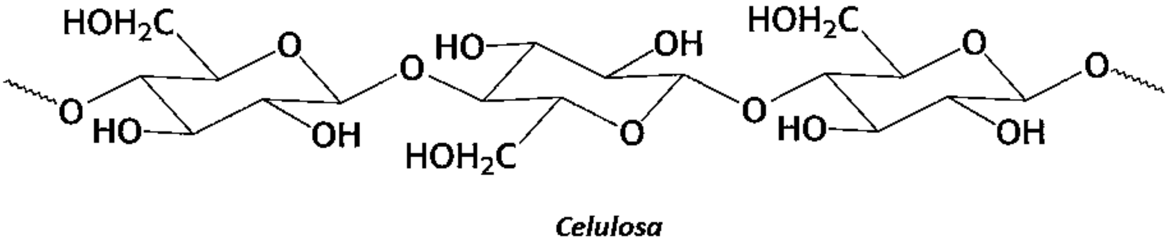

Figura 1. Estructura de celulosa

1.5.2. Hemicelulosa

Junto con la celulosa, la hemicelulosa está también presente en la pared celular de las plantas. Consta de polímeros formados por más de un tipo de azúcares tales como la D-xilosa, L-arabinosa, D-manosa, D-glucosa, D-galactosa, D-xilulosa, etc., y por varios ácidos urónicos, como el ácido glucurónico y galacturónico mostrados en la Figura 2. Su función principal es proporcionar la unión entre la celulosa y la lignina (Sun y col., 1998).

Generalmente está constituida por una cadena principal que puede consistir en una única unidad (homopolímero) p. ej. Xilanos, o estar formada por dos o más unidades (heteropolímero). De forma general, en función del azúcar dominante en la cadena principal, se pueden agrupar en: xilanos, mánanos, glucanos y galactanos (Fengel y Wegener, 1983). Dichas cadenas, a diferencia de la celulosa, pueden presentar ramificaciones y sustituciones mediante enlaces covalentes.

Existen diferencias en la composición y estructura de las hemicelulosas entre los diferentes tipos de biomasas. En las maderas latifoliadas el xilano es el polímero más abundante, está altamente sustituido por grupos acetilos y puede representar hasta un 30% del peso total (Dahlman y col., 2003, Fengel y Wegener, 1983). Por el contrario, el polímero más abundante en las hemicelulosas de las maderas coníferas es el galactoglucomanano y presenta mayor cantidad de unidades de manosa y galactosa que las maderas duras (Dahlman y col., 2003).

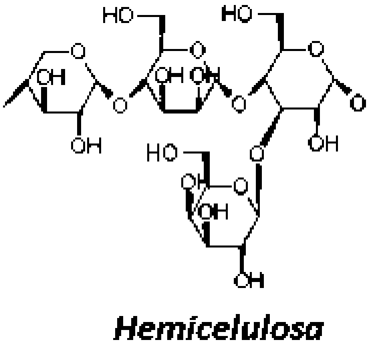

Figura 2. Estructura de hemicelulosa

1.5.3. Lignina

La lignina es el tercer componente mayoritario en los materiales lignocelulósicos, es por ello el tercer polímero natural más abundante en la naturaleza tras la celulosa y hemicelulosa (Buranov y Mazza, 2008). Es un polímero tridimensional amorfo cuyos monómeros básicos, llamados alcoholes cinamílicos, son: el alcohol ρ-cumarílico, el alcohol coniferílico y el alcohol sinapílico (Higuchi, 1990) que se muestran en la Figura 3. Estos tres alcoholes aromáticos dan lugar a unidades ρ-hidroxifenilo (H), unidades guayacilo (G), y unidades siringilo (S) respectivamente, cuya proporción también varía en las maderas duras, maderas blandas y biomasa herbácea (Evers y col., 1999, Fengel y Wegener, 1983).El contenido en lignina, al igual que en el caso de las hemicelulosas, difiere entre los diferentes materiales lignocelulósicos.

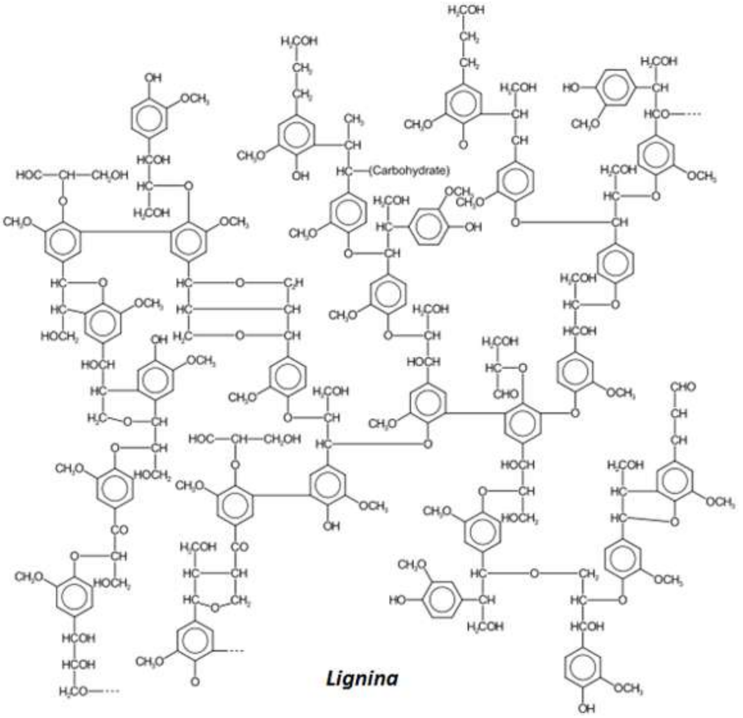

Figura 3. Estructura de lignina

1.6 Pretratamientos

La principal problemática que presentan los residuos lignocelulósico como el bagazo de agave y los lodos de papel son los pretratamientos para posteriormente llevar a cabo el procedimiento enzimático el cual está limitado por la presencia de lignina y la cristalinidad de la celulosa ((Millett y col., 1976); (Marten y col., 1996); (Åkerholm y Salmén, 2001); (McLean y col., 2002); (Schell y col., 2003)), por ello se han venido utilizando diversos procedimientos de pretratamiento, entre los que destacan el alcalino, el ácido, la explosión con vapor y otros como la explosión de vapor con amoniaco y organosolv (Marten y col., 1996); (Åkerholm y Salmén, 2001, Akerholm y Salmén, 2002). Cada uno produce cambios particulares en las materias primas; así, el pretratamiento con ácido diluido hidroliza principalmente a las hemicelulosas, dejando una estructura porosa formada principalmente por celulosa y lignina, la cual es más accesible a la acción de las enzimas (Wyman, 1994). La explosión con vapor rompe la estructura del material y aumenta la accesibilidad de la celulosa al ataque enzimático (Shimizu, 1991); (Gregg y Saddler, 1996), el pretratamiento con hidróxido de sodio (NaOH) rompe los enlaces lignina–carbohidrato, retira parte de la lignina y de las hemicelulosas, abre las estructuras del material, incrementa el área interfacial y reduce el grado de polimerización y cristalinidad de la celulosa, favoreciendo su sacarificación enzimática (Tuor y col., 1995), la explosión de vapor con amoniaco es eficiente para sustratos con poca lignina, logrando hasta el 90% de la hidrólisis de la celulosa y hemicelulosa, no se producen inhibidores ni se requiere que el material lignocelulósico sea triturado (Holtzapple y col., 1991), el proceso organosolv es usado para romper los enlaces lignina hemicelulosa (Thring y col., 1990). La biomasa lignocelulósica deberá ser pretratada para elevar los rendimientos (Wyman y col., 2005); no obstante, este es uno de los pasos más costosos, de manera que deberá prestársele particular atención a fin de ganar ventaja de esta operación (Martínez y col., 1997). Encontraron que es posible producir cambios muy similares en la composición química del sustrato lignocelulósico durante el pretratamiento, utilizando diferentes combinaciones de las principales variables de operación (temperatura, tiempo y concentración); entre los pretratamientos más usados y de mayores rendimientos se encuentra la explosión de vapor y la combinación de pretratamientos (Martínez y col., 1997).

1.6.1 Pretratamiento Alcalino-Oxidativo

Consiste en un primer paso alcalino, que solubiliza principalmente la hemicelulosa y hace que el material sea más accesible para ataque químico adicional, y una segunda etapa oxidativa (durante 24 h), que solubiliza y oxida la lignina a compuestos menos contaminantes. Todo el proceso se lleva a cabo a temperatura ambiente (20-30ºC) utilizando una baja concentración de productos químicos, dando como resultado un coste relativamente bajo y licores residuales que contenían sólo trazas de contaminantes peligrosos derivados de la lignina.

La oxidación en húmedo (WO) es un método de pretratamiento eficaz para el fraccionamiento de lignocelulosa en una fracción de hemicelulosa solubilizada y una fracción sólida de celulosa susceptible de hidrólisis enzimática y fermentación (Bjerre y col., 1996, McGinnis y col., 1983)

La química del pretratamiento puede involucrar la modificación química, despolimerización y/o solvatación, redistribución física de la lignina y hemicelulosa, y potencialmente alterar la cristalinidad de la celulosa (Banerjee y col., 2011). Diferentes tipos de pretratamientos físicos, químicos, fisicoquímicos y biológicos de la biomasa lignocelulósica han sido ampliamente estudiados para mejorar la producción de bioetanol. Todos estos métodos pueden hacer la lignocelulosa más accesible a la hidrólisis enzimática, donde la cristalinidad de la celulosa, el área superficial accesible, y la disponibilidad de lignina y hemicelulosa son los principales factores que afectan la hidrólisis. Sobre los diferentes métodos de pretratamiento, los químicos y termoquímicos son los más comúnmente efectivos e incluyen las tecnologías más promisorias para aplicaciones industriales (Alvira y col., 2010; Taherzadeh y Karimi, 2008).

1.6.2 Explosión de vapor

El material lignocelulósico se somete a temperaturas entre 190-230 ºC, mediante la inyección directa de vapor saturado, durante un intervalo de tiempo entre 1 y 10 minutos. Tras el tiempo de tratamiento, se somete el material a una rápida despresurización. El efecto del pretratamiento sobre la biomasa es una combinación de alteraciones físicas (desagregación y ruptura de las fibras) y químicas (despolimerización y rotura de enlaces). El efecto mecánico está causado por la rápida despresurización que provoca una evaporación del agua interna, creando unas fuerzas de corte que producen la separación de las fibras, principalmente de las regiones más débiles (celulosa amorfa). El efecto químico se debe a la hidrólisis de los restos acetilos de las hemicelulosas produciendo ácido acético, que a la temperatura del proceso, cataliza la hidrólisis de la hemicelulosa (autohidrólisis). Durante el tratamiento se destruyen parcialmente los enlaces lignina-carbohidrato. Como resultado, se obtiene un producto fibroso cuya celulosa es más accesible a la hidrólisis enzimática. La hemicelulosa se despolimeriza en mayor o menor medida dependiendo de las condiciones del tratamiento, siendo fácilmente recuperada por lavado. La lignina, prácticamente sin alterar puede ser extraída y utilizada con diferentes fines.

Las variables más importantes en el pretratamiento de explosión por vapor son la temperatura, el tiempo de residencia, el tamaño de partícula y la humedad (Duff y Murray, 1996).

La explosión por vapor ha sido reconocida como un método muy efectivo para el pretratamiento de maderas duras y residuos agrícolas. Este pretratamiento se ha aplicado a diferentes materiales como chopo (Ballesteros y col., 1998, Excoffier y col., 1991, Ramos y col., 1992), eucalipto (Ramos y col., 1992), pino (Ballesteros, 2001, Negro y col., 2003, San Martin y col., 1995), paja de arroz (Moniruzzaman, 1996), residuos herbáceos (Ballesteros y col., 2002), residuos oleícolas (Fernandez-Bolanos y col., 2001), bagazo (Martín y col., 2002) etc. Sin embargo, este pretratamiento se ha mostrado menos efectivo con las maderas blandas, debido a su estructura mucho más rígida y su mayor contenido en lignina. Además, el contenido de grupos acetilados es mucho menor que en las maderas duras, con lo que el proceso de autohidrólisis no ocurre en la misma medida. En el caso de las maderas blandas es deseable añadir un catalizador ácido. El ácido sulfúrico ha sido el más estudiado por su coste y efectividad (Nguyen y col., 2000, Tengborg y col., 1998, Torget y col., 1996). El SO_2 también ha sido empleado en numerosos trabajos (Clark y Mackie, 1987, Stenberg y col., 2000). Tiene la ventaja de no ser tan corrosivo como el sulfúrico y es fácil y rápido de introducir en el material.

Su principal desventaja es su alta toxicidad. Una diferencia muy importante que se produce al utilizar en el pretratamiento uno u otro catalizador es la diferente fermentabilidad de las fracciones obtenidas tras el pretratamiento. Así, el material obtenido tras el tratamiento con sulfúrico muestra una baja fermentabilidad, debido a las altas concentraciones de productos tóxicos que se forman.

Entre las ventajas del pretratamiento con explosión por vapor debe citarse que el tamaño de partícula del material requerido (15-30 mm) es considerablemente superior a los utilizados en otros pretratamientos, reduciéndose costos en la molienda (Ballesteros, 2001). Además, no emplea catalizadores ácidos (en el caso de las maderas duras) con lo que se reducen los efectos medioambientales.

Entre sus limitaciones se encuentran, la destrucción de una parte de los xilanos de las hemicelulosas, la incompleta rotura de la matriz lignina-carbohidratos y la generación de compuestos que pueden resultar tóxicos para los microorganismos empleados en el proceso de fermentación tras la hidrólisis enzimática. Debido a la formación de estos compuestos, el material pretratado debe ser lavado con agua para eliminar estos productos inhibitorios (McMillan, 1994).

Para maximizar la recuperación de azúcares en el pretratamiento de explosión por vapor se requieren diferentes condiciones de pretratamiento para la recuperación de los azúcares celulósicos y hemicelulósicos. Algunos autores (Stenberg y col., 1998, Stenberg y col., 1998) han sugerido un pretratamiento en dos etapas empleando sulfúrico en condiciones suaves en una primera etapa, seguido de una impregnación con SO_2 bajo unas condiciones más severas. De esta forma es posible conseguir un incremento de un 7% en el rendimiento global de azúcares después de la hidrólisis enzimática comparándolo con los procesos en una etapa.

La explosión por vapor en dos etapas tiene una serie de ventajas como son, mayor rendimiento en bioetanol, mejor aprovechamiento de la materia prima y menor consumo de enzimas en la fase de hidrólisis. Sin embargo, es necesario una evaluación económica para determinar si estas ventajas justifican una explosión a vapor adicional (Galbe y Zacchi, 2002).

1.6.3 Pretratamiento Organosolv

El pretratamiento Organosolv es uno de varios pretratamientos investigados y utilizados hoy en día para diferentes variedades de biomasa lignocelulósica (Balat, 2011). Este pretratamiento emplea disolventes orgánicos, principalmente alcoholes primarios, o sus soluciones acuosas, que pueden recuperarse y reutilizarse posteriormente para extraer lignina de la biomasa (Pan y col., 2005). El desarrollo del proceso de organosolv se puede remontar a la Universidad de Pensilvania ya la Compañía General Electric en los años 70 para hacer un biocombustible limpio para generadores de turbina (Pan y col., 2005). Comúnmente el pretratamiento de organosolv es conducido a altas temperaturas (185-210 °C) sin necesidad de acción de ácido como catalizador porque el ácido liberado de la biomasa actúa como catalizadores para la escisión del complejo carbohidrato-lignina (Duff y Murray, 1996). Las reacciones asociadas con el procedimiento del alcohol organosolvido son la hidrólisis del enlace inter-nal en las ligninas así como las uniones de lignina-hemicelulosas (éter y enlaces de éster de ácido 4-O-metilglucorónico a los carbonos de las unidades de lignina), hidrólisis del enlace glicosídico en hemicelulosas y en menor medida en la celulosa y la deshidratación catalizada por ácido de los monosacáridos en furfural y 5-hidroximetil furfural seguidos por las reacciones de condensación entre la lignina y los aldehídos activos (Zhao y col., 2009). Cuando se utilizan ácidos minerales tales como ácido sulfúrico, clorhídrico y fosfórico como catalizador para el pretratamiento de organosolv, se acelera la deslignificación y degradación de la hemicelulosa (Zhao y col., 2009).

1.7 Hidrólisis de carbohidratos

La hidrólisis es una reacción química, catalizada por enzimas o por compuestos químicos, como el ácido sulfúrico, que tiene como objetivo convertir a los polisacáridos complejos en moléculas de azúcares simples como se observa en la Figura 4. La hidrólisis del enlace glucosídico ocurre cuando una molécula de agua se disocia y el hidrógeno liberado del agua se une al oxígeno del extremo de una de las moléculas de azúcar y el OH se une al carbono libre del otro residuo de azúcar. El resultado de esta reacción, es la liberación de monosacáridos y/o oligosacáridos de menor peso molecular si se trata de un polisacárido complejo (Vázquez-Contreras, 2003).

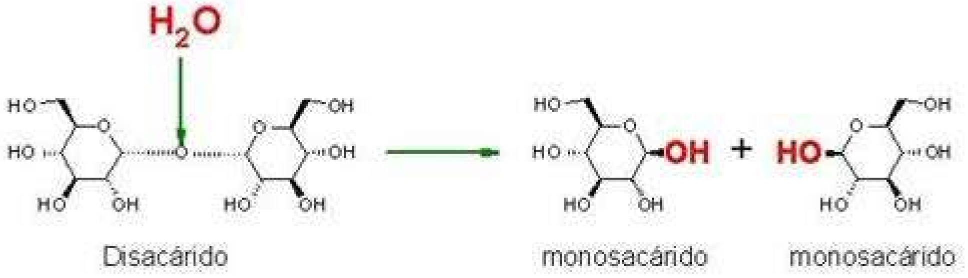

Figura 4. Esquema característico del mecanismo de hidrólisis.

1.8 Fermentación alcohólica

Se puede definir como una serie de reacciones químicas llevadas a cabo por microorganismos en condiciones de anaerobiosis para la conversión de azúcar en alcohol y dióxido de carbono. El mecanismo de la fermentación fue cuantificado por primera vez por Gay-Lussac (Figura 5). Basándose en la estequiometria de la conversión de una hexosa en bioetanol y anhídrido carbónico, el rendimiento teórico obtenido es de 51.1 kilogramos de bioetanol + 48.9 kilogramos de anhídrido carbónico por 100 kilogramos de hexosa (Bu'lock y Kristiansen, 1987).

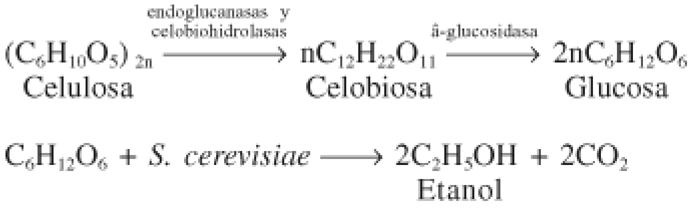

Figura 5. Esquema característico del mecanismo de hidrólisis.

Este rendimiento se denomina coeficiente de Gay-Lussac y representa el dato básico para el cálculo de la eficiencia de conversión. Posteriormente Pasteur determinó experimentalmente que solo es posible alcanzar el 94.19% del rendimiento teórico (Bu'lock y Kristiansen, 1987) esto es el 48.39 kg de bioetanol/100 Kg de hexosa o 61.31 L de bioetanol absoluto/100Kg de hexosa fermentada (Tabla 1).

Tabla 1. Rendimiento de bioetanol ideal según Pasteur (%).

Bioetanol	**48.4**
Anhídrido carbónico	46.6
Glicerol	3.3
Ácido succínico	0.6
Materia Celular	1.2
Total	100

1.8.1 Microorganismos utilizados en la fermentación alcohólica

La síntesis de bioetanol por vía microbiana tradicionalmente ha sido llevada a cabo por levaduras, siendo la más utilizada *Saccharomyces cerevisiae*. Sin embargo, ha sido reportado en la literatura la producción de bioetanol por *Zymomonas mobilis* bajo diferentes condiciones, resultando en procesos altamente ventajosos comparado con *S. cerevisiae*.

Zymomonas mobilis es una bacteria gram negativa, anaerobia facultativa. Se encuentra predominante como parte de la flora natural en fermentación de las savias, por ejemplo el agave, la palma y la caña de azúcar, donde están presentes altas concentraciones de glucosa; así como en las cervezas, peras y las sidras, donde actúa como flora de los desechos (Coton y col., 2005),

comparado con *S. cerevisiae* y otras levaduras, se ha reportado que puede producir bioetanol con rendimientos hasta cinco veces mayores por la vía Entner-Doudoroff (Figura 6) bajo condiciones anaerobias (Tao y col., 2005). Produciendo 1 mol de ATP por mol de glucosa, a diferencia de la vía Embden-Meyerhoff-Parnas en S. cerevisiae (Figura 7), que produce 2 mol de ATP por mol de glucosa, este decremento permite que más energía sea usada para la formación de bioetanol, (Davis y col., 2006, Wilkins, 2009). Además, *Zymomonas mobilis* tiene la capacidad de crecer en un medio con concentraciones de bioetanol por arriba de 13% (p/v). Esta tolerancia al bioetanol se debe a la alta cantidad de hopanoides en la membrana citoplasmática (30 mg g-1 de peso seco celular). Sin embargo, su capacidad catabólica se limita a pocos sustratos como la glucosa, fructosa y sacarosa, y este exigente microbio requiere estrictamente el nitrógeno orgánico o medios semi-definidos con minerales.

Dentro de los factores más críticos que contribuyen a un aumento de los rendimientos de producción de bioetanol por *Z. mobilis*, se encuentran la concentración de sustrato (sacarosa, glucosa o fructosa), temperatura, velocidad de agitación, pH, concentración de inóculo, tiempo de fermentación, y algunas sustancias nutritivas y compuestos minerales como extracto de levadura, $(NH_4)_2SO_4$, $MgSO_4$, fosfato monopotasio, $FeCl_3$ (Ruanglek y col., 2006).

$$\text{Glucosa (C6)} + \text{ADP} + \text{NAD} + \text{NADP}^+ \Rightarrow 2\ \text{piruvato (C3)} + \text{ATP} + \text{NADH} + \text{NADPH} + 2\text{H}^+$$

Figura 6 Ruta metabólica de Entner-Doudoroff

Los NADH (poder reductor) se dirigen a la cadena transportadora de electrones, produciendo por cada mol de NADH 1.5 mol de ATP α-D-glucosa + 2NAD+ + 2ADP + 2Pi ==> 2(piruvato) + 2NADH + 2ATP + 2H+ + $2H_2O$

Figura 7. Ruta metabolica vía de Embden-Meyerhof

1.9 Sacarificación y fermentación simultánea

En los procesos en una etapa la hidrólisis y fermentación simultánea (HFS) se realizan en el mismo reactor. La principal ventaja de estos procesos, es que se reduce la inhibición por producto final que se produce en la operación en dos etapas HFS, ya que la presencia de microorganismos fermentadores, junto con las enzimas celulolíticas reducen la acumulación de azúcar en el fermentador. Por ello se consiguen mayores tasas de hidrólisis que en el proceso de hidrólisis y fermentación por separado, necesitándose una menor cantidad de enzimas y obteniéndose como resultado un aumento de los rendimientos de bioetanol (Ballesteros, 2001).

Otra ventaja es que se realiza en un mismo reactor con lo cual se reducen los costos de inversión. Las principales desventajas de este proceso son las diferentes condiciones óptimas de pH y temperatura de la etapa de hidrólisis y fermentación. Por ello es necesario realizar el proceso en unas condiciones que sean compatibles con ambas etapas. Puesto que la temperatura óptima de hidrólisis está próxima a los 50 ºC y que las levaduras productoras de bioetanol convencionales trabajan en torno a los 37 ºC, es aconsejable la utilización de microorganismos termo tolerantes cuando se quieran realizar procesos de obtención de bioetanol en una sola etapa; En términos productivos, el tiempo de operación es mucho menor (50% menos) que la HFS, ya que la etapa de hidrólisis y de fermentación toman, por separado, 6 días, lo que se reduce a 3 días, en el caso de la SFS. Las altas concentraciones de bioetanol que pueden estar presentes en el medio, evitan la presencia de otros Microorganismos (MO) contaminantes (Tomas-Pejo y col., 2008), lo que contribuye al rendimiento final de bioetanol.

Los procesos en una etapa se pueden dividir en dos grupos: procesos en los que el mismo microorganismo produce los enzimas y realiza la fermentación, proceso conocido como conversión directa por el microorganismo (CDM); y procesos sacarificación y fermentación simultánea (SFS), en los que se emplean celulasas provenientes de un microorganismo celulolítico (normalmente un hongo), junto con la presencia de un microorganismo productor de bioetanol.

En los procesos CDM se pueden emplear monocultivos, en los que un único microorganismo hidroliza y fermenta los azúcares a bioetanol. Se han empleado bacterias del género *Clostridium* (Zeikus 1980, Ng y col. 1981, Ahring, J. et al. 1996, Lynd 1996). Los rendimientos en bioetanol son bajos debido a la formación de subproductos, a la baja tolerancia del microorganismo al bioetanol y al crecimiento limitado del microorganismo en los hidrolizados debido a la existencia de productos tóxicos. También pueden emplearse co-cultivos, en los que se utilizan dos microorganismos. Uno de ellos realiza la hidrólisis de la celulosa (*Clostridium thermocellum)* y una bacteria bioetanologénica fermenta los azúcares producidos (Zeikus y col. 1983). En la actualidad el proceso de SFS es el que ofrece las mejores expectativas. Las celulasas provienen de hongos celulolítico, normalmente *Trichoderma reesei,* y el microorganismo fermentador es una levadura o bacteria como *Zymomonas mobilis o Saccharomyces cerevisiae*.

2. ANTECEDENTES

Existen varias investigaciones sobre el tema pero están relacionadas y enfocadas para obtener bioetanol a partir de fermentación de azucares como caña de azúcar y granos comestibles que son los más ampliamente difundidos y conocidos desde la antigüedad, pero estos compiten con el consumo humano encareciendo el grano y por lo tanto también la alimentación humana.

El empleo de los biocombustibles data desde hace varias décadas a nivel internacional, pues fueron los combustibles de los primeros motores; sin embargo, su uso se abandonó con la aparición de las gasolinas. Actualmente, se emplean como oxigenantes de las gasolinas o como sustitutos de éstas (Bravo, 2007). En México ha comenzado el desarrollo en la producción de biocombustibles, aunque los proyectos que en la actualidad existen en la materia son pocos y a pequeña escala, es decir, sólo para satisfacer necesidades de autoconsumo de algunas empresas y procesos locales; lo que representa una desventaja ante países como Brasil, quién lleva más de treinta años aplicando una política de bioenergía tendiente a lograr la seguridad energética, emprendiendo acciones para el fomento de investigación, tecnología y operación (Infocana, 2008).

En México, sólo 9.5% de la oferta total de energía es renovable, mientras que en Brasil 38.7% de su energía es de fuentes renovables. Además, habría que aclarar que la poca energía renovable que se produce en México, a diferencia de Brasil, es fundamentalmente hidráulica, solar y eólica, pero su utilización es limitada hasta el momento.

En México se ha trabajado en las siguientes normas sobre biocombustibles: Ley para el Fomento de la Caña de Azúcar, Iniciativa de Ley de Desarrollo y Promoción de los Bioenergéticos e Iniciativa de Ley para el Fomento de las Energías Renovables (SENER, 2013).

En Abril del 2011, M&G y Chemtex comenzó con la producción de una planta con una producción de 13.4 Mega Galones por año de bioetanol lignocelulósico. En esta planta, ubicada en Cresentino, Italia, se generan 15 Mega Watts de “energía verde” de la lignina residual del proceso de bioetanol, esto a partir de un proceso de torrefacción de residuos y la quema en las centrales cogeneradoras de electricidad (Chiaramonti, 2013).

3. JUSTIFICACIÓN

Los materiales lignocelulósicos constituyen una fuente de materia prima importante para la obtención de productos de amplia utilización. Dentro de estos materiales se encuentran el bagazo de agave que resultan desechos de la industria del tequila. En el mundo se desarrollan cada día nuevas tecnologías para dar un uso racional a estos residuos, que además contribuyen con su acumulación a la contaminación del entorno. En el presente trabajo se exponen la viabilidad del proyecto de “aprovechamiento de bagazo de agave para la producción de bioetanol” ya que estos residuos no están siendo aprovechados e incluso generan contaminación al ambiente.

4. HIPÓTESIS

Los residuos agroindustriales, entre ellos el bagazo de agave de la industria tequilera, es factible para la conversión a azúcares fermentables para su posterior obtención de bioetanol, después de un apropiado pretratamiento e hidrólisis enzimática.

5. OBJETIVO GENERAL

Aprovechar el bagazo de agave de la industria del tequila, para maximizar la obtención de azúcares con diversos pretratamientos, seguido de un método de sacarificación y fermentación para obtención de productos de valor agregado como bioetanol.

6. OBJETIVOS PARTICULARES

Objetivo 1. Determinar la composición química del bagazo de agave.

Objetivo 2. Selección de los pretratamientos para la transformación del bagazo de agave en azúcares fermentables.

Objetivo 3. Identificar las variables que influyen en el rendimiento de azúcares fermentables en la hidrólisis enzimática de bagazo de agave pretratado.

Objetivo 4. Obtener las condiciones óptimas para la producción de azúcares fermentables por hidrólisis enzimática del bagazo de agave pretratados.

Objetivo 5. Obtención de bioetanol por sacarificación y fermentación simultánea.

7. MATERIALES Y MÉTODOS

7.1 Materiales

7.1.1 Material lignocelulósico

Bagazo de agave obtenido de la industria mezcalera “Ex hacienda Zacapendo” localizado en el municipio de Indaparapeo, Michoacán México, con tamaño de partícula malla 40.

7.1.2 Enzimas

Se utilizan enzimas comerciales que incluyen Cellic CTec3 de Novozymes (ß-glucosidasas y Hemicelulasas) y Cellic HTec3 (Endoxylanase).

7.1.3 Microorganismos fermentativos

Se emplea *Saccharomyces cerevisiae* como levadura fermentativa para bioetanol.

7.2 MÉTODOS

7.2.1 Caracterización química de biomasa Inicial

Determinación de la composición química del bagazo de agave en cada una de las etapas del proceso: biomasa cruda, biomasa pretratada y biomasa hidrolizada. Se determina, % de humedad (AOAC, 1990), % de cenizas (TAPPI, 2000), % de lignina (Runkel y Wilke, 1951), % de extraíbles (extracciones sucesivas con cuatro solventes: ciclohexano, acetona, etanol y agua), % de holocelulosa (Wise y col., 1946) y % alfa celulosa (ASTM, 1977).

7.2.1.1 Porcentaje de humedad

Se determina colocando 5 gramos de muestra (en un crisol previamente secado a 105°C), en una estufa de secado a 105 °C por 5 horas y trasladando a un desecador para su enfriado por 30 minutos y su posterior pesado (AOAC, 1990).

7.2.1.2 Contenido de cenizas

Se efectúa de manera gravimétrica, después de quemar cuidadosamente las muestras en las placas de calentamiento, para posteriormente calcinar en una mufla a 525 ºC utilizando crisoles de níquel, esto de acuerdo a la norma T 211 om-93 (TAPPI, 2000).

7.2.1.3 Determinación lignina

El contenido de lignina presente en la madera se desarrolla siguiendo la técnica Runkel y Wilke (1951), en la cual se procede agregando ácido sulfúrico al 72 % y ácido bromhídrico al 40 % a la muestra, agitándola y dejándola reposar por 2 horas, posteriormente se le agregaron 100 ml de agua destilada y se deja hervir por 5 minutos, finalmente se filtra en embudos Buchner y se lavan las muestras en repetidas ocasiones para no tener residuos de ácido. Para finalizar se lleva a peso constante en una estufa a 105 °C (Runkel y Wilke, 1951).

7.2.1.4 Determinación de holocelulosa

Se colocan las muestras en matraces Erlenmeyer adicionando una solución de clorito de sodio y ácido acético, manteniendo los matraces tapados y en reacción en baño de agua a 75ºC. Añadiendo cada hora, los químicos señalados, por un periodo de 4 horas. Después se vierten las muestras en filtros de fondo poroso, se procedieron a lavar en repetidas ocasiones con agua destilada fría y acetona, finalmente se llevaron las muestras a una estufa a 40°C hasta peso constante (Wise y col., 1946).

7.2.1.5 Determinación de alfa celulosa

Se efectúa siguiendo la técnica ASTM-D 1103-60 (1977), en la cual se agrega hidróxido de sodio al 17.5 % y una solución de sosa al 8.3 % dejándola reposar por un lapso de 2 h. Después de esto, las muestras se colocan en vasos de vidrio porosidad media para en estos hacer una lavado y filtrado. Una vez terminada esta etapa se pasaron a la estufa a 105 °C por 8 horas.

7.2.2 Pretratamientos para la transformación del bagazo de agave y los lodos de la industria papelera en azúcares fermentables.

7.2.2.1Pretratamiento Alcalino-Oxidativo

El material seco y molido se trató con diferentes combinaciones de reactivos, las cuales se estudiaron en un diseño factorial 2^3 utilizando como limites superior e inferior: 2-10 de NaOH (% p/v), 2-10 de H_2O_2 (% p/v). En todos los casos se utilizaron 10g de bagazo seco y molido, se colocaron en un matraz Erlenmeyer de 500 mL, se le agregaron 100 mL de hidróxido de sodio a la concentración especificada por el diseño. Se trató dicha mezcla a 121°C en autoclave por 60 minutos. Transcurrido ese tiempo se dejó enfriar a temperatura ambiente y se le agregaron 100 mL de la solución de peróxido de hidrogeno especificada por el diseño. Esta mezcla se dejó reposar por 24 horas a 30°C en una estufa bacteriológica marca Precision; Una vez transcurrido ese tiempo, se acidificó la mezcla con ácido clorhídrico concentrado (al 36.9%) hasta un pH de 5.0, para el proceso de hidrólisis, la materia prima se almaceno húmeda para evitar los cambios de estructura por efecto del secado (Luo y Zhu, 2011). A cada uno de los materiales resultantes de los pretratamientos se les determinó su contenido de lignina por el método antes mencionado.

7.2.2.2 Pretratamiento con explosión de vapor y ácido

La materia prima se secó a 80 °C y sin reducción de tamaño o molienda, se pretrató usando explosión de vapor acidificada en un equipo con un volumen de 1300 ml diseñado para una presión de trabajo de 16 kg/cm^2. Aplicando un diseño experimental de superficie de respuesta con 2 puntos estrella para la experimentación con el bagazo de agave proveniente de la industria.

Los valores usados en el diseño fueron altos de 0,5% de H_2SO_4, relación líquido/sólido 4, presión 15,85 kg/cm^2 y bajo de 0,1% de H_2SO_4, líquido/sólido relación 2, presión 6,3 kg/cm^2) se introducen los valores de los datos experimentales en el programa Software Statgraphics Centurion Versión XV (Statistical Graphics Corp., Manugistics, Rockville, MD), para el diseño de superficie de respuesta de con 2 puntos centrales. Se utilizó para la experimentación una muestra de 40 g de bagazo seco, se colocó en un matraz Erlenmeyer de 500 ml, impregnando la muestra durante 24 h con ácido sulfúrico en la proporción dada por el diseño. Después del periodo de 24 h, la mezcla fue tratada en el equipo de explosión por vapor a la presión indicada por el diseño, después, una descompresión súbita el sistema, el bagazo se enfrió, se detoxificó con hidróxido de calcio, y se almacenó para su uso posterior en la hidrólisis enzimática; la materia prima previamente tratada fue utilizada en húmedo para evitar cambios en la estructura por el efecto de secado de acuerdo con Luo y Zhu, (2011).

7.2.2.3 Pretratamiento organosolv con etanolamina

Se pesan muestras de 10 g de biomasa seca, molida y libre de extraíbles, se coloca en un recipiente cilíndrico de teflón con una capacidad de 100 ml, se le adiciona una mezcla de etanolamina-agua con una relación L/S dada por el diseño taguchi L9 de la Tabla 14; este recipiente se introduce en otro metálico el cual a su vez se calienta en un baño de aceite para mantener una temperatura (°C) constante y tiempo (min) también dada por el diseño. Transcurrido este tiempo se sumerge en un baño de aceite frio, se abre, y al material pretratado en su interior se le adicionan 20 ml de agua, se filtra en un embudo Buchner con papel Wathman del número 5, se seca y pesa. Las muestras pretratadas se almacenan a 4 °C en recipientes de vidrio herméticamente cerrados.

7.2.3 Identificar las variables que influyen en el rendimiento de azúcares reductores en la hidrólisis enzimática de bagazo de agave pretratado.

Toda la materia prima resultante de los pretratamientos se somete a la hidrólisis enzimática, en la cual se utilizaron las enzimas Cellic ctec 3 y Cellic htec 3 a razón de 6% (el proveedor Novozymes recomienda el uso de la enzima 6% en biomasa seca) de biomasa seca. A una muestra de 2 gramos de biomasa pretratada se le agrega la solución de la enzima, y un tampón 0,1 M de acetato a pH = 5,0 en una proporción de 1:5. Esta mezcla se mantiene en agitación durante 96 horas a 50 °C con 150 rpm en un agitador orbital Polyscience. Al final de este período la muestra se filtró, se lavó y se secó el residuo sólido. El sobrenadante se analizó usando una técnica estándar en un HPLC Varian Pro star usando una columna Metacarb 87 C a 65 °C y un detector de índice de refracción a 50 °C con flujo de agua isocrática a 0.6 ml/min para determinar azúcares simples

7.2.3.1 Azúcares reductores e hidrólisis enzimática

7.2.3.1.1 Determinación de azúcares reductores

Se colocan 3 ml de reactivo DNS y 1 mL de muestra en un tubo de ensayo de 25 ml, se homogenizo la solución y se calienta en un baño María a 100°C, durante 5 minutos, inmediatamente después se enfría la solución en un baño de agua fría durante 10 minutos. Se determina la absorbancia a 540 nm. El blanco utilizado se somete a las mismas condiciones de reacción de la muestra. El resultado se lee en una curva patrón de glucosa previamente realizado.

7.2.3.1.2 Curva patrón de glucosa

Se preparan 50 mL de glucosa con una concentración de 5 g/L, posteriormente se preparan diluciones (Tabla 2), agregando buffer de acetatos con una concentración 0.1 molar preparado con anterioridad. Se determina el contenido de azúcares reductores, utilizando el método antes descrito. Se elabora la curva patrón (Tabla 3), se grafica las absorbancias vs las concentraciones de glucosa a 540 nm, se obtiene la ecuación que relaciona los dos parámetros.

Tabla 2. Diluciones para la curva patrón de glucosa.

Solución stock (mL)	Buffer acetatos (mL)	Concentración en g/L de glucosa
0.1	0.9	0.5
0.2	0.8	1
0.3	0.7	1.5
0.4	0.6	2
0.5	0.5	2.5
0.6	0.4	3
0.7	0.3	3.5
0.8	0.2	4
0.9	0.1	4.5
1	0	5

7.2.3.1.3 Determinación de la actividad enzimática del extracto comercial.

Se determina la actividad de Cellic CTec3 de Novozymes. La actividad enzimática es la potencia requerida para liberar 1 μm de celulosa por minuto y se determina por el método del papel filtro (Mandels y Sternberg, 1976), llamado así ya que usa papel filtro como sustrato para la medición de la actividad celulósica. La unidad internacional de medición de la actividad enzimática utilizada para este método, es conocida como unidad de papel filtro (UPF). Los azúcares reductores se determinaron por el método DNS y HPLC antes mencionado.

La actividad enzimática del extracto se calcula mediante la siguiente ecuación:

$$FPU = \frac{Concentración\ de\ glucosa\ (mg)}{\left(0.18\frac{mg}{min}\right) * (0.5\ mL) * (60\ \min)} unidades\ mL^{-1}$$

(Ec. 1)

7.2.3.1.4 Hidrólisis enzimática

Se pesa 2 gramo de las muestras pretratadas, se colocan en recipientes de plástico de 20 ml, se mezclaron con 4 ml de azida de sodio al 0.1% m/v y el volumen requerido de solución enzimática (Cellic CTec3 y Cellic HTec3 de Novozymes) hasta obtener las UPF/g de celulosa óptimas calculadas con anterioridad. Se ajusta el volumen de reacción a 10 ml con buffer de acetatos 0.1M a un pH de 5, Se incuban a 50°C y 150 rpm durante 72 horas. Se hace una cinética tomando 100 µl de sobrenadante cada 12 horas, se le agregaron 3 ml de reactivo DNS, se calientan a ebullición durante 5 minutos y se lee la absorbancia a 540 nm. Transcurridas las 72 horas se toma una muestra de 2 ml de sobrenadante, se centrifuga a 5000 rpm durante 10 minutos, se toman 100 µl de la muestra centrifugada y se repite el procedimiento anterior para azúcares reductores. El contenido de azúcares se determinó por medio de la curva de calibración realizada con anterioridad.

7.2.3.1.5 Cinética de hidrólisis enzimática

La materia prima pretratada en las condiciones dadas se somete a hidrólisis enzimática por incubación a 50°C, 150 rpm y una carga enzimática apropiada durante 0, 4, 8, 12, 24, 48, 72, 96, 168, y 200 h. Se determina su contenido de azúcares utilizando el método del DNS (Chaplin y Kennedy, 1994) y/o HPLC antes mencionado.

Los resultados obtenidos permiten seleccionar las condiciones de pretratamiento y tiempo de hidrólisis enzimática que producen la concentración más alta de azúcares reductores.

7.2.4 Obtener las condiciones óptimas para la producción de azúcares reductores por hidrólisis enzimática del bagazo de agave pretratado

Evaluar la participación de las variables temperatura, tiempo y concentración de ácido o álcali mediante un método estadístico de optimización como el Diseño Central Compuesto con 2 puntos intermedios y 1 replica, el diseño Taguchi L9 o factoriales, variando los factores de concentración de ácido o álcali, temperatura y tiempo. Se identifica el orden de ejecución y se corren los experimentos, se analizan estadísticamente los resultados y con éste modelo se obtienen las condiciones óptimas de proceso.

El material pretratado optimo se somete a hidrólisis enzimática, empleando para ello Cellic Ctec3 y Cellic Htec3 durante 72 horas y 50 °C de temperatura.

7.2.5 fermentación para obtención de bioetanol

Se realiza el proceso de fermentación en las condiciones óptimas del proceso previamente determinadas. Para ello se utiliza la cepa de *Saccharomyces cerevisiae.* Esta se conserva útil en medio YPD (levadura, peptona, glucosa) a un pH de 5.0. El inóculo se prepara con 100 ml de medio YPD, inoculado e incubado a 30°C durante 12 horas. En el caldo producido se determina la cuenta total de levaduras en cámara de Neubauer (1 ml de inóculo). Se lleva a cabo en frascos de vidrio de 50 mL de medio YP (extracto de levadura y peptona), $1X10^7$ cel/ml de *Saccharomyces cerevisiae.* Se ajusta el volumen de reacción a 20 mL con agua des ionizada. La mezcla se incuba a 150 RPM a una temperatura de 30 °C durante 54 horas, tomándose muestras cada 8 horas. Después de este periodo, la biomasa celular será separada por filtración del fermentado, y se cuantificará la concentración de glucosa y bioetanol por HPLC (Agilent Technologies). Se determinará la productividad del proceso (PP), que expresa la cantidad de bioetanol producido por litro y por hora; el rendimiento del proceso es ($Y_{P/S}$), que es la cantidad de bioetanol producido por azúcar consumida; y la eficiencia de conversión de acuerdo al valor teórico. Estos parámetros serán obtenidos de acuerdo a las ecuaciones 2, 3 y 4:

$$\boldsymbol{Productividad\ (PP) = \frac{Concentración\ final\ de\ etanol\ producido\ (g/L)}{h}} \qquad (\boldsymbol{Ec.2})$$

$$\boldsymbol{Rendimiento\ (Y_{P/S}) = \frac{Concentración\ final\ de\ etanol\ producido\ (g)}{Concentración\ de\ glucosa\ consumida\ (g)}} \qquad (\boldsymbol{Ec.3})$$

$$\boldsymbol{Eficiencia\ de\ converción\ (\%) = \frac{Rendimiento}{Rendimiento\ máximo\ teórico} * 100} \qquad (\boldsymbol{Ec.4})$$

*Rendimiento máximo teórico de bioetanol: 0.51 g bioetanol/g glucosa

8. RESULTADOS

8.1 Composición química

8.1.1 Bagazo de *Agave tequilana*

La composición del material lignocelulósico se muestra en la Tabla 3 (o artículo 2 del anexo) realizada con la metodología antes mencionada.

Tabla 3. Composición del material lignocelulósico inicial.

Componente	Cantidad (% w/w)		
Material Soluble	44.29	±	0.93
Lignina	13.57	±	0.18
Cenizas	2.07	±	0.08
Alfa celulosa	26.81	±	0.32
Hemicelulosa	13.33	±	0.12
Holocelulosa	40.14	±	0.77

Se observa que en el bagazo de agave obtenido como residuo de la producción tequilera hay una gran cantidad de material soluble (sustancias extraíbles), el cual se puede remover por simple ebullición con agua; esta materia soluble está constituida principalmente por inulina, esto se corroboró analizando la muestra del sobrenadante del proceso de remoción de material soluble por medio de HPLC con el método estandarizado. Los resultados obtenidos experimentalmente de la composición del bagazo de agave corresponden con los datos obtenidos por otros autores como Yang y col., (2015) quien reporta 13.8±1.3% de lignina, 43.8 ± 1.3 % de holocelulosa y 6 % de cenizas y Flores-Sahagun y col., (2013) quien reporta 21.10 ± 1.13 % de lignina, 73.6 ± 0.01 % de holocelulosa y 5.3 ± 0.07 % de cenizas en base seca y libre de solubles, estas pequeñas variaciones pueden ser atribuidas a la zona de crecimiento y época de cosecha del *Agave tequilana*.

8.2 Pretratamiento Alcalino-Oxidativo

8.2.1 Maximización de azúcares obtenidos

Para esta parte se realizó un análisis de experimentos factorial 2^3 y se llegó al diseño mostrado en la Tabla 4 tomando como mínimo y máximo para NaOH (% p/v) y H_2O_2 (%p/v) 2 y 10 respectivamente.

Tabla 4. Análisis de experimentos factorial 2^3

Número	NaOH (% p/v)	H_2O_2 (%p/v)
1	2	2
2	2	6
3	2	10
4	6	2
5	6	6
6	6	10
7	10	2
8	10	6
9	10	10

Los resultados de la hidrólisis de cada uno de los pretratamientos por el método alcalino-oxidativo se muestran en la Tabla 5.

Tabla 5. Resultados de la hidrólisis de los pretratamientos por método alcalino-oxidativo

Número	NaOH (%p/v)	H_2O_2 (%p/v)	Azúcares (g/l)	% Rendimiento
1	2	2	33.82 ± 1.04	34.36 ± 1.04
2	2	6	38.30 ± 0.74	40.57 ± 0.74
3	2	10	35.65 ± 0.57	37.10 ± 0.57
4	6	2	49.03 ± 0.67	47.60 ± 0.67
5	6	6	56.02 ± 0.92	55.50 ± 0.92
6	6	10	54.92 ± 1.16	52.95 ± 1.16
7	10	2	12.33 ± 0.97	11.83 ± 0.97
8	10	6	16.78 ± 0.82	16.28 ± 0.82
9	10	10	13.68 ± 0.77	13.13 ± 0.77

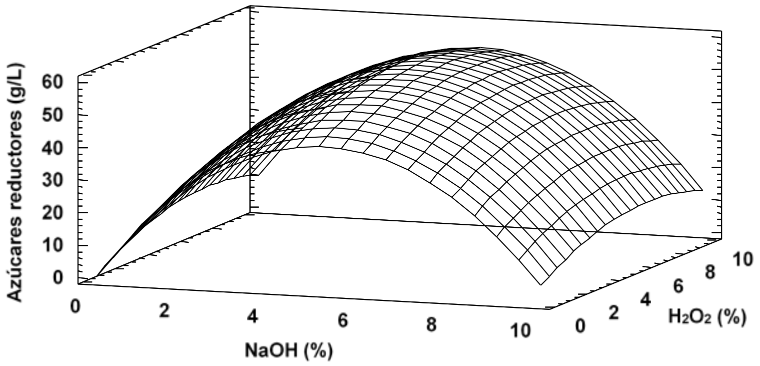

Figura 8. Gráfica de superficie de respuesta de hidrólisis enzimática

Nótese que el máximo de hidrólisis se encuentra en intermedia de hidróxido de sodio y de peróxido de hidrogeno como se observa en la Figura 8.

Para una base de 10 g de material lignocelulósico libre de solubles, utilizando el pretratamiento elegido y posteriormente hidrolizando con el procedimiento estándar; se obtuvieron 4.07 gramos de azucares simples y 2.85 g de oligosacáridos (determinados como celobiosa), resultando 2.42 gramos de sólido residual. El efluente del pretratamiento contiene todo el material restante, el cual se calculó por la diferencia del material sólido posterior al pretratamiento y el material inicial, el cual corresponde a 2.59 gramos. De esta manera es posible calcular la cantidad de azucares reductores a obtener los cuales serían **407 g de azucares por kilogramo de material** lignocelulósico libre de solubles y un porcentaje de eficiencia de **56.56%.**

8.2.2 Variación enzimática para obtención de azúcares

Se estudió el efecto sinérgico de una mezcla de varias enzimas: Celluclast 1,5 L como celulasa, Novoenzyme 188 como beta-glucosidasa, y Viscozyme L como xilanasa tal como la Tabla 6 con el pretratamiento de la Tabla 4 con la cual se obtuvo el resultado de azúcares más alto.

Tabla 6. Diseño de experimentos de enzimas

	Amount of enzyme (ml/g material)			Reducing sugars obtained (g/l)		
No.	**Viscozyme L**	**Celluclast 1.5 L**	**Novozyme 188**	**72 h**		
1	0.1	0.1	0.1	106.64	±	2.04
2	0.1	0.1	0.5	98.65	±	1.96
3	0.1	0.5	0.1	128.07	±	1.15
4	0.1	0.5	0.5	121.95	±	1.57
5	0.5	0.1	0.1	112.79	±	2.45
6	0.5	0.1	0.5	110.46	±	1.05
7	0.5	0.5	0.1	118.07	±	2.58
8	0.5	0.5	0.5	116.98	±	0.89

En esta tabla se puede observar diseño de experimentos para probar diferentes enzimas y sus combinaciones partiendo desde 0.1 mL de enzima por gramos de materia prima pretratada hasta 0.5 mL y se obtiene resultados máximos de 128.07 g/L de azucares a las 72 horas. Usando el mejor pretratamiento de la experimentación de la tabla 5 como estándar a hidrolizar y reduciendo la relación líquido-sólido de 1:10 usada en la experimentación a **1:5** obteniendo los resultados tal como lo muestra la Tabla 6. Partiendo de estos resultados se realiza los siguientes experimentos y diseños.

Se probaron nuevas enzimas y se compararon con las antiguas, realizando una combinación de Cellic Ctec 3 y Htec 3 en diferentes proporciones como lo muestra la Tabla 7 usando el mejor pretratamiento de la experimentación de la tabla 5 como estándar a hidrolizar y reduciendo la relación líquido-sólido de 1:10 usada en la experimentación anterior a **1:5** obteniendo los resultados tal como lo muestra la Tabla 7.

Tabla 7. Experimentos y combinación de enzimas

Experimento	*Ctec 3	*Htec 3	**Azúcares obtenidos (g/l)**
1	6	3	126.92
2	6	6	165.67
3	9	3	151.81
4	9	6	142.24

* Concentración de enzima (% en peso de material a hidrolizar)

Los resultados de los diseños experimentales condujeron combinaciones de enzimas que son óptimas en su correspondiente serie, sin embargo, sólo con la utilización de las enzimas Cellic se logra minimizar la cantidad de enzima utilizada, ya que para hidrolizar el bagazo de *Agave tequilana* con estas enzimas se requieren casi seis veces menos carga que con el conjunto Viscozyme L / Celluclast 1.5 L / Novozyme 188. Por tal motivo, se concluye que para hidrolizar un gramo de material pretratado con el método alcalino oxidativo, se requieren 60 mg de Cellic Ctec 3 y 60 mg de Cellic HTec 3 con una conversión de 80.49 % y un rendimiento de 614.09 g de azúcares por kilogramo de bagazo de *Agave tequilana* libre de solubles con una concentración de 165.67 g/l de azúcares reductores (136.44 g/l of glucosa and 29.23 g/l of xylosa). Estos rendimientos son altos comparados con Farías-Sánchez y col., (2015) quien obtuvo una concentración de 46.24 g/l de azúcares reductores con un pretratamiento de 7.5 % de ácido nítrico a 120 °C, seguido de 1 % hidróxido de sodio a 90 °C y una hidrólisis enzimática con 25 UPF de Celluclast 1.5 L, de NOVOZYME por gramo de muestra pretratada a partir de aserrín de pino; también comparados con Saucedo-Luna y col., (2011) quien obtuvo 41 g /l de azúcares fermentables, correspondiente al 73.6% de rendimiento con 2% de ácido sulfúrico a 147°C por 15 minutos y una hidrólisis enzimática por 72 horas a 40°C a partir de bagazo de agave o comparados con Cheng y col., (2008) quien obtuvo 32.5 g/l de azucares fermentables a partir de bagazo de caña con un pretratamiento alcalino oxidativo con 1% NaOH y 0.6% H_2O_2 y una hidrólisis enzimática con 4% de celulasas 50 °C, pH of 4.5, 140 rpm y por último comparado con Rana y col., (2014) quien obtuvo 130.94 g/L de azúcares totales y un rendimiento de 81% con rastrojo de maíz.

8.2.3 Cinética de reacción

Una vez que se ha encontrado un óptimo en la combinación de enzimas, es necesario aproximar los resultados experimentales a un modelo matemático para un futuro escalamiento del proceso. Debido a que las soluciones comerciales de enzimas utilizadas son en sí mismas combinaciones de las cuales no se conocen las composiciones y tipos de enzimas que la forman, no es posible determinar la cinética de Michaelis Menten. Por otro lado, en la hidrólisis ocurren al menos las siguientes reacciones:

- La ruptura de las cadenas de celulosa en oligosacáridos de longitud variable.
- La conversión de las cadenas de oligosacáridos en celobiosa.
- El desdoblamiento de las moléculas de celobiosa en glucosa.
- La hidrólisis de la Hemicelulosa.

Cada reacción es catalizada por un tipo de enzima específica y la concentración de cada uno de los reactivos en cuestión es difícil de medir en un instante determinado. Así que para la construcción del modelo cinético, únicamente se tomaran en cuenta el efecto global del conjunto de enzimas, el cual consiste en la ruptura de la holocelulosa en azúcares simples (o reductores).

En la Tabla 8, se enlistan las concentraciones de azúcares totales obtenidas en este experimento, se muestran los promedios de las tres corridas.

Tabla 8. Datos para la construcción de la cinética de las enzimas Cellic.

Tiempo (h)	1	6	24	48	72
Azúcares totales (g/L)	8.98 ± 1.56	59.68 ± 2.44	123.56 ± 3.67	151.75 ± 3.37	170.34 ± 3.08

Debido a que la velocidad de las reacciones enzimáticas está relacionada con la concentración del sustrato, entonces se calculó la concentración de polisacáridos [S] según la ecuación (5)

$$[S] = \frac{\{w_0 * (1 - f_{lig})\} - \{[P]/1.1\}}{V_R} \qquad \textbf{\textit{(Ec 5)}}$$

Donde w_0, es el peso de material pretratado inicial en gramos; f_{lig}, es la fracción de lignina en el sólido inicial, [P], es la concentración en gramos por litro de azúcares totales en el instante en el que se quiere calcular [S]; V_R, es el volumen total de reacción que incluye la humedad contenida entre las fibras de material pretratado y el buffer de citratos agregado hasta completar la relación de solido-liquido deseada. El factor 1.1 es adimensional y representa el hecho de que por cada mol de azúcares que se hidroliza es necesario un mol de agua.

Haciendo este cálculo para cada instante con una base de cálculo de 1 litro de volumen total de reacción y una relación 1:5 con respecto al sólido añadido, se obtuvieron los datos de la Tabla 9. Los cuales fueron ajustados a una cinética de segundo orden, de la forma de la ecuación (6).

$$\frac{d[S]}{dt} = -k[S]^2 \qquad \textbf{\textit{(Ec. 6)}}$$

Tabla 9. Variación de la concentración de sustrato con el tiempo.

t (h)	0	1	6	24	48	72
[S] (g/l)	188.58	180.42	134.33	76.25	50.63	33.73

Donde [S] es la concentración de sustrato (polisacáridos), t es el tiempo de reacción en horas y k es la constante de velocidad por determinar en litro por gramo-hora (L/g*h). Integrando la ecuación (3) se llegó a:

$$\frac{1}{[S]} = kt + \frac{1}{[S_0]} \qquad \textbf{\textit{(Ec. 7)}}$$

La cual puede ser transformada a una recta con la constante de reacción como pendiente y al inverso de la concentración inicial como intersección al origen. Numéricamente, la linealización se hizo graficando el tiempo contra los inversos de la concentración.

Con esta transformación matemática se realizó un ajuste de mínimos cuadrados llegándose a un valor de $k = 3.29 \times 10^{-4}$ L/g*h. Con un coeficiente de correlación de 0.9952.

8.2.4 Conclusiones

Esta investigación demuestra la eficacia de la oxidación alcalina como pretratamiento ya que hubo una pérdida de carbohidratos estructurales igual al 3,84% mientras que el 82,62% de la lignina en bruto es eliminada. Además, la estructura cristalina de la celulosa fue destruida efectivamente como lo demuestra el hecho de que el 98,09% de la celulosa en el material pretratado se despolimerizó; Sin embargo, sólo el 47,69% de la hemicelulosa se hidroliza. Al probar las combinaciones de complejos enzimáticos (Celluclast 1,5 L, Viscozyme L y Novozyme 188), las interacciones y funcionamientos sinérgicos alcanzados. Estas combinaciones incrementan los rendimientos obtenibles en comparación con un complejo de una sola enzima (Celluclast 1,5 L). Al comparar estos resultados con la generación final con los complejos de enzimas (Cellic Ctec 3 y Cellic HTec 3), el rendimiento se incrementó mucho más que para la enzima común, de 128 ,07±1,15 a 165,67±2,26 g/l de azúcares, lo que representa un incremento del 29,35%. Todos estos resultados indican que la última generación de complejos enzimáticos Cellic Ctec3 y Cellic HTec3 son viables para su uso a nivel industrial y aumentar el rendimiento de azúcar con una reducción del 82,85% de enzima -necesario comparado con las enzimas viejas.

Debido a que las soluciones comerciales de las enzimas utilizadas son de una composición desconocida, por lo tanto, no es posible determinar la cinética de Michaelis-Menten, por lo que los datos fueron aproximados a un modelo cinético de segundo orden, que debería ser útil para escalar el proceso (todos estos datos se pueden encontrar en el artículo 2 de los anexos).

8.3 Explosión por vapor ácido con bagazo de agave

Se lleva a cabo la experimentación como se observa en la Tabla 10 la cual se obtiene del programa Startgraphics centurion version XV al ingresar los datos mas altos y mas bajos para obtener el diseño de superficie de respuesta.

Tabla 10. Diseño de superficie de respuesta con 2 puntos estrella

	Ácido Sulfúrico (%)	Relación L/S	Presión (kg/cm^2)
1	0.25	3.00	10.60
2	0.10	2.00	6.30
3	0.25	3.00	10.50
4	0.50	3.00	10.22
7	0.10	2.00	6.30
8	0.25	3.00	15.85
9	0.00	3.00	10.22
10	0.10	4.00	6.30
11	0.25	4.68	10.22
12	0.25	1.32	10.22
13	0.10	4.00	15.85
14	0.40	2.00	15.85
15	0.40	4.00	6.30
16	0.40	2.00	6.30
17	0.4	4	15.85
18	0.25	3	4.50

Con los datos obtenidos de la experimentación (Tabla 11) se llevan a cabo el analisis de experimentos en el programa Startgraphics centurion versión XV arrojandonos las Figuras 9, 10 y 11 en las cuales se puede observar que no hay un optimo definido si no solo un maximo de los valores usados en la matriz.

Tabla 11. Experimentos del diseño de superficie de respuesta y resultados obtenidos

	Ácido Sulfúrico (%)	Relación L/S	Presión (kg/cm2)	Azucares g/l
1	0.25	3.00	10.60	36.82
2	0.10	2.00	6.30	20.00
3	0.25	3.00	10.50	34.33
4	0.50	3.00	10.22	20.00
7	0.10	2.00	6.30	38.20
8	0.25	3.00	15.85	16.44
9	0.00	3.00	10.22	27.12
10	0.10	4.00	6.30	20.54
11	0.25	4.68	10.22	31.60
12	0.25	1.32	10.22	21.47
13	0.10	4.00	15.85	33.01
14	0.40	2.00	15.85	31.11
15	0.40	4.00	6.30	34.98
16	0.40	2.00	6.30	24.26
17	0.4	4	15.85	34.91
18	0.25	3	4.50	19.47

En los datos especificos se puede ver una variedad de y variación grande por lo cual se procede a trabajar con los gráficos y datos óptimos que arroja el programa de diseño estadistico.

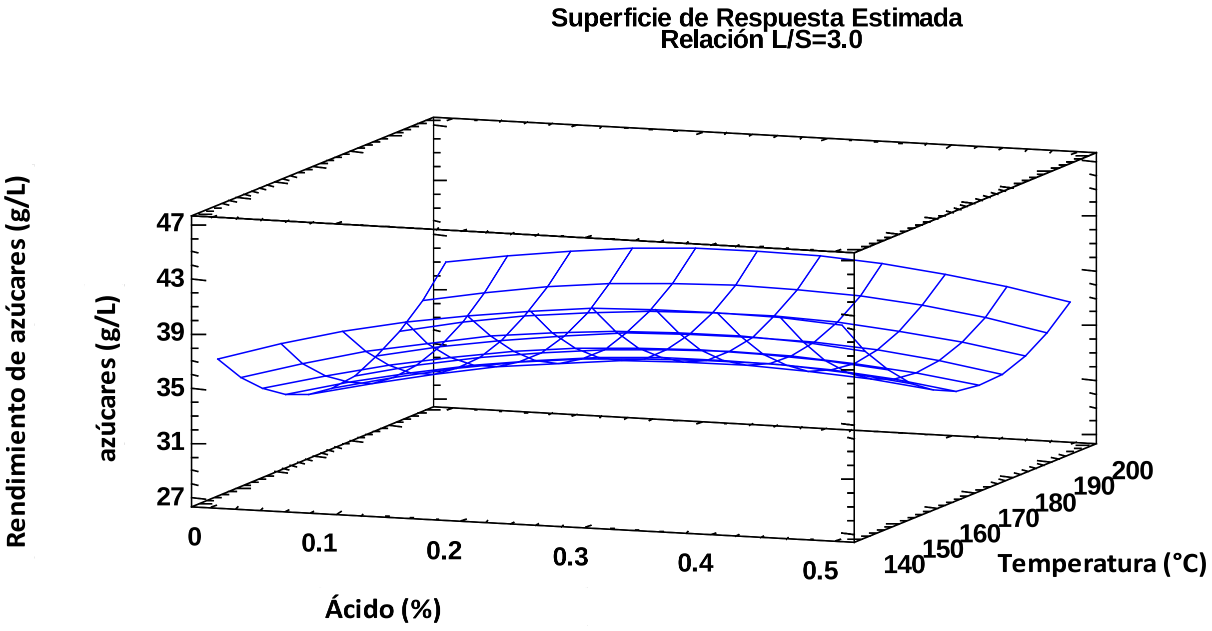

Figura 9. Gráfico de superficie de respuesta Ácido vs Temperatura

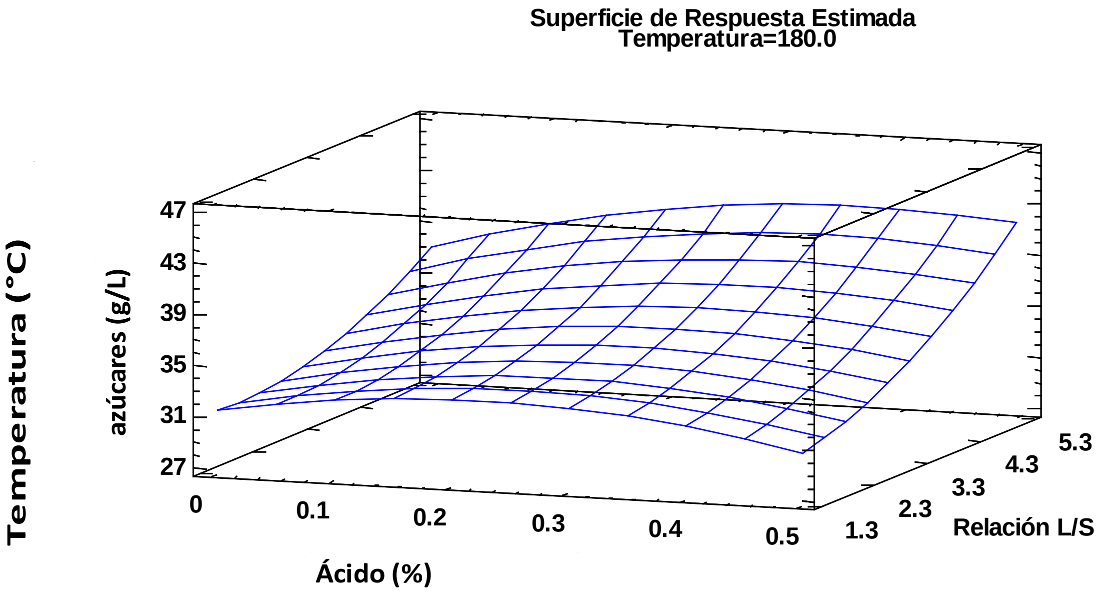

Figura 10. Gráfico de superficie de respuesta Ácido vs Relación L/S

En esta gráfica es muy notorio el impacto que tiene la relación Líquido/Sólido sobre las demás variables, está claro que entre más grande es la relación hasta llegar a 4.68, aumentan la concentración de azúcares obtenida por el tratamiento. De igual manera pero de manera más gradual al aumentar el porcentaje de ácido en el tratamiento aumenta de manera proporcional la concentración de azúcares hasta llegar a una aproximación de 0.4% y baja muy levemente al llegar a 0.5% todo esto manejando una temperatura de 180 °C como constante.

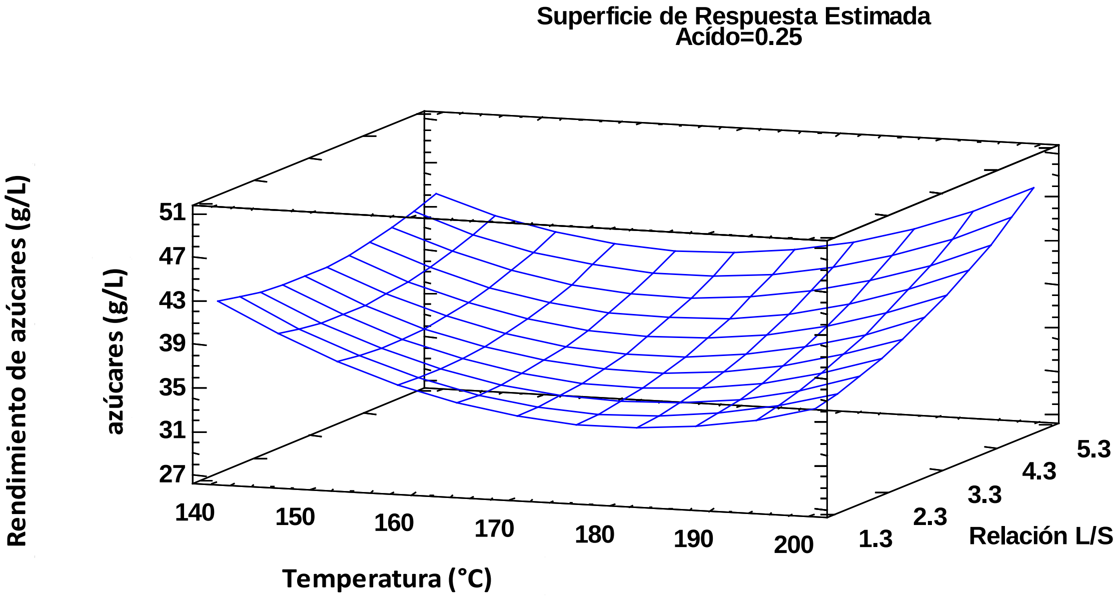

Figura 11. Gráfico de superficie de respuesta Temperatura vs Relación L/S

Usando una constante de ácido de 0.25% se hizo esta gráfica para poder comparar el comportamiento de la variación de la temperatura contra la relación Líquido/Sólido y así poder darnos cuenta que tenemos varios máximos locales en cada esquina de la gráfica excepto en la temperatura alta y relación L/S bajo, y en contraste un máximo de la gráfica en la temperatura alta de 200°C y relación S/L alta de 4.68, por todo esto vamos a la Tabla 12 que muestra los resultados arrojados del programas para obtener las variables óptimas.

Tabla 12. Variables para optimización de respuesta

Optimizar Respuesta			
Meta: maximizar Azúcares			
Valor óptimo = 45.0346			
Factor	Bajo	Alto	Óptimo
Ácido	0	0.5	0.359945
Temperatura	146.36	200	200
Relación L/S	1.32	4.68	4.65806

Como se observa en los datos dados por el software, el valor experimental más alto usado para la temperatura en la matriz es el dato óptimo, de la misma forma el dato de la relación L/S por lo cual podemos corroborar que no es un óptimo sino un máximo.

Por todo lo anterior se hacen otras pequeñas corridas experimentales con las condiciones de la Tabla 13 manteniendo la relación de ácido con 0.5%, relación líquido sólido de 5 y cambiando el tamaño de partícula por una reducción de tamaño a malla 40.

Tabla 13. Experimentos aislados y comparados con los máximos de la matriz de superficie de respuesta

Hidrolizados					
Pretratamiento	Azúcares (g/l)	Temperatura (°C)	Ácido (%)	Tamaño de partícula	Relación L/S
3	11.39	160	0.5	Malla 40	5
4	33.22	180	0.5	Malla 40	5
5	62.83	200	0.5	Malla 40	5
Comparados con los valores más altos de la matriz de Explosión de vapor anterior					
EV 17	31.15	200	0.4	N/A	4
EV 8	21.57	200	0.25	N/A	3
EV 3	32.71	180	0.25	N/A	3

Al ver que los datos obtenidos de los azúcares son más consistentes, se observa que al reducir el tamaño de partícula a malla 40 sin pasar malla 60 y aumentar la cantidad de ácido obtenemos una mayor concentración de azúcares reductores, esto se lo atribuimos a que las variables que utilizamos no reaccionan con la materia prima por ser una matriz muy grande pero al reducir su tamaño exponemos una mayor área superficial al contacto de las variables utilizadas y aumentamos la cantidad total de materia prima que reacciona en este proceso con lo cual aumentamos significativamente la cantidad de azúcares del proceso obteniendo así **62.83 g/l y con eficiencia de 71.26%,** estos rendimientos son un poco bajos comparados con Negro y col., (2014) quien obtuvo una concentración de 72.8 g/l de azúcares y una eficiencia del 88% con un pretratamiento de explosión de vapor a 195°C, con ácido fosfórico al 1% del árbol de olivo y Sharma y col., (2015) quien obtuvo una eficiencia del 81.8% con un pretratamiento de explosión de vapor a 200°C, con ácido sulfúrico al 0.5% con paja de arroz.

Nuestra preocupación principal es que se encuentra en el límite superior de la presión utilizada, al ver que no podemos hacer experimentación con una mayor presión se decide tomar en cuenta el diseño de un reactor con especificaciones para soportar hasta 25 kg/cm^2 de presión y utilizando vapor sobrecalentado para la presurización y calentamiento de la muestra a tratar, esto porque el reactor utilizado actualmente se usa en la cocción de astillas de madera para pulpeo con condiciones de alrededor de 160 °C y 6.3 kg/cm^2 de presión pero fue llevado hasta 200 °C o 15.85 kg/cm^2 confiando en el sobredimensionamiento que este tiene.

8.3.1 Conclusiones

Los resultados de esta investigación muestran que bagaso de *Agave tequilana* (BAT) es una materia prima muy interesante para producción del bioetanol y para el desarrollo de un concepto de biorrefinería. El BAT, tiene una gran cantidad de extractivos (azúcares) que podría ser utilizado para el mismo propósito. Con el pretratamiento por explosión de vapor acidificado, a 15,85 kg/cm^2, 0,5% de sulfúrico ácido y malla 40 seguido de hidrólisis enzimática con Cellic Ctec 3 y Cellic Htec 3 fue posible obtener un lıquido hidrolizado de 71,1 g/l de azúcares reductores (59,29% glucosa, 29,05% de xilosa, 11,66% de fructosa), desgraciadamente por cuestiones de equipo los análisis no pueden ser llevados más a fondo ya que se requiere un aumento en la severidad de los tratamientos la cual no es posible. Pero podría buscarse o diseñarse un equipo que cumpla las características requeridas para llevar a cabo un nuevo diseño y análisis y así poder obtener mejores resultados de los ya obtenidos. (La información de explosión de vapor se encuentra recabada y extendida en el artículo 5 de los anexos).

8.4 Pretratamiento organosolv con etanolamina

Primeramente, se planteó establecer las condiciones adecuadas del proceso organosolv, mediante un diseño Taguchi L9, evaluando el porcentaje de etanolamina, la temperatura, el tiempo y la relación liquido-solido, tal como se muestra en la Tabla 14.

Tabla 14. Diseño Taguchi L9 y resultados obtenidos del pretratamiento organosolv con etanolamina

	Etanolamina	Relación L/S	Temperatura (°C)	Tiempo (min)	% de MPF	g/l (g+x)	% Rend. Total
1	50%	3:1	160	30	67.32%	122.20	41.13
2	50%	5:1	180	60	62.83%	96.05	30.17
3	50%	7:1	200	90	53.86%	130.96	35.27
4	65%	3:1	180	90	60.93%	72.40	21.76
5	65%	5:1	200	30	57.95%	97.68	28.30
6	65%	7:1	160	60	65.25%	97.02	31.06
7	80%	3:1	200	60	54.32%	130.18	35.82
8	80%	5:1	160	90	64.03%	92.80	28.83
9	80%	7:1	180	30	62.22%	130.20	40.51

En la Tabla 14 se puede observar una diversidad de resultados con los 4 factores y tres niveles utilizados para el diseño, el experimento destacado con una menor cantidad de perdida de materia prima inicial es el experimento #1 con una pérdida de únicamente 32.68%, continuando con los experimentos #6 y #8 con 34.75% y 35.97% respectivamente, con el experimento #2 y #9 con alrededor de un 38% y subiendo.

En el gráfico de efectos principales (Figura 12) nos ayuda para ver rápidamente la tendencia de los datos de la materia prima final (MPF), en el cual las medias de porcentaje de etanolamina apuntan a que es factible usar el factor bajo ya que el promedio de estos es el que obtiene mejores resultados con menor porcentaje de etanolamina, en la relación L/S el factor bajo tiene un menor rendimiento que el factor medio y el factor alto más bajo que los dos primeros, lógicamente se escogería el factor medio, para la temperatura no hay mucho que decir es mucha la diferencia entre los tres factores, dando como máximo el factor bajo de esta variable y por último pero no menos importante el tiempo, que de la misma manera que la variable de temperatura el valor con los resultados más altos es el nivel bajo.

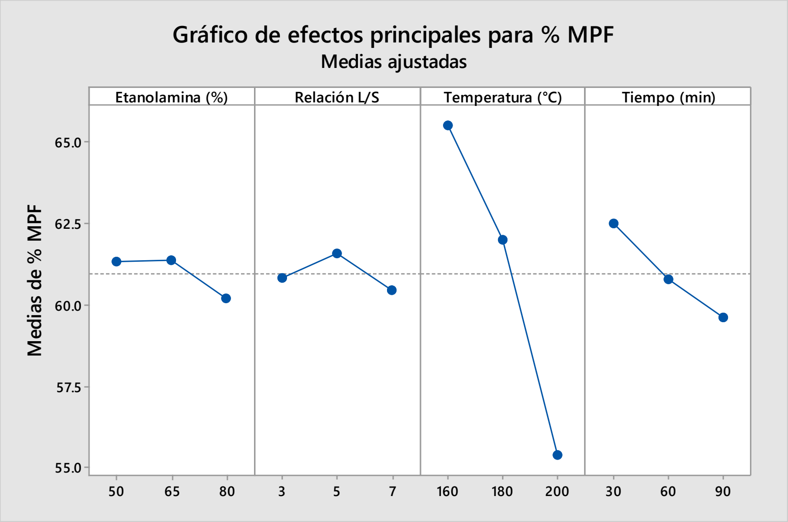

Figura 12. Gráfico de efectos principales para % de MPF

La Tabla 15 es la respuesta numérica de las medias de cada factor, en la cual es fácilmente visible las diferencias y los deltas de cada factor así como la tendencia y el promedio de cada uno de los niveles, siendo estos con los mejores resultados en forma general el nivel 1 o bajo.

Tabla 15. Tabla de respuesta para medias de % MPF

Nivel	Solvente (%)	Relación L/S	Temperatura (°C)	Tiempo (min)	Promedio
1	61.34	60.85	65.53	62.5	62.55
2	61.37	61.6	61.99	60.8	61.44
2	60.19	60.44	55.38	59.61	58.9
Delta	1.18	1.16	10.16	2.89	

Las interacciones de la media de todos los niveles con todos los factores se puede ver en la Figura 13.

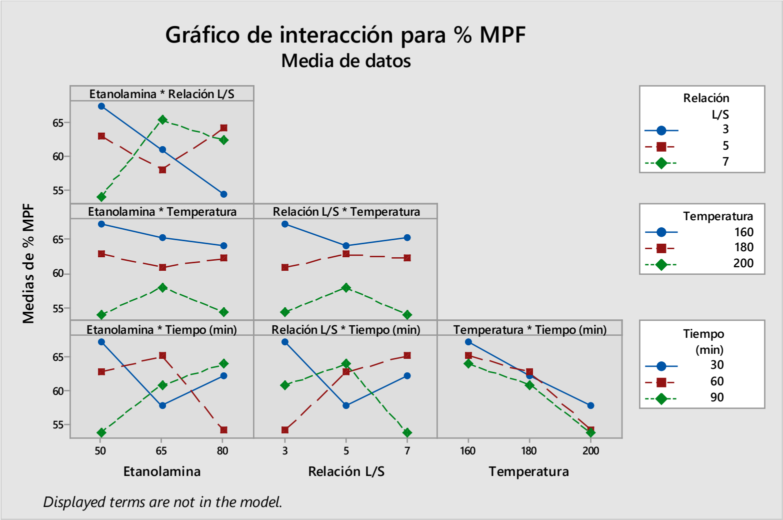

Figura 13. Gráfico de interacción para % de MPF

Esta Figura muestra el comportamiento de cada una de las medias de cada nivel con cada uno de los factores para darnos cuenta del comportamiento individual de cada uno de estos, corroborando de manera genera los resultados mostrados en la tabla y Figura anterior pero con un poco más de detalle.

Tabla 16. Maximización de la respuesta de % de MPF

Meta	Bajo	Alto
Maximizar	53.8631	67.3173

Tabla 17. Tabla de factores y niveles con resultado esperado

Solución	Tiempo (min)	Temperatura (°C)	Relación L/S	Etanolamina (%)	
1	30	160	5	65	**68.1056**

Con los diferentes gráficos y tablas mencionadas y explicadas anteriormente llegamos a estas últimas Tablas 16 y 17, las cuales son el resumen de todo lo anterior y nos dice cuáles son los factores y niveles necesarios para maximizar la MPF hasta llegar a 68.10% la cual es un poco mayor a lo obtenido experimentalmente, se puede ver que 2 de los niveles son bajos y dos son nivel medio, con esto podemos concluir que el factor de severidad se encuentra debajo de la media y con esto hay menor degradación de materia prima y por lo tanto mayor MPF.

Por otro lado en la Tabla 14, se obtuvieron concentraciones máximas de 130 g/l de azúcares totales en 3 experimentos, seguido por solo una concentración de 122 g/l y las restantes con valores abajo de 100 g/l.

Todo lo anterior mencionado se reduce a la columna de % de rendimiento total la cual consiste en el total de azúcares que se pueden obtener a partir de la materia prima antes de ser pretratada; esto es, por ejemplo: a partir de los 10 gramos de materia inicial para el pretratamiento 1 se pueden obtener 4.11 gramos de azúcares fermentables después del pretratamiento y la hidrólisis enzimática.

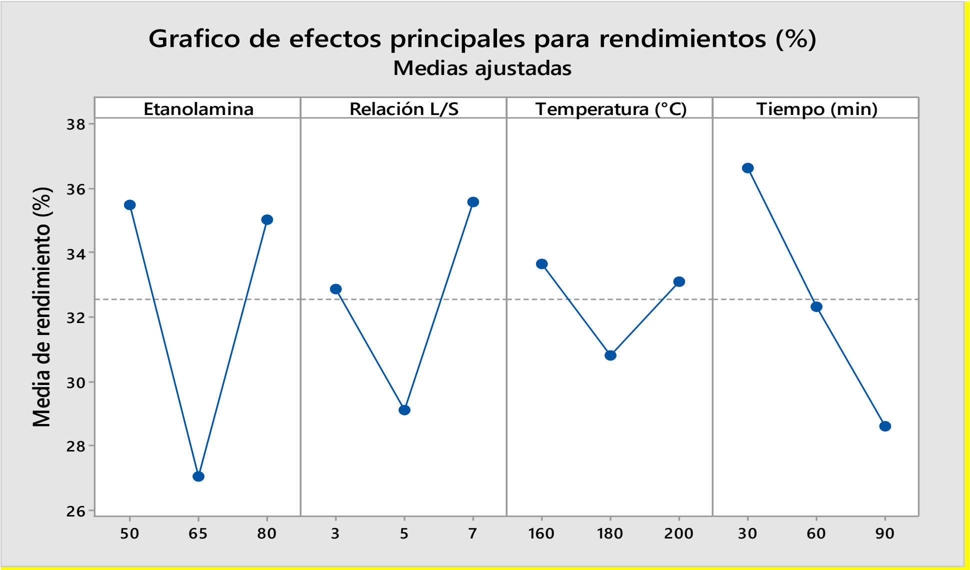

Figura 14. Gráfico de efectos principales para rendimientos (%)

En la Figura 14 encontramos la media de cada factor con sus tres niveles de forma gráfica en la cual es fácilmente visible y en la Tabla 18 se encuentran las medias con valores puntuales; en los factores de etanolamina y temperatura los valores de los niveles 1 y 3 son muy similares con 35 y 33, respectivamente, esto indica que no hay una diferencia significativa entre esos niveles y factores; por otro lado en el factor de tiempo es inversamente proporcional a los niveles, esto es que en el nivel más bajo obtenemos la media de rendimientos más alta.

Tabla 18. Tabla de respuesta para medias de rendimiento (%)

Level	Etanolamina (%)	Relación L/S	Temperatura (°C)	Tiempo (min)
1	35.53	32.9	33.68	36.65
2	27.04	29.1	30.81	32.35
3	35.05	35.61	33.13	28.62
Delta	8.48	6.51	2.86	8.03

También estos resultados podemos obtenerlos con la ecuación de regresión 8 para probar otros factores y niveles sin necesidad de hacerlos experimentalmente

Rendimiento (%) =

32.54 + 4.108 Tiempo (min)_30 - 0.1883 Tiempo (min)_60 - 3.920 Tiempo (min)_90
+ 1.137 Temperatura (°C)_160 - 1.727 Temperatura (°C)_180 + 0.5900 Temperatura (°C)_200
+ 0.3618 Relación L/S_3 - 3.436 Relación L/S_5 + 3.075 Relación L/S_7 + 2.986 Etanolamina_50
- 5.498 Etanolamina_65 + 2.512 Etanolamina_80 ***(Ec 8)***

Con todos los datos obtenidos con anterioridad y con el uso del programa MINITAB se lleva a cabo una optimización del cual la meta es maximizar la obtención de azúcares partiendo de los datos obtenidos como valor bajo de 21.75% de rendimiento y 41.13% como valor alto obtenidos de los experimentos 4 y 1 respectivamente el programa nos arroja el uso de un tratamiento con un tiempo de 30 minutos, con 160 °C de temperatura, una relación líquido-sólido de 7:1 y 50% de etanolamina para buscar obtener un rendimiento de 43.84% como lo muestra la Tabla 19.

Tabla 19. Optimización de la respuesta de rendimiento %

Solución	Tiempo (min)	Temperatura (°C)	Relación L/S	Etanolamina (%)	
1	30	160	7	50	**43.8443**

8.4.1 Conclusiones

Este trabajo tiene por objetivo obtener la mayor cantidad de azúcares fermentables a partir de materia prima de bagazo de agave, con los resultados obtenidos podemos concluir que a partir de 1 kg de bagazo de agave libre de solubles y molido se pueden obtener 438.24 g de azúcares, esto es una eficiencia de 54.06% basado en el óptimo arrojado por el programa MINITAB del 43.84% de rendimiento y el 81.06% de holocelulosa contenido en la materia prima inicial para el bagazo de agave.

9. CONCLUSIONES

Cada una de la metodología y pretratamientos usados en esta tesis tienen ventajas y desventajas entre las que destacan:

Ventajas:

Pretratamiento Alcalino-Oxidativo

La concentración alta de azúcares que se puede obtener del orden de 165 g/L.

La materia libre soluble puede ser susceptible para la conversión a azúcares por métodos térmicos o enzimáticos.

No lleva molienda para su posterior uso, lo cual implica un ahorro de trabajo y energético considerable.

Tiene un bajo costo de los equipos ya que no se necesita un equipo sofisticado que soporte una presión y temperatura alta.

Explosión de vapor

Se puede usar la materia prima sin hacerle un lavado previo (quitarle el material soluble)

Tiene la mayor eficiencia total.

El consumo de agua de lavado es nulo ya que no se lava en ningún paso del proceso, solo se detoxifica con $Ca(OH)_2$.

La lignina y los residuos celulósicos pueden ser utilizados para procesos posteriores como la producción de pellets y briquetas, y por el tipo de materias primas de que se trata, podrían tener un poder calorífico mayor al aserrín de pino utilizado por el mercado en la actualidad.

Proceso Organosolv con Etanolamina

Tiene una concentración de azúcares 122.7 g/L

Hay un bajo consumo de químicos ya que puede ser reciclada en el proceso y solo agregar las pérdidas que se van teniendo al paso del tiempo.

Tiempos bajos de proceso (30 minutos)

Se obtiene una lignina de alto valor y pureza la cual se puede comercializar con un valor agregado alto y así poder hacer más costeable el proceso.

Desventajas

Pretratamiento Alcalino-Oxidativo

La materia prima debe ser libre de extraíbles, esto es que antes del pretratamiento la materia prima tiene que pasar por un proceso de calentamiento en agua para poder liberar toda la materia soluble contenida en esta.

La cantidad de químicos usados en este pretratamiento es alta por lo tanto entraríamos en el problema de “¿qué hacer con los residuos químicos de este proceso?” aunque esta podría ser una área de oportunidad para futuros investigadores ya que esta mezcla es rica en lignina despolimerizada y carbohidratos los cuales podrían ser susceptibles para venta como un producto de valor agregado.

Tiempo de residencia muy alto de alrededor de 24 horas, en este punto podría ser solucionado con un equipo de agitación para una reacción más rápida del pretratamiento con los químicos y así disminuir el tiempo del proceso.

Consumo de agua para lavados muy alto.

Explosión de vapor

Se requieren equipos muy sofisticados para el proceso ya que se requieren altas presiones y temperaturas del orden de 200°C y 17 kg/cm^2

Se requiere una molienda y tamizado a malla 40 sin pasar malla 60, lo cual es una molienda muy fina y requiere de una gran cantidad de energía.

Proceso Organosolv con Etanolamina

Se requiere materia prima libre de extraíbles, esto es que antes del pretratamiento la materia prima tiene que pasar por un proceso de calentamiento en agua para poder liberar toda la materia soluble contenida en esta.

El consumo de agua de lavado es alto, esta parte podría ser solucionada con algún compuesto para neutralizar la función de la etanolamina y así evitar el uso de agua.

Se requiere una molienda y tamizado a malla 40 sin pasar malla 60, lo cual es una molienda muy fina y requiere de una gran cantidad de energía.

Poniendo en una balanza las ventajas y desventajas de estos pretratamientos tan variados, la decisión de un pretratamiento especifico o mejor que otro es complicado ya que entrarían una infinidad de variables técnicas a tomar en cuenta para elegir la mejor opción; pero, si, para nuestra elección solo tomamos en cuenta los resultados de azúcares obtenidos en este trabajo entonces podríamos decidir que el mejor tratamiento es el proceso organosolv ya que finalmente por cada tonelada de materia prima seca obtenemos 286.21 kg de azúcares fermentables, a la cual le sigue la explosión de vapor 285.53 kg de azúcares fermentables y por último el alcalino-oxidativo con 265.81 kg de azúcares fermentables.

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Anexos

EXCLI Journal 2015;14:430-438 – ISSN 1611-2156
Received: September 27, 2014, accepted: February 11, 2015, published: March 13, 2015

Original article:

COMPARISON OF FIVE PRETREATMENTS FOR THE PRODUCTION OF FERMENTABLE SUGARS OBTAINED FROM *PINUS PSEUDOSTROBUS* L. WOOD

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http://dx.doi.org/10.17179/excli2014-613

ABSTRACT

To benefit from the use of a waste product such as pine sawdust from a sawmill in Michoacán, Mexico, five different pretreatments for the production of reducing sugars by enzymatic hydrolysis were evaluated (sodium hydroxide, sulfuric acid, steam explosion, organosolv and combined method nitric acid / sodium hydroxide). The main finding of the study was that the pretreatment with 6 % HNO_3 and 1 % NaOH led to better yields than those obtained with sodium hydroxide, dilute sulfuric acid, steam explosion, and organosolv pretreatments. Also, HNO_3 yields were maximized by the factorial method. With those results the maxima concentration of reducing sugar found was 97.83 ± 1.59, obtained after pretreatment with 7.5 % HNO_3 at 120 °C for 30 minutes; followed by 1 % of NaOH at 90 °C for 30 minutes at pH 4.5 for 168 hours with a load enzyme of 25 FPU/g of total carbohydrates. Comparing the results obtained by the authors with those reported in the literature, the combined method was found to be suitable for use in the exploitation of sawdust.

Keywords: Reducing sugars, enzymatic hydrolysis, acid hydrolysis, pretreatments, pine sawdust, *Pinus pseudostrobus*

INTRODUCTION

Ethanol can be produced by fermentation of sugars presents in vegetables products (cereals, beet, cane, sorghum, and other biomasses); these sugars are present in the forms of saccharose, starch, hemicelluloses, and cellulose. The product of this fermentation process is hydrated alcohol containing approximately 5 % of moisture. After a dehydration step, the alcohol can serve as a vehicle fuel (SEMARNAP, 2000).

Received: September 27, 2014, accepted: February 11, 2015, published: March 13, 2015

Forest lands, and particularly timberlands, have the potential to sustainably produce close to 370 million dry tons of biomass annually. This estimate includes the residues generated in the manufacture of various forest products and the residues generated in the use of manufactured forest products. It also includes the harvest of wood for various residential and commercial space-heating applications. With the exception of urban wood residues, most of these sources of forest biomass are currently being utilized and there are significant efforts under way to use these resources much more efficiently (Perlack et al., 2005). Environmentally speaking, biomass generated in forest industrial processes, such as sawdust and crusts, is highly polluting. In Mexico, the sawmill industry generates around 0.35 m^3 of pine sawdust per cubic meter of processed wood (SEMARNAP, 2000). This is equivalent to an annual average sawdust production of 206 thousand cubic meters (INEGI, 2008). Considering an average density of 500 kg/m^3, such annual production is equivalent to 103 thousand tons (Valencia and López, 1999). On the one hand, the above mentioned by-products reduce the available space in wood transformation centers and therefore, productivity. By-products can also be the cause of an accumulation of dust in the air, with the consequent health risks to sawmill workers and inhabitants of the nearby zones, since dust may bring on a number of respiratory diseases, such asthma, chronic bronchitis, and allergies (Malmström et al., 1999). In addition, dust might be the cause for dermatitis and different kinds of cancer, namely pulmonary, gastrointestinal and nasal (Seguros de Texas, 2004). Moreover, dust has been claimed responsible for other environmental problems, such as fires and spontaneous ignition.

On the other hand, both sawdust and other waste products originated while working with coniferous woods may be raw material for the production of ethanol fuel and other chemical products (Palonen et al., 2004). That is to say that the use of the previously mentioned 103 thousand tons of sawdust would lead to an approximate production of $33x10^6$ L of ethanol fuel. However, the main restriction for producing ethanol fuel from sawdust lies in recovering the sugars that the latter contains. Thus, the recovery stage is determined by the hydrolysis procedure being used (acid or enzymatic). The most investigated pretreatment processes for woody biomass include dilute acid, steam explosion, organosolv, and sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) (Zhu et al., 2009). Sodium hydroxide treatment breaks the lignin-carbohydrate bonds, partially removes lignin and hemicelluloses, opens the material structure, increases interface area (Tuor et al., 1995). Ethanol organosolv pretreatment can effectively remove there calcitrance of woody biomass for enzymatic cellulose saccharification (Pan et al., 2005, 2006; Pan, 2008). Dilute acid pretreatment is able to mainly hydrolyze hemicelluloses, which turn into a porous material formed by cellulose and lignin (Wyman et al., 2005). Similarly, steam explosion breaks down the structure of the material in order to enhance enzyme access to cellulose (Gregg and Saddler, 1996; Shimizu et al., 1998). The sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) is an acidic pretreatment and is very similar to diluted acid pretreatment, in terms of process flow configuration, but with the addition of sulfite or bisulfite. This pretreatment, breaks down the structure of the material and mainly hydrolyze hemicelluloses (Tian et al., 2011). While the HNO_3 process hydrolyzes and removes lignin from the structure (Wyman et al., 2005), a HNO_3 and NaOH combination treatments remove a great deal of lignin and hemicellulose and, in a smaller proportion, cellulose; hence a remnant of cellulose material is obtained.

This study reports the results of the evaluation of different pretreatment methods, seeking for the one that leads to higher yields and that enhances both pretreatment conditions and enzymatic hydrolysis to obtain glucose from pine sawdust. The analysis also demonstrates that it is possible to use pine sawdust, considered as a potentially risky pollutant, for the fermentable sugars production, which in turn might be used for producing ethanol fuel.

MATERIAL AND METHODS

Lignocellulosic material and chemical composition

The sawdust used in this study came from *Pinus pseudostrobus* trees that had just been felled under authorization for forest harvesting in the Indigenous Community of San Juan Nuevo Parangaricutiro, in Michoacán, Mexico (19°21´00´´ N. Lat., 102°08´15´´ W. Long.), at 1674 mamsl. The sawdust samples were milled and sieved using a 20-fraction mesh (841 µm) retained on 40 mesh screen.

The following chemical features were determined in duplicate in this research work: moisture percentage (TAPPI, 2000), pH (Sandermann and Rothkamm, 1959), and ashes (TAPPI, 2000). The total amount of extractives was determined using a Soxhlet extraction sequence with cyclohexane, acetone, methanol, and water. Whereas Runkel lignin (Runkel and Wilke, 1951), holocellulose (Wise et al., 1946), and alpha cellulose (ASTM, 2000) were all determined in extractive-free wood meal.

Pretreatments for hydrolysis

Sawdust samples were treated according to five pretreatment methods at the specified operating conditions shown in Table 1. Samples of pure cellulose and pine sawdust, without any pretreatment at all, were used as controls. Most articles are likely to have only two levels of headings. All experiments were performed in duplicate.

Alkaline pretreatment

The procedure of González et al. (2011) was performed in this step using a 3 % NaOH concentration, instead of one of 0.5 % NaOH. 10 g samples of sawdust were mixed with 100 mL of sodium hydroxide, ACS grade, in 250-mL-Erlenmeyer flasks. The mixture was then heated in a sterilizer at 121 °C for 78 minutes. The mixture was next set aside to cool to room temperature and after that filtered through a nylon filter cloth. Finally, the solid fractions were squeezed and dried out.

Acid pretreatment

10 g sawdust samples were mixed with 100 mL of 5 % H_2SO_4 in 250-mL-Erlenmeyer flasks. After that the mixture was heated in a sterilizer at 121 °C for 60 minutes, set aside to cool to room temperature, and filtered. The solid fractions were squeezed, dried out and weighed (Cortez, 2010).

Steam explosion

10 g sawdust samples were mixed with 100 mL of distilled water in 250-mL-Erlenmeyer flasks, which were capped and left to stand for 48 hours at room temperature. Next, the samples were heated in a sterilizer (at 121 °C), and a sudden decompression of the system was made. Flasks were allowed to cool to room temperature. The solid fraction was filtered through a nylon cloth, dried out, and weighed (Oliva, 2003).

Organosolv pretreatment

This pretreatment was performed using a reflux equipment in which the 10 g sawdust samples was treated with watery ethanol and acetone (60/40 w/w), with a solid-liquid ratio of 7:1, at 80 °C for 90 minutes. At the end of the treatment the samples were allowed to cool to room temperature and filtered through nylon cloth; the solid fraction was then squeezed, dried out, and weighed (Martinez, 2011).

Combined nitric acid and sodium hydroxide

20 g sawdust samples were mixed with 120 mL of 6 % nitric acid in a ball flask. The mix was kept under reflux conditions at 100 °C for 60 minutes in an oil bath. The solid fraction was washed out with distilled water, allowed to dry, and later mixed with 120 mL of 1 % sodium hydroxide. The mixture was heated at 90 °C for 30 minutes under reflux conditions (Alhasan et al. 2010).

Received: September 27, 2014, accepted: February 11, 2015, published: March 13, 2015

Enzymatic hydrolysis

Pretreated materials were hydrolyzed using a commercial enzymatic complex obtained from *Trichoderma reesei* (Celluclast 1.5 L, a product of NOVOZYME Corp.). For the hydrolysis step, 1 g samples of pretreated material were mixed with the appropriate amount of enzymatic extract in 20-mL-containers to provide 25 FPU. Meanwhile, the mix pH was adjusted to 4.5 by adding the required volume of 0.1 molar buffer acetate solutions until reaching 10 mL. The mixture was later shaken for 72 hours, at 80 rpm and 45 °C. The following step was taking samples of the hydrolyzed material every twelve hours, determining their reducing sugar content using Chaplin and Kennedy´s DNS method (Chaplin and Kennedy, 1994).

The yield results were used to evaluate the pretreatments procedures. A variance analysis was conducted with a 95 % confidence level. Both the LSD method and the multiple rank tests were applied. Data were processed with STATGRAPHICS Centurion XVI software, version 16.1.18.

Identification of the hydrolysis variables

Out of the evaluated pretreatments, the one with the greatest yield was the combined pretreatment. After applying a 2^3 factorial design, the factors and levels obtained were as following:

factor A: time (30 and 60 min);
factor B: nitric acid concentration (3 and 6 %); and,
factor C: temperature (50 and 100 °C).

The dependent variable was the yield of fermentable sugars. The collected data were used for an analysis of variance (ANOVA), with a 95 % confidence level. The software used was STATGRAPHICS Centurion XVI, version 16.1.18.

Maximizing reducing sugar yield

The results from the 2^3 factorial design showed that the pretreatment that yielded to the highest concentration of reducing sugar (36.55 g/L) was the one performed with 6 % nitric acid at 100 °C for 30 minutes. Hence, to maximize the hydrolysis yield a 3^2 factorial design was used and all the experiments were run in duplicate. The factors and levels were:

factor A: Nitric acid concentration (4.5, 6 and 7.5 %); and,
factor B: temperature (80, 100 and 120 °C); pretreatment time was kept constant at 30 minutes.

Collected data were used for an analysis of variance (ANOVA), with a 95 % confidence level. The software used was STATGRAPHICS Centurion XVI, version 16.1.18.

Calculations

The yield obtained for each pretreatments procedure was expressed in terms of weight by means of Eq. 1,

$$RP = [(W_i - W_f) / W_i] \times 100 \qquad [1]$$

where: W_i (g) is the weight of lignocellulosic material subjected to pretreatment, W_f (g) is the weight of pretreated material, and *RP* is the pretreatment yield (% on a mass basis).

Table 1: Pretreatments and specified conditions

Pretreatment	Catalyst	Temperature (°C)	Time (min)	Concentration (%)	Sample (g)	Ratio L/W
Alkaline	NaOH	121	78	3	10	10:1
Acid	H_2SO_4	121	60	5	10	10:1
Steam explosion	H_2O	121	30	100	10	10:1
Organosolv	Methanol/Acetone	80	90	60/40	10	7:1
Combined	HNO_3	100	60	6	20	6:1
	NaOH	90	30	1	-	6:1

The production of reducing sugars obtained by means of enzymatic hydrolysis of pretreated materials was quantified by Eq. 2.

$$\% RA = [(A \times W_1 \times 0{,}9) / B\,] \times 100 \qquad [2]$$

where: *A* (g) is the amount of reducing sugars in the hydrolyzed material, W_1 (g) is the initial weight of the sample that underwent hydrolysis, B (g) is the amount of cellulose on untreated wood, and *RA* is the cellulose hydrolysis yield (%).

RESULTS AND DISCUSSION

Chemical composition

Table 2 shows the results of the chemical composition of pine sawdust. The observed results match those data reported by other authors for the same wood species (Rutiaga-Quiñones, 2001; Lima-Rojas, 2013).

Pretreatments for hydrolysis

Table 3 shows different yields obtained in each pretreatment tested. The reducing sugars yield in the liquor produced during the acid pretratment was low (18.2 % or 18.2 g/L). The results displayed in Table 3 on column “Reducing sugar yield (%)” are based on the 6 carbon carbohydrates on untreated wood (Equation 2) and clearly show that the pretreatment with the highest hydrolysis yield of reducing sugars was the combined pretreatment, performed with 6 % nitric acid at 100 °C, for 60 minutes. There was some considerable weight loss of material (59.16 %), though.

Data collected for the alkaline pretreatment in this work were similar to those obtained by Saha and Cotta (2006) for pretreated wheat straw (8.6 % w/v) with alkaline peroxide (2.15 % of H_2O_2 v/v, pH 11.5, 35 °C, 24 h) and for an enzymatic saccharification (45 ºC, pH 5.0, 120 h) with three commercial enzyme preparations (cellulase, beta-glucosidase, and xylanase).

Sugar yields obtained by hydrolysis with sulfuric acid were lower than those reported by Saha et al. (2005), who used rice husk pretreated with sulfuric acid (7.83 % w/v, 0.75 % of H_2SO_4 v/v, 60 min, 121°C) prior to hydrolysis with a mixture of cellulase and glucosidase (Celluclast, Novozyme 188), for 72 h at 121 °C. However, the results obtained in this work were higher to the ones reported by Awasthi et al. (2013), who treated water lilies with sulfuric acid (4 % v/v, 121 °C, 15 minutes) and reported 12.63 mg of reducing sugars per gram of water (carbohydrates in watery solution).

Table 3: Sugar yields in studied pretreatments and controls (w/v)

Pretreatments and controls	Reducing sugar yield (%)
Alkaline	27.78 ± 0.19
Acid	3.60 ± 0.63
Steam explosion	22.24 ± 0.99
Organosolv	20.04 ± 1.46
Combined	77.32 ± 2.07
Control 1: Sawdust	22.64 ± 1.79
Control 2: Cellulose	22.40 ± 0.80

Results are mean values ± SD (n=2).

Table 2: Chemical composition of pine sawdust (w/w)

Component (%)	Experimental values (%)	Lima (2013) (*P. pseudostrobus*)	Rutiaga (2001) (*P. pseudostrobus*)
Ash	0.19 ± 0.06	0.35 ± 0.08	0.16 ± 0.02
Lignin	28.94 ± 0.17	25.39 ± 1.09	26.6 ± 0.22
Holocellulose	66.53 ± 0.85	71.02 ± 0.27	67.8 ± 0.17
Hemicellulose	23.55 ± 0.85	17.85 ± 0.27	
α-cellulose	42.98 ± 4.96	53.17 ± 1.35	
Extractives	5.11 ± 0.32	11.35 ± 5.93	4.4 ± 0.20
Total	100.77 ± 1.40	108.11 ± 7.37	98.96 ± 0.61

Data represented as means ± SD (n=2)

Pretreatment using steam explosion led to lower yields than those obtained by López-Miranda et al. (2009), who reported an 8.15 % of reducing sugars after pretreating sawdust pine with steam explosion for 30 minutes 120 °C, at hydrolysis pH of 4.5, for 240 h, with an enzymatic load of 25 FPU/g of total carbohydrates and lower than Shuai et al. (2010) who reported 77.7 % at 180 °C for 30 min using steam explosion and sulfuric acid loading of 5 % on oven-dry wood and a 5:1 liquor to-wood ratio whit spruce wood and enzyme loading of 15 FPU.

Regarding organosolv pretreatment, Mesa et al. (2011) reported higher yield than those obtained in this study. They treated sugarcane bagasse (29.1 % glucose) with 30 % (v/v) ethanol at 195 °C, for 60 minutes. The difference is due to: 1) this process was previously pretreated with diluted acid; 2) there was a later addition of NaOH; and 3) the difference in temperature at which the process was carried out.

Finally, the yields obtained with combined pretreatment were a little lower than those obtained by Alhasan et al. (2010) with rubber tree wood pretreated with 6 % HNO_3 at 100 °C for 60 minutes, followed by 1 % NaOH plus cellulase along with β-glucosidase with 25 FPU and 60 UBC, respectively. Our sugar yields obtained with combined pretreatment was also a little lower than those obtained by Luo et al. (2010), who used the SPORL treatment with the following experimental conditions: a chemical charges sulfuric acid (2.21 %) and sodium bisulfite (8 %) on oven dry pine wood, enzimatic charge of Celluclast 1.5 L (15 FPU/g substrate) and Novozyme 188 (22.5 CBU/g substrate).

On the other hand, sugars yield at the specified conditions using the sodium hydroxide pretreatment was 24.76 % higher than the sawdust control sample without pretreatment. The sulfuric acid pretreatment led to the second lowest reducing sugars results after enzymatic hydrolysis (Table 3). Nevertheless, while performing this pretreatment, the amount of reducing sugars in the aqueous solution was 18.2 %, which was 87.05 % greater than the yield of the sawdust control sample in the solution at the start of the pretreatment. As can be seen from Table 3, organosolv and steam explosion pretreatments, as well as the sawdust sample without pretreating (control sample) exhibited very close results in their corresponding yields of reducing sugars. Therefore, steam explosion and organosolv were not effective pretreatment methods for pine sawdust prior to an enzymatic hydrolysis. The combined pretreatment led to the highest production of reducing sugars after hydrolysis (Table 3), 341.5 % greater than the sawdust sample pattern and 47.8 % greater than the cellulose control sample.

When comparing the saccharification yield obtained with the combined pretreatment and the other methods used in this study, it was found that such production was higher in all the cases. With sodium hydroxide it was 273.7 % higher, with sulfuric acid pretreatment is was 182.6 % higher, and with steam explosion it was 385.5 % higher.

This seems to suggest that a consecutive pretreatment with nitric acid and sodium hydroxide facilitates enzymatic hydrolysis of the pretreated material, since nitric acid helps to partially hydrolyze lignin and hemicelluloses, and sodium hydroxide favors the release of lignin and an increasing amount of amorphous cellulose content in the material. All of the above contribute to a higher reducing sugar concentration in the final product.

Identification of hydrolysis variables

It follows from the factorial design 2^3 results that factor A, time, did not have any significant statistical effect over sugars yield (p=0.1345), whereas factors B and C, concentration of nitric acid and temperature, did have an effect (p=0.0000). These results show that the highest sugars yield (91.26 %) was obtained using the 6 % nitric acid pretreatment, at 100 °C for 30 minutes (7th run) (Table 4). As observed, acid concentration and operating temperature were the same as those reported in Table 1 (before actually beginning the experiments), but in this case time was reduced to 30 minutes, which means a 50 % saving in processing time. Hence, an operating cost reduction opportunity can be spotted when processing this raw material.

Maximization 3^k

The factorial design 3^2 results indicate that the maximum reducing sugars yield was obtained pretreating sawdust with 7.5 % nitric acid, at 120 °C for 30 minutes (Table 5). Such yield is even higher than that obtained with the pure cellulose control sample (Table 1).

Table 4: Design matrix (2^3) of hydrolysis and results

Run	HNO_3 (%)	Temperature (°C)	Time (min)	Reducing sugar yield (%) (w/v)
1	3	50	30	17.98 ± 0.44
2			60	18.63 ± 0.73
3		100	30	56.05 ± 1.17
4			60	51.14 ± 2.04
5	6	50	30	21.05 ± 1.23
6			60	27.6 ± 0.94
7		100	30	91.26 ± 1.41
8			60	93.87 ± 0.07
Control 1 (Sawdust)		-	-	23.37 ± 0.46
Control 2 (Cellulose)		-	-	45.15 ± 0.04

The results have been expressed as means ± SD (n=2).

Table 5: Design matrix (3^2) of hydrolysis and results

Run	HNO3 (%)	Temperature (°C)	Time (min)	Reducing sugar yield (%) (w/v)
1	4.5	80	30	23.24 ± 0.72
2		100	30	42.93 ± 0.74
3		120	30	67.86 ± 0.38
4	6	80	30	55.08 ± 2.27
5		100	30	64.34 ± 1.85
6		120	30	73.95 ± 0.91
7	7.5	80	30	49.93 ± 0.85
8		100	30	81.86 ± 2.12
9		120	30	97.83 ± 1.59
Control 1 (Sawdust)		-	-	21.26 ± 0.58
Control 2 (Cellulose)		-	-	46.39 ± 0.56

The results have been expressed as means ± SD (n=2)

CONCLUSIONS

After comparing the pretreatment methods evaluated in this study, the combined method (with nitric acid and sodium hydroxide) yielded to the best results and it went up to the maximization of getting to pretreatment, the second finding was that the maximum reducing sugars yield (97.83 ± 1.59 %) from raw material was obtained when performing the 7.5 % nitric acid pretreatment, at 120 °C, followed by the 1 % sodium hydroxide at 90 °C, according to the 3^2 factorial design applied.

Acknowledgments

The authors are grateful both to CIC-UMSNH (Project 21.3-JGRQ) and CONACYT (Project 16644) for all support provided, and deeply appreciate the study material donated by the Indigenous Community of San Juan Parangaricutiro, Michoacán.

Declaration of interest

The authors declare that they have no conflict of interest. The authors are alone responsible for the content and writing of the paper.

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Bioenerg. Res.
DOI 10.1007/s12155-016-9757-8

Tequilana weber Agave Bagasse Enzymatic Hydrolysis for the Production of Fermentable Sugars: Oxidative-Alkaline Pretreatment and Kinetic Modeling

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Abstract As a way to mitigate the harmful effects of fossil fuel utilization, the use of second-generation ethanol has been proposed. However, the microorganisms responsible for its production are not able to degrade structural polysaccharides, so their hydrolysis is necessary. Previously to this work, a factorial experimental design was carried out to investigate the relation between the NaOH and H_2O_2 concentrations with the yield of carbohydrates, and then this variable was optimized by using a response surface method. A study of the hydrolysis process was performed using enzymes to establish a process that maximizes the depolymerization of *Agave tequilana* fibers after an alkali-oxidative pretreatment with optimal reagents concentrations, this pretreatment was selected because it can remove almost the total content of lignin and destroys efficiently the crystallinity of cellulose fibers with a lower sugar losses and no production of toxic compounds. An orthogonal array using the novel enzymes Cellic CTec 3 and Cellic HTec 3 was performed to determinate the optimal combination of them, which has resulted in a concentration of 165.67 g/L at the supernatant with 82.21 % conversion and a yield of 352.18 g reducing sugars per kilogram of lignocellulosic material in dry basis. These results are 29 % better in comparison with the previous generation of enzymes with a reduction in the enzymatic charge of 82 %.

Keywords Enzymatic hydrolysis · *Agave tequilana* · Kinetic modeling

Introduction

At present, it is impossible to imagine daily activities without energy; all the development humanity has achieved as a species is linked to energy transformations. Multiple sources of energy have been used for this purpose, among which the most widely used has been fossil energy, including coal, natural gas, and petroleum, due to its low production cost and relative abundance. Petroleum is the most widely exploited energy source in the world. Approximately 80 % of the energy consumed comes from this source, and it is calculated that each person on the planet consumes 4.8 barrels of oil per year [1]. Oil is a nonrenewable resource, and its cost will be always uncertain because its availability is limited to certain territories. Despite ventures into biofuel production, effective technologies to produce advanced biofuels from biomass feedstock are still lacking compared with the well-established fossil fuel technologies [2]. Therefore, proposals have been made for the utilization of alternative fuels, such as ethanol, which is currently produced from food crops, such as sugarcane and corn, thus reducing their availability for human consumption. It is feasible to produce cellulosic ethanol from all plant parts that are not likely to be used as food, eliminating the utilization of resources oriented toward human consumption to produce fuel. Ethanol produced in this way is known as second-generation ethanol.

Mexico is considered the central origin of the Agave genus [3, 4], which according to Granados [5], includes 272 species, while other authors report from 136 to 150 species [3, 6]. Agave species have been used to meet and complement a number of basic needs, such as food, fiber, fodder, medicine, construction, and the production of alcoholic beverages [7]. The Agaves are widely distributed in limiting environments (shallow, infertile, and dry soils), which makes them attractive for use as energetic crops.

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Agave tequilana is a succulent plant species belonging to the ancient family of the agavaceas, now subfamily *Agavoideae*. It is monocarpic, blooms once in its life and then dies. Its flowering occurs after a decade. The plant produces a flower stalk a few meters high, with many umbels of greenish tubular and vertical flowers. Pollination depends exclusively on the *Leptonycteris nivalis* species of nectarivore bat.

This plant is used mainly for tequila production in the western part of Mexico. Under the laws of the country, only *Agave tequilana azul weber* can be used to produce the spirit drink [1]. Other species may be used to produce mezcal. The part of the plant with the major commercial interest is the stem of the plant, called the "pineapple," which is used to obtain, through a cooking process, a fructose-rich syrup that is fermented to produce tequila. Afterward, a material with no important application is obtained, the bagasse, which is considered a possible residue for use in the second-generation biofuel production of bioethanol. In the western state of Jalisco, Mexico, approximately 1.7 million tons of annual *Agave tequilana* are produced, according to the INEGI [2].

In previous studies, such as Saucedo-Luna et al. [11], the hydrolysis of lignocellulosic materials of *Agave tequilana* was conducted. However, the results were unsatisfactory, both in sugar concentration in the hydrolysate and the total yield. A similar study used ammonia fiber explosion (AFEX) to treat the lignocellulosic material of prairie grass taken from several stations and locations [3]. This study used the enzymes Acellerase, Novozyme 188, Multieffect Xilanase, and Multieffect Pectinase. This configuration achieved between 400 and 500 g of reducing sugars per kilogram of lignocellulosic material, depending on the station and the place where the samples were taken. On the other hand, the work of Equihua S. [4] reported reducing sugar concentrations near 80 g/L using the Acellerase 1500 enzyme acting on sweet sorghum bagasse pretreated with the same conditions as in this study. Therefore, it is necessary to continue the use of synergies between different types of enzymes, as proposed in Hu et al. [5], where Celluclast 1.5 L, Novozyme 188, and Multieffect Xilanase were used in combination to achieve near 70 % conversion.

Based on this information, this work studied the synergistic effect of a mixture of several enzymes: Celluclast 1.5 L as cellulase, Novoenzyme 188 as beta-glucosidase, and Viscozyme L as xylanase. Furthermore, the new generation enzymes Cellic CTec3 and Cellic HTec3 (enzyme complexes including all necessary components to achieve high levels of fiber depolymerization) from Novoenzyme were used in the enzymatic hydrolysis of the *Agave bagasse tequilana weber* previously treated by an alkaline-oxidative method.

Table 1 Two-level orthogonal L8 array to study enzymatic hydrolysis

	Amount of enzyme (mL/g material)		
Experiment	Viscozyme L	Celluclast 1.5 L	Novozyme 188
1	0.1	0.1	0.1
2	0.1	0.1	0.5
3	0.1	0.5	0.1
4	0.1	0.5	0.5
5	0.5	0.1	0.1
6	0.5	0.1	0.5
7	0.5	0.5	0.1
8	0.5	0.5	0.5

Materials and Methods

Raw Material Conditioning

Lignocellulosic material, a residue of the cooking of *Agave tequilana* pineapples, was obtained from the mezcal industry "Ex hacienda de Zacapendo," located in the municipality of Indaparapeo, Michoacan, Mexico. It was dried at 80 °C for 24 h in a Novatech electric furnace and then partially reduced in size in a generic blade mill at 1500 W to obtain heterogeneous fibers ranging from 15 to 3 mm long with diameters of 0.3 mm. The experiments were performed with this fiber size, which is higher than the size usually used, mesh 40 spherical particles.

Fiber Composition Analysis

A sample of the conditioned material was subjected to leaching with saturated water at 120 °C for 1 h to remove the water-soluble compounds, which were determined by weight difference. The remaining material was analyzed to determine the lignin content through total hydrolysis of the polysaccharides with sulfuric and hydrobromic acid [6]. Holocellulose [7] and alpha cellulose [8] were all determined in extractive-free bagasse. Finally, the ash content was determined by calcination [9]. The differences between holocellulose and alpha cellulose were considered as hemicellulose.

Table 2 Two-level orthogonal L4 array to study enzymatic hydrolysis

	Amount of enzyme (as a percentage of material to be hydrolyzed)	
Experiment	CTec 3	HTec 3
1	6	3
2	6	6
3	9	3
4	9	6

Table 3 Composition of the initial lignocellulosic material

Compound	(% *w/w*)
Soluble matter	44.29 %
Lignin	13.57 %
Holocellulose	40.14 %
Alpha cellulose	26.81 %
Hemicellulose	13.33 %
Ash	2.00 %

Pretreatment

The dried and ground lignocellulosic material was treated by an alkaline-oxidative method consisting of two stages. In the first stage, a solution of sodium hydroxide was added, 6 % by weight dry basis, in a 1 to 5 proportion in relation to the solid, and the mixture was placed in an autoclave at 120 °C and 2 atm for 1 h. Then, the mixture as left to cool, and a solution of 6 % hydrogen peroxide by weight dry basis was added at the same solid-liquid ratio, then maintained at 30 °C without agitation and protected from light for 24 h. At the end, the material suspensions were acidified with hydrochloric acid to a pH of 5 before filtration. The material intended for enzymatic hydrolysis was not dried to avoid the structural changes induced by dryness [10]; however, samples were dried to determine process' efficiency and the residual lignin by total hydrolysis with sulfuric acid and hydrobromic acid, according to the procedure of Runkel, Wilke [6].

Enzymatic Hydrolysis

To maximize the depolymerization of the cellulose fiber and the hemicelluloses present in the pretreated material, the combination of enzymes was proposed and tested in an orthogonal L8 array of two levels and three factors. Each factor was one of the three enzymes used: Viscozyme L, Celluclast 1.5 L, and Novozyme 188. The lowest level was 0.1 mL/g material dry basis, and the highest was 0.5 mL/g of material dry basis. Details of the orthogonal array are shown in Table 1.

At the same time, hydrolysis of the same pretreated material was performed using a combination of ultimate generation enzymes, which were generously donated by Novoenzymes: Cellic CTec3 and Cellic HTec3. The manufacturer recommends that the amount to be used should be between 5 and 10 % of the weight dry basis of the material to be hydrolyzed. For this reason, an orthogonal L4 array of two factors and two levels was proposed to analyze the combined effect of those two enzymes. In this case, the highest level was 9 % enzyme in relation to the total amount of material to be hydrolyzed, and the lowest level was 3 %. The series of experiments is shown in Table 2.

For the enzyme addition, we carry out the next process; we dilute the needed commercial product in the solution of citrate buffer 0.05 M with a pH = 5.0 until the reaction volume was complete. So the total reaction volume was the same for every experience. All experiments were performed in triplicate on a Polyscience orbital shaker at 50 °C and a pH of 5 in 0.05 M citrate buffer. The solid/liquid ratio was in all cases 1:5. The time of the reaction was 72 h. At the end of this period, a sample was taken for analysis by a standardized method in a high-resolution liquid chromatography (HPLC) Varian Pro Star instrument, using a Metacarb 87 C column at 70 °C and a refraction index detector at 50 °C with isocratic water flux to 1 mL/m to determine the simple sugars. Data were analyzed by a signal to noise ratio method.

Reaction Kinetics

To determine the kinetics of the enzymatic reaction, a hydrolysis experiment was conducted in triplicate in 0.05 M citrate buffer, pH 5. The pretreated material/liquid ratio was 1:5. The optimal combination of enzymes was used (6 % Cellic CTec 3 y 6 % HTec 3), and the temperature was maintained at 50 °C, with stirring at 150 rpm in a Polyscience orbital shaker.

Table 4 Experimental design results with the enzymes Viscozyme, Celluclast 1.5 L, and Novozyme 188

No.	Amount of enzyme (mL/g material)			Reducing sugars obtained (g/L)		
	Viscozyme L	Celluclast 1.5 L	Novozyme 188	72 h		
1	0.1	0.1	0.1	106.64	±	2.04
2	0.1	0.1	0.5	98.65	±	1.96
3	0.1	0.5	0.1	128.07	±	1.15
4	0.1	0.5	0.5	121.95	±	1.57
5	0.5	0.1	0.1	112.79	±	2.45
6	0.5	0.1	0.5	110.46	±	1.05
7	0.5	0.5	0.1	118.07	±	2.58
8	0.5	0.5	0.5	116.98	±	0.89

Table 5 Experimental design results using the Cellic enzymes from Novoenzymes

	Enzyme concentration (% material to be hydrolyzed)		Obtained sugars (g/L)		
Experiment	Ctec 3	HTec 3	72 h		
1	6	3	126.92	±	2.34
2	6	6	165.67	±	2.26
3	9	3	151.81	±	1.98
4	9	6	142.24	±	2.55

Samples were taken at 1, 6, 24, 48, and 72 h and analyzed by HPLC.

Results and Discussion

Composition of *Agave tequilana* bagasse

The composition of the lignocellulosic material is shown in Table 3.

It is observed that in agave bagasse obtained as residue from the tequila production is a large amount of soluble material, which can be removed by simple boiling water; this soluble material consists mainly of inulin, this was confirmed by analyzing the sample supernatant removal process soluble material by HPLC with the standardized method. Experimental results on the composition of agave bagasse correspond with the data obtained by other authors as Yang et al. [11], who reports 13.8 ± 1.3 % lignin, 43.8 ± 1.3 % of holocellulose and 6 % ash and Flores-Sahagun et al. [12], who reports 21.10 ± 1.13 % lignin, 73.6 ± 0.01 % of holocellulose and 5.3 ± 0.07 % ash in dry soluble free base, these small changes can be attributed to the growth zone harvest time and *Agave tequilana*.

Analysis of the Experimental Designs

Table 4 shows the results of the enzymatic hydrolysis with the ternary combination of enzymes (Celluclast 1.5 L, Novozyme 188, and Viscozyme L), and Table 5 shows the results of the experiment with the ultimate generation enzymes (Cellic CTec 3 and Cellic HTec 3). Data obtained throughout the experiment are shown as averages of triplicate experiments.

The total sugar concentration data at 72 h was analyzed by the methodology described in Taguchi et al. [13] for the signal to noise index. The best combination of enzymes to maximize the amount of simple sugars released was found to be represented by experiment number 3, i.e., for each gram of the sample dry basis, it is optimal to add 0.5 mL of Celluclast 1.5 L, 0.1 mL of Viscozyme L, and 0.1 mL of Novozyme 188 to obtain the highest content of 128.07 ± 1.15 g/L of sugars. Saha, Cotta [14], reported a concentration of sugar equal to 41.5 g/L using a commercial enzyme pretreatment (cellulase, α-glucosidase, and xylanase) with a 24 h alkaline-oxidative pretreatment of wheat straw with NaOH 8.6 %, *w*/*v* and H_2O_2, 2.15 % *v*/*v* at 35 °C. The solid-liquid ratio in the hydrolysis was not reported.

When analyzed by the same methodology, the results obtained using the Cellic enzymes show that the superior combination of enzymes is experiment number 2, with 6 % Cellic CTec3 and 6 % Cellic HTec3.

The results of the experimental designs identified the optimal combinations of enzymes in their corresponding series; however, only through using the Cellic enzymes could the amount of enzyme used be decreased, as hydrolyzing the *Agave tequilana* bagasse with these enzymes requires almost six times less than the set load of Viscozyme L, Celluclast 1.5 L, and Novozyme 188. Therefore, it is concluded that to hydrolyze 1 g of the pretreated material dry basis by the alkaline-oxidative method, 60 mg of Cellic CTec 3 and 60 mg of Cellic HTec 3 are required, with a conversion of 80.49 % and a yield of 614.09 sugars per kilogram of *Agave tequilana* removals-free bagasse with a concentration of 165.67 g/L of reducing sugars (136.44 g/L of glucose and 29.23 g/L of xylose). These yields are high compared to the results of Farías-Sánchez et al. [15], who obtained a concentration of 46.24 g/L reducing sugars with a pretreatment using 7.5 % nitric acid at 120 °C, followed by 1 % sodium hydroxide at 90 °C and enzymatic hydrolysis with 25 UPF of Celluclast 1.5 L from NOVOZYME per gram of pretreated sample from pine sawdust, this study was carry out with a solid-liquid ratio of 1:10. Saucedo-Luna et al. [16] obtained 41 g/L of fermentable sugars, corresponding to a yield of 73.6 %, with 2 % sulfuric acid at 147 °C for 15 min and

Table 6 Data to construct the kinetics of the enzymatic reaction of the Cellic enzymes

Time (h)	1	6	24	48	72
Total sugars (g/L)	8.98 ± 1.56	59.68 ± 2.44	123.56 ± 3.67	151.75 ± 3.37	165.67 ± 3.08

Table 7 Variation of the substrate concentration versus time

t (h)	0	1	6	24	48	72
[S] (g/L)	188.58	180.42	134.33	76.25	50.63	33.73

enzymatic hydrolysis of agave bagasse for 72 h at 40 °C using a solid-liquid ratio of 1:10. Cheng et al. [17] obtained 32.5 g/L of fermentable sugars from sugarcane bagasse using an alkaline-oxidative pretreatment with 1 % NaOH and 0.6 % H_2O_2 and enzymatic hydrolysis with 4 % cellulase at 50 °C, pH 4.5, stirring at 140 rpm with a solid liquid ratio of 1:20. Finally, Rana et al. [18] obtained 130.94 g/L of total sugars and a yield of 81 % using Cellic CTec2 to hydrolyze corn stover with a solid liquid ratio of 1:5. As shown in the above comparisons solid-liquid ratio is higher with all the authors except Rana et al. [18] who use 1:5, the hydrolysis yields remain higher in this article.

Kinetics Reaction

After optimizing the combination of enzymes, it is necessary to approximate the results by a mathematic model that allows future scaling. Because the commercial solutions of enzymes used are themselves combinations of unknown composition and enzyme types, it is not possible to determine the Michaelis-Menten kinetics. However, during hydrolysis, at least the following reactions occur:

- The breaking of the cellulose chains into variable length oligosaccharides.
- The conversion of oligosaccharides chains into cellobiose.
- The cleavage of molecules of cellobiose to glucose.
- The hydrolysis of hemicellulose.

Each reaction is catalyzed by a specific type of enzyme, and the concentration of each reagents involved is difficult to measure in a given time. Thus, to construct the kinetic model, only the overall effect of all enzymes was considered, which consists of breaking the holocellulose into simple sugars (or reducing sugars).

In Table 6, the total sugar concentrations obtained in this experiment are listed along with their averages (using 60 mg Cellic CTec3 and 60 mg Cellic HTec3 per gram of raw material pretreated in dry basis).

Because the enzymatic reaction rate is related to the substrate concentration, the polysaccharide concentration [S] was calculated according to Eq. (1)

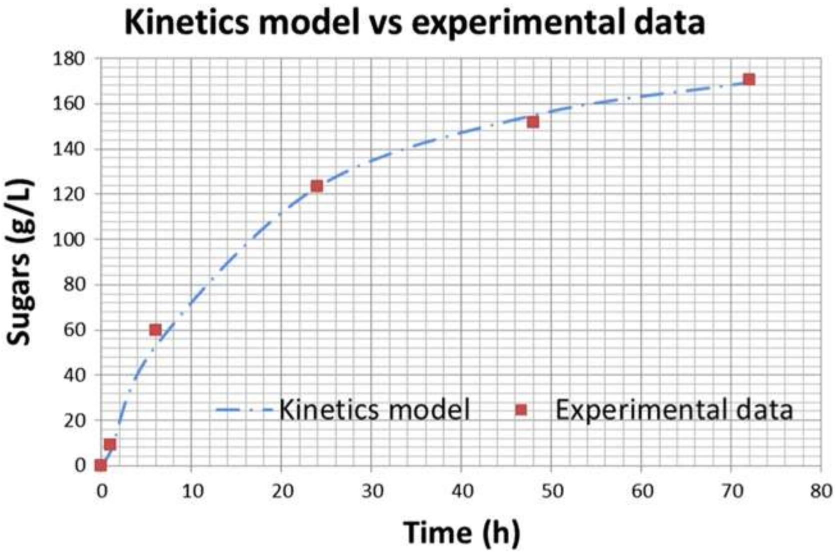
$$[S] = \frac{\left\{w_0 * \left(1 - f_{lig}\right)\right\} - \left\{[P] / 1.1\right\}}{V_R} \tag{1}$$

where the variable meanings are as follows: w_0, weight of the initial pretreated material in grams; f_{lig}, fraction of lignin in the initial solid; $[P]$, concentration in grams per liter of total sugars at the time calculated $[S]$; V_R, total reaction volume, which includes the moisture contained in the fibers of the pretreated material and the citrate buffer added to produce a desirable solid-liquid proportion. The factor 1.1 is dimensionless and represents the fact that for each mole of sugar hydrolyzed, a mole of water is necessary.

By performing this calculation for each instant with a calculation basis of 1 L as a total reaction volume and a 1:5 ratio to the added solid, the data in Table 7 were obtained and fitted to a second-order kinetics model in the form expressed by Eq. (2):

$$\frac{d[S]}{d_t} = -k[S]^2 \tag{2}$$

Fig. 1 Graphic representation of kinetics model vs experimental data



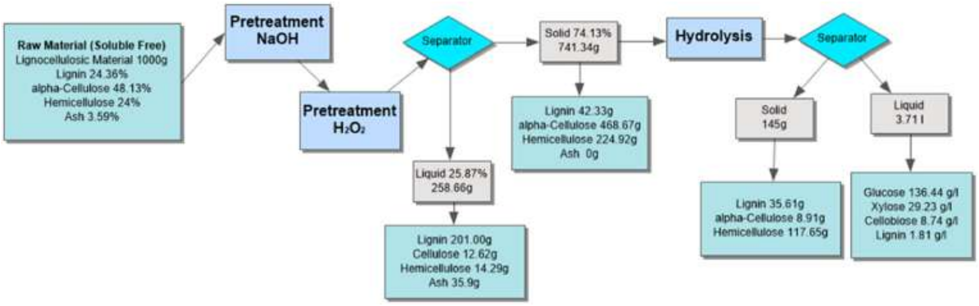


Fig 2 Diagram showing mass balance for the complete process

where [*S*] is the substrate concentration (polysaccharides), *t* is the reaction time in hours, and *k* is the rate constant to be determined in liters per gram-hour (L/g*h).

After integration and mathematical transformation, it was possible to adjust the data by the least squares method, finding that $k = 3.29 \times 10^{-4}$ L/g*h with an $R^2 = 0.9952$. The actual modeling is very similar to the real experimental data, as shown in Fig. 1. The dotted line represents the kinetics model, and the single red squares indicate the experimental data.

In order to describe the actual process completely, a matter balance was established and it is showed in Fig. 2. The raw material was considered soluble free basis because the soluble matter is mainly inulin, which is removed from the fibers by the pretreatment. This mass balance does not show any inlet stream in order to simplification of the diagram, but the reader must take into account that in the pretreatment process, there is two inlet streams, 5 L of 6 % *w*/*w* sodium hydroxide solution and 5 L of 6 % *w*/*w* hydrogen peroxide solution. After the pretreatment, the reaction volume (10 l) is neutralized with concentrated HCl until pH = 5.0. Then, the solid is separated by filtration and moisture is determinated. Before the hydrolysis, another stream made up mainly by the mixture of citrate buffer (0.05 M, pH = 5.0) and the necessary amount of Cellic enzymes (6 % of CTec3, 6 % of HTec3; dry basis) until the 1:5 solid-liquid ratio is complete. For the calculus base showed in the diagram, the total volume of reaction is about 3.71 L. The concentration of reducing sugars must be considerate in this base (3.71 L). There is an important assumption we made writing this mass balance, the solid-liquid separations are showed as every liquid can be separated from the solid phase.

During the pretreatment, there is not a significant loss of carbohydrates (approximately 3.7 %); on the other hand, an 82.62 % of lignin was solubilized by the effect of pretreatment. This lignin removal can be seen visually in the Fig. 3. The (a) part is a microphotograph at 50× of the raw material and the (b) part is the pretreated material seen at the same magnification, in fact the black color observed is an indicator of lignin content.

On the other hand, the pretreated matter did not need any detoxification procedure further to the neutralization until pH = 5.0, this fact suggest the idea that alkaline-oxidative pretreatment does not produce toxic compounds like furfural. Also, because the pretreated material did not wash with fresh water, this pretreatment could be environmentally feasible.

After the hydrolysis process a small amount of solid material remained, this residual matter contents approximately the 3 % of structural carbohydrates of the raw material.

Conclusion

This research demonstrates the effectiveness of alkaline oxidative pretreatment since there was a loss of structural carbohydrates equal to 3.84 % while 82.62 % of a lignin in the raw material was removed. Furthermore, the crystal structure of the cellulose was destroyed effectively as evidenced by the fact that 98.09 % of the cellulose in the pretreated material

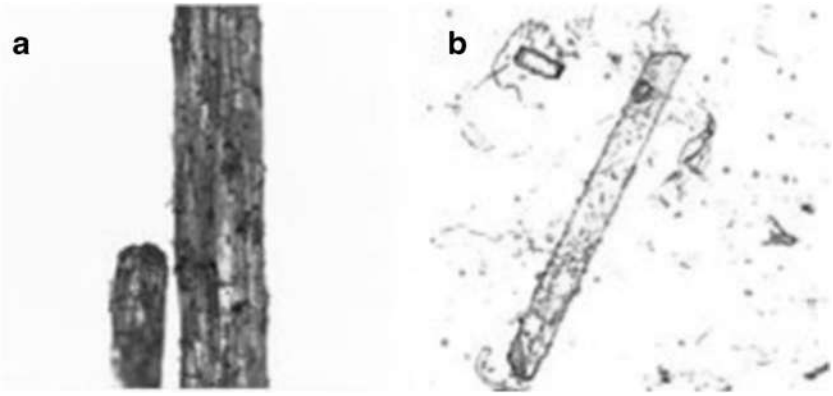


Fig. 3 Optical microphotograph at 50× of *A. tequilana* bagasse **a** before the pretreatment and **b** after pretreatment

was depolymerized; however, only 47.69 % of the hemicellulose is hydrolyzed. By testing combinations of enzyme complexes (Celluclast 1.5 L, Viscozyme L y Novozyme 188), the interactions and synergetic workings achieved can be observed. These combinations increase the obtainable yields compared to a single-enzyme complex (Celluclast 1.5 L). In addition, upon comparing these results with ultimate generation enzyme complexes (Cellic Ctec 3 and Cellic HTec 3), the yield was enhanced by much more than for common enzyme complexes, from 128.07 ± 1.15 to 165.67 ± 2.26 g/L of sugars, which represents an increase of 29.35 %. All of these results indicate that the ultimate generation enzyme complexes Cellic Ctec3 and Cellic HTec3 are viable for use at an industrial level to increase the yield of sugar with a reduction of 82.85 % of enzyme required than for the old enzymes.

Because the commercial solutions of enzymes used are themselves combinations of unknown composition and enzyme types, it is not possible to determine the Michaelis-Menten kinetics, so the data were approximated by a second-order kinetics model, which should be useful for scaling up the process. The reaction constant obtained by that procedure is small compared with the catalytic chemical process; however, it is not comparable because it is a biochemical reaction and no data have been reported on this enzyme complex.

Acknowledgments This work was supported by the Mexican Council of Science and Technology (CONACYT) through the Bioenergy Thematic Network ("Red Temática de Bioenergía").

This paper could not be possible without the generous donation of Cellic CTec 3 and HTec 3 made by Novozymes.

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Olefins and Ethanol from Polyolefins: Analysis of Potential Chemical Recycling of Poly(ethylene) Mexican Case

DOI 10.1515/ijcre-2015-0217

Abstract: Plastic solid waste (PSW) presents challenges and opportunities to society regardless of their sustainability awareness and technological advances. A special emphasis is paid on waste generated from polyolefin sources, which makes up a great percentage of our daily commodities' plastic products. In Mexico 7.6 millions of tons of plastic in 2012 were wasted, which low density polyethylene LDPE, and high density polyethylene HDPE were the most abundant. Increasing cost, and decreasing space of landfills are forcing considerations of alternative options for PSW disposal. Years of research, study and testing have resulted in a number of treatment, recycling and recovery methods for plastics that can be economically, and environmentally viable. The following work studies the possibilities of polyethylene recycling. Nowadays, non-catalytic thermal cracking (Pyrolysis) is receiving renewed attention, due to the fact of added value on a crude oil barrel and its very valuable yielded products, but a fact remains that advanced thermo-chemical recycling of polyolefin still lacks the proper design, and kinetic background to target certain desired products and/or chemicals. On the other hand some research have shown a good performance that can be used in a real plant. ASPEN Plus is used to simulate a non-catalytic thermal cracking process. The process behavior of simulation is similar to the experimental data from other authors. Using gibbs free energy to identify the chemical equilibrium in system, its global minimization allows identifying the amount of substances present in the process. The simulation results demonstrate that it could be produced 49 % and 34 % wt of ethylene and propylene respectively from gas yield at 850 °C. Then scale the plant to produce ethylene and propylene from the pyrolysis and ethanol from a direct hydration of ethylene. Aspen Process Economics Analyzer is used in order to find the feasibility of the pyrolysis and ethanol production. The total sales/total production cost ratio obtained for the integrated process approaches was 2.55.

Keywords: ASPEN, pyrolysis, polyethylene, ethylene, propylene, ethanol

1 Introduction

Polyethylene (PE) have excelled among many other materials due to its properties, such polymer is used as containers for different products, mainly in food, agricultural, construction, textile, automotive among others. Plastics have accelerated their output by continuously adding new products to be emerging in market. China leads world production with 15 % of all world plastic followed by Germany with 7 %; Mexico produces only 2 % of the 241 million tons produced globally. Figure 1 shows the production of plastics worldwide (Plasticseurope 2013).

The Plastic products industry is important in Mexico since nationwide is to 3.6 % of gross domestic product manufacturing (GDP). About 5.2 million ton were consumed in 2012, of which for its chemical, thermal and mechanical properties, the high density polyethylene (HDPE) and low density polyethylene (LDPE) were the most used (Conde Ortiz 2013).

At 2013, NOM-161-SEMARNAT-2011, was published in Mexico establishing some criteria for classify PSW and other wastes that are generated in high level. In that year, 4.2 million tons of plastic were discarded and only 11 % were recycled to produce plastics again, 2 % were used for energy recovery, discarding more than 3.6 million tons of plastic waste.

It has to be analyzed which can be an alternative in the reuse of waste sources, that can replace fossil and agricultural sources. In this sense it is necessary to propose methods for recycling or reuse plastics generated by different products.

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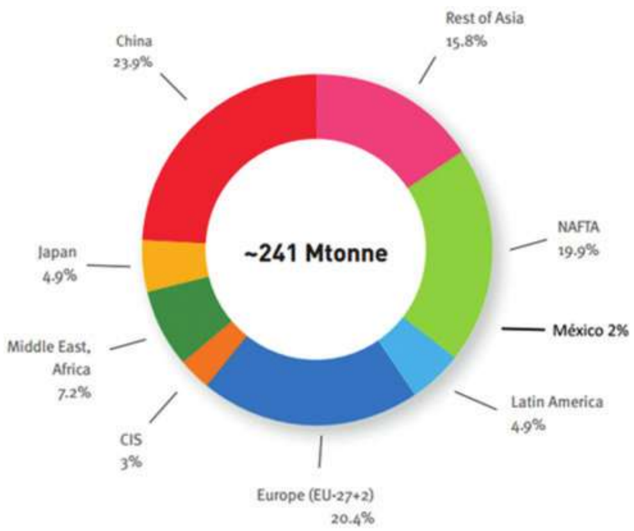
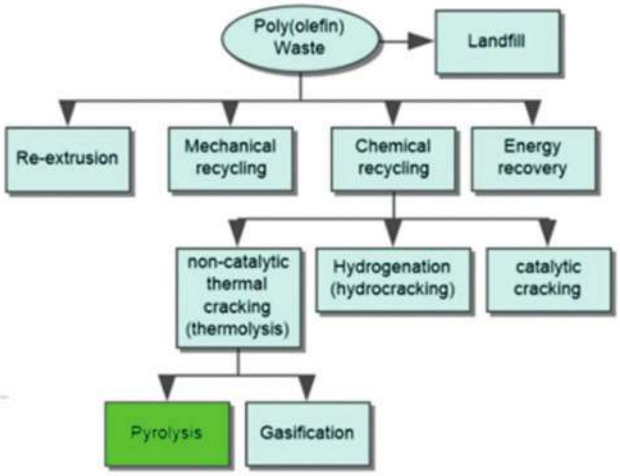


Figure 1: World plastics materials production 2012.

PSW from commercial grade resins have been successfully recycled from a number of end-products, including: automobile parts, appliances, textiles, mulches, greenhouses and films (Al-Salem, Lettieri, and Baeyens 2009). PSW treatment and recycling processes could be allocated to four major categories (Mastellone 1999): re-extrusion (primary), mechanical (secondary), chemical (tertiary) and energy recovery (quaternary) as shown in Figure 2. Each method provides a unique set of advantages that make it particularly beneficial for specific locations, applications or requirements. Mechanical recycling (i. e. secondary or material recycling) involves physical treatment, whilst chemical recycling and treatment (i. e. tertiary encompassing feedstock recycling) produces feedstock chemicals for the chemical industry. Energy recovery involves complete or partial oxidation of the material (Troitsch 1990), producing heat, power and/or gaseous fuels, oils and chars besides by-products that must be disposed of, such as ash.



Q2 **Figure 2:** Recycling options of plastics solids wastes.

Pyrolysis provides a number of other advantages, such as operational and environmental, moreover financial benefits. Pyrolysis requires no flue gas clean up as flue gas produced is mostly treated prior to utilization. Environmentally, pyrolysis provides an alternative solution to landfilling and reduces greenhouse gas (GHGs) and CO_2 emissions. Financially, pyrolysis produces a high calorific value fuel that could be easily marketed and used in gas engines to produce electricity and heat (Al-Salem, Lettieri, and Baeyens 2009).

In particular, PE has been targeted as a potential feedstock to add valued product produced from pyrolysis process. Many authors (6–13) have studied the thermal cracking behavior of HDPE, LDPE and others polyolefins. It was reported that PE thermally cracks into gases, liquids, waxes, aromatics and char (Al-Salem, Lettieri, and Baeyens 2009a). Non catalytic thermal cracking product varies from gas, liquid, char or mixtures depending upon the type of pyrolysis mode such a variety of reactors, which include fixed bed (Williams and Williams 1999), fluidized bed (Kaminsky, Predel, and Sadiki 2004), ultrafast (Milne, Behie, and Berruti 1999), free-fall reactor (FFR) (Bilgesu et al. 2006), and autoclave (Mastral et al. 1999), under different process conditions. Although pyrolysis can be performed under various modes, the ease in operation and commercial scale up favor a fluidized-bed operation evidenced from the majority of the work using a fluidized-bed pyrolyzer. From all this studies is certain conclude that as the pyrolysis temperature increases, the gas yield increases with a simultaneous decrease in the oil and wax content. The trend is markedly significant at temperatures above 650 °C and until 850 °C. The residence time has an effect only at low and moderate temperatures (<730 °C). At very high temperatures (above 780 °C), the yield of gases remains unaffected by residence times. However, there is not sufficient data available in the literature to distinguish the effects of the residence time from the pyrolysis temperature.

Among the known methods for converting biomass to user-friendly fuels, fast pyrolysis is a promising technique (Ghodke, Ganesh, and Mahajani 2015). Most of the studies for monomer recovery have mainly focused on gas production process, Milne et al. employed temperatures ranging from 780 to 860 °C and residence times of about 400–600 ms for fast pyrolysis of LDPE. Pyrolysis has a gas yield bigger than 90 wt % and an alkenes-rich (as high as 75 wt %) gas stream. Sodero, Berruti, and Behie (1996) reported that the maximum C2–C4 olefin production (ca. 48 wt % and 30 % ethylene) was obtained at reaction conditions of 900 °C. Kannan, Shoaibi, and Srinivasakannan (2014) has the most high yield of gas from the literature at a temperature around 950–1,000 °C and recovers up to 48 % ethylene monomer with a gas yield of >99 %.

Ethylene is used not only as a monomeric raw material for polymerization but also as a starting material for the synthesis of important industrial chemicals, as ethanol, produced from a direct hydration of this compound (Fernelius, Wittcoff, and Varnerin 1979). Anhydrous ethanol is one of the fuels produced today and it is a subset of renewable energy. It is considered to be an excellent clean-burning alternative fuel for gasoline (Kumar, Singh, and Prasad 2010). Ethanol, ethylene and propylene has several applications, but considering the established biofuel worldwide and the potential increase of plastics market, these uses are the most promising (Rodrigo and Alvarenga 2013). In this sense, the objective of this study was to evaluate process that produce raw material for the market needs as is show in the Figure 3.

There are many products that can be obtained from pyrolysis and others materials from that products, however there is no probe of its feasibility. The challenge of this work is to simulate a thermochemical process in order to find a technical and economical choice that can mitigate the PSW generation and probe that this kind of technology can be used in Mexico and other countries.

2 Material and methods

Aspen Plus is basically a program simulation of chemical processes, which in addition to flowcharts simulations, it can perform: (1) estimation of properties of compounds, (2) sensitivity analysis of process variables, (3) obtain process design's specifications, and (4) synthesis and analysis of chemical processes, among other tasks of process design and equipment (Mitta et al. 2006). Many authors have worked in gasification and pyrolysis of LDPE and HDPE, in such research demonstrates the efficiency of experimental chemical processes (Kannan, Shoaibi, and Srinivasakannan 2011). In this work HDPE are studied by thermochemical pyrolysis method using Aspen 8.8 simulation software. Experimental test has proved that this pyrolysis process produces mainly ethylene and propylene from polyolefin (Tukker et al. 1999).

Figure 4 illustrates the process flow sheet of the simplified model. The first stage corresponds to the pyrolysis, where the polyethylene sample was processed to crack into monomers of ethylene and propylene. The polymer stream labeled as "PE" was defined whit pure polyethylene as an "oligomer" stream and the monomer ethylene-r is used to obtain the polymer properties from Van Krevelen equations (Van Krevelen 2009). Polymer NRTL equation of state with Henry's law "POLYNRTL" was chosen as parameter models to calculate the thermophysical properties of the component.

At first, the polymer stream was introduced into a pyrolysis unit "PYROLYS", which was modeled in Aspen Plus using an RGibbs module. The stream leaving the pyrolysis, labeled "PYROGAS" contains mainly ethylene, propylene, but-1-ene, cis-but-2-ene, trans-but-2-ene, 2-methylpropene and is in gas phase.

This stream, at 850 °C, was fed to a heat exchanger unit that recovers the thermic energy of outlet stream of

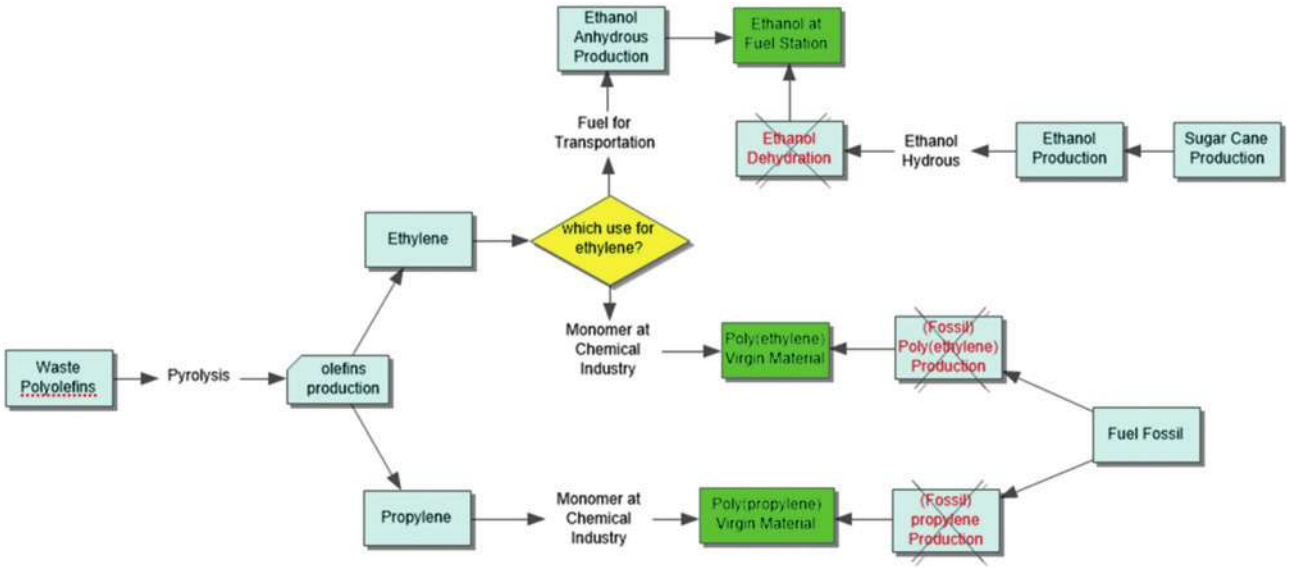


Figure 3: Simplified flowchart of three possible uses for polyolefin considered in this study.

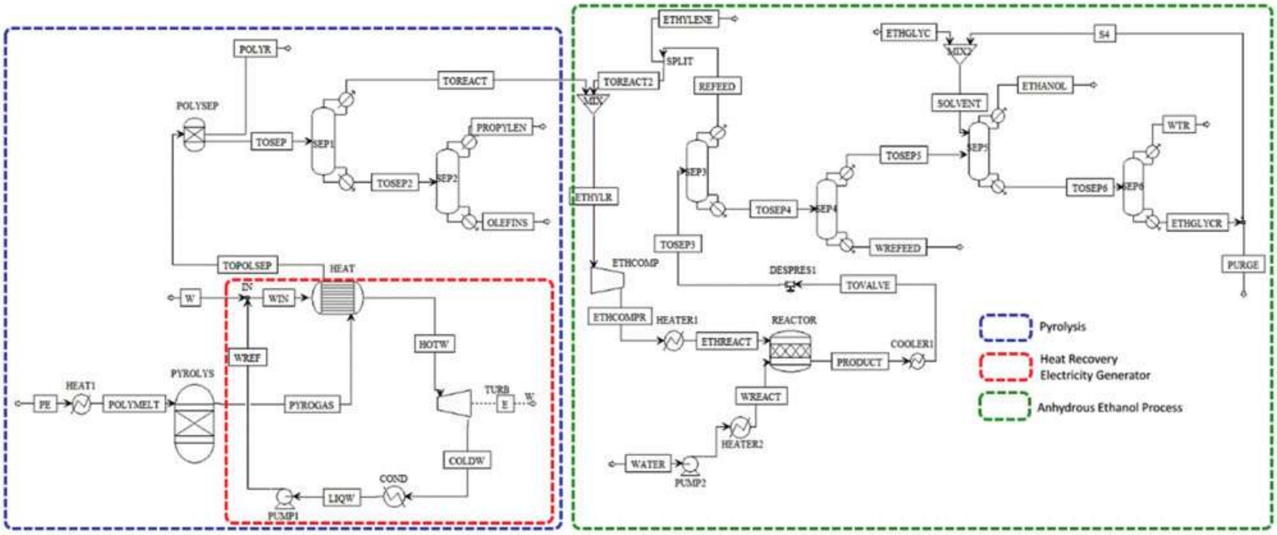


Figure 4: Process flow diagram of polyethylene pyrolysis and ethanol production in Aspen plus.

pyrolysis process and generates water steam that is used to produce electricity in a turbine. After that, the cold stream “TOPOLSEP” is fed to olefins separation section. The first column, separates ethylene in the overhead product and the rest of the components outlet in the bottom product and go to another distillation column that separates propylene of heavier gases. Peng Robinson thermodynamic model is used to calculate equilibrium phase.

In a direct hydration process ethanol is manufactured by reacting ethylene with steam. The reaction is reversible, and the formation of the ethanol is exothermic. Only 5 % of the ethylene is converted into ethanol at each pass through the reactor. By removing the ethanol in another distillation column from the equilibrium mixture and then recycling the ethylene into the reactor, in the same stream have ethylene product. It is possible to achieve an overall 95 % conversion from this process. Ethanol at 55 % from the bottom is feed into another column and a 91 % pure goes into an extractive distillation to achieve an ethanol 99.5 %.

The entire process was modeled using Aspen’s built-in unit operation library in three stages; pyrolysis, ethanol production and energy recovery. The three stages are discussed separately in the following sections.

2.1 Pyrolysis description

The Pyrolysis process models available in literature can be generally classified under steady state or quasi-steady state or transient state models (Al-Salem, Lettieri, and Baeyens 2009). The steady state models do not consider the time derivatives and are further classified as kinetics free equilibrium models or kinetic rate models (Paviet, Chazarenc, and Tazerout 2009). From other authors it can be probe that residence time do not affect at high temperatures.

A Gibbs reactor called “PYROLIS” was implemented in software for the gas production, which was fed with only polymer. Ten levels of pressure were tested (from 1 to 10 atm) and the analyzed temperature range was from 350 to 1,000 °C.

The following assumptions are made in the current study for developing the process model:

- All the chemical reactions were assumed to have reached equilibrium within the pyrolysis.
- The raw material that enters into the reactor is pure polyethylene.
- Only ethylene, propylene, but-1-ene, cis-but-2-ene, trans-but-2-ene, 2-methylpropene, methane, ethane, propane, butane, hydrogen and polyethylene were considered to be present in the product stream in the pyrolysis reactor (Kaminsky, Schlesselmann, and Simon 1995).
- Preliminary research prove that LDPE and HDPE have similar behavior in pyrolysis reactor.
- The main products should be ethylene and propylene.

The Gibb’s free energy is a thermodynamic variable used to identify the chemical equilibrium in a system, its global minimization allows identifying the amount of substances present in the process. There are other identified products from pyrolysis but alkanes and alkenes are the most important in this process, furthermore from experimental data the introduced compounds have no relevant selectivity.

The gas from “PYROLIS” reactor goes to a RadFrac column called “SEP1” which separate ethylene from other olefins, getting 99.9 % ethylene in distillate stream. Bottom stream goes to another column called “SEP2” and reach 99.8 % propylene in distillate stream.

In this work, a response surface (obtained by a sensitivity analysis) was proposed to investigate the performance characteristics of the polymer pyrolysis process in order to compare with the experimental data.

2.2 Ethanol production modeling

In ethanol production step, the ethylene is separated from the other components by using column RadFrac called “SEP1”. The stream “TOREACT” is compressed and heated, then is feed into RStoic reactor with water to produce ethanol. The product labeled “PRODUCT” contains water, ethylene and ethanol. The stream reach in ethylene is separated in a RadFrac called “SEP3” reaching pure ethylene in distillate stream and 55 % ethanol water mix in bottom. The bottom stream go to another distillation column called “SEP4” in order to get the azeotrope. The production of anhydrous ethanol has been made by extractive distillation using a conventional solvents ethylene-glycol (Ethane-1,2-diol) in the column called “SEP5”. One last column called “SEP6” separate the remain ethylene-glycol in order to refeed to “SEP5” column. Thermodynamic parameters for the ethanol/water/ethylene-glycol (Ethane-1,2-diol) system were calculated using UNIFAC model (Ravagnani et al. 2010).

2.3 Energy recovery

The stream “W” contains liquid water that is heated with the thermic energy of the pyrolysis stream outlet. A turbine is used to generate electricity and the cooler water is sent to a condenser then the water is pumped and refeed to the exchanger in a hypothetical Rankine cycle.

2.4 Process analysis

In this work, a response surface (obtained by a sensitivity analysis) was proposed to investigate the performance characteristics of global process. For each step: polyethylene depolymerization, olefins separation, ethanol production and ethanol separation different variables are changed to generate results to make the response surface.

2.5 Process economics analysis

The simulator Aspen Plus has a useful tool, Economic Analyzer, for calculate the cost of a process. Some of the features of this tool are: (i) Generate conceptual and detailed estimates of costs, (ii) Estimate the cost of the product from the process, (ii) Optimizes the process design finding better routes.

The total capital investment (TCI) is computed from the total equipment cost and cash flow is analyzed. With these costs, cash flow analysis is used to determine a finite internal rate of return (IRR) required to obtain a zero net present value (NPV) at product selling price (MPSP).

Moreover, specific parameters regarding some Mexican conditions such as income tax (35 %), annual interest rate (8 %), labor salaries, among others, were incorporated in order to calculate the unitary production costs for the different obtained products. Table 1 shows prices for utilities, main raw materials and products in plastic recycling for the Mexican context in recent years. This analysis was estimated in US dollars for a 20-year period. The depreciation of capital was calculated using the straight line method. Utilities, civil works, pipelines, man hours, and many different parameters were estimated using the same software.

Table 1: Prices used in the economic analysis.

Item	Unit	Price
HDPE mixed color	USD/ton	452.94
Ethylene glycol	USD/kg	1.43
Electricity	USD/kWh	0.1
Water	USD/m3	1.252
Fuel	USD/MWh	26.81
Refrigerant	USD/MJ	0.00271
Anhydrous Ethanol	USD/kg	1.14
Ethylene	USD/kg	1.7
Propylene	USD/kg	1.57
Operator labor	USD/h	3.08
Supervisor labor	USD/h	4.41

3 Results and discussion

3.1 Pirolysys

As already explained for pyrolysis simulation an important issue is the mass fraction of olefin production in the reactor, it is the reason that it is used as a response variable. The effects of pressure and temperature on the gas (olefins like ethylene and propylene) production were studied in this work. The best operating conditions that improve the ethylene and propylene production for pyrolysis were determined as it is explain next.

With the sensitivity analysis the process was analyzed from 1 to 10 atm and a temperature from 340 to 1,000 °C, whereby it was found that the ethylene production decreases when the pressure increases and increases with temperature. Kannan et al exhibit same results reaching up to 48 % wt of ethylene with a gas yield bigger than 99 %. There is no data for different pressure performance, but it can be assumed that is not a vacuum process, thus yield must be at a higher pressure. Response surface (Figure 5) shows results of mass fraction of ethylene at variable set conditions. It should be noticed that ethylene production exhibits a behavior directly proportional to temperature and inversely proportional with respect to pressure: at 850 °C and 4 atm shows 49.56 % of ethylene, 58.19 % at 950 °C and has a maximum of 79 % at 1,000 °C and 1 atm. These results confirm the previously reported data, the difference can be explained for the reason that simulation assumed to have reached the equilibrium within the pyrolysis. Besides that it must be assumed that in fast pyrolysis reactions olefin production is favored.

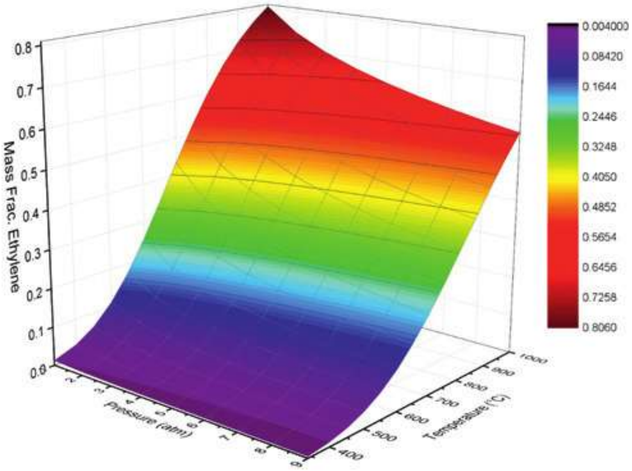


Figure 5: Output mass fraction of ethylene from pyrolysis reactor.

The behavior is different for propylene: production increases proportionally with the temperature until a maximum and then it drops. Between 600 and 800 °C, the maximum propylene production can be reached. In fast pyrolysis, Kannan has the same behavior for propylene's yield within a range from 700 to 900 °C in maximum reach, and then drops. Figure 6 shows the response surface for propylene mass fraction at variable condition.

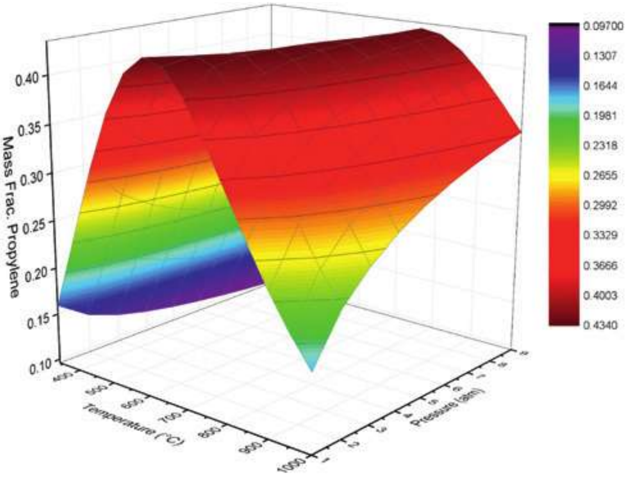


Figure 6: Output mass fraction of propylene from pyrolysis reactor at different temperature and pressure.

It is important to note that the conditions in which the propylene production is maximum are different for the ethylene production, so any ethylene/propylene mass ratio in gas has to be observed as a response variable, allowing to choose which production to take; this will be discussed for economic issue.

Figure 7 shows the different olefins that are obtained from the pyrolysis reactor. It can be observed, as long as the temperature is higher, it gets lower production of but-1-ene, cis-but-2-ene, trans-but-2-ene, and 2-methylpropene.

For this reason it can be concluded that as the temperature grows up the polymer chain get cracked and the product is a smaller molecule like ethylene, and the but-1-ene, cis-but-2-ene, trans-but-2-ene, and 2-methylpropene gets cracked and contributes to the formation of ethylene at this conditions too. Once again the results from Kannan's work have the same performance for butylene's yield.

The response surfaces show the importance of the temperature effects to produce ethylene and propylene. The pressure effect seems to be not as relevant as the temperature, but however pressure is an important variable in real pyrolysis reactors, for this reason it should be considered. As it is to be expected and is reported in Figure 8, as the temperature grows up the energy demand in the process is greater. However, pressure do not affect as much as temperature.

3.2 Ethanol production

The results for ethanol production process step by ethylene hydration are reported in the Figure 9. This reaction depend

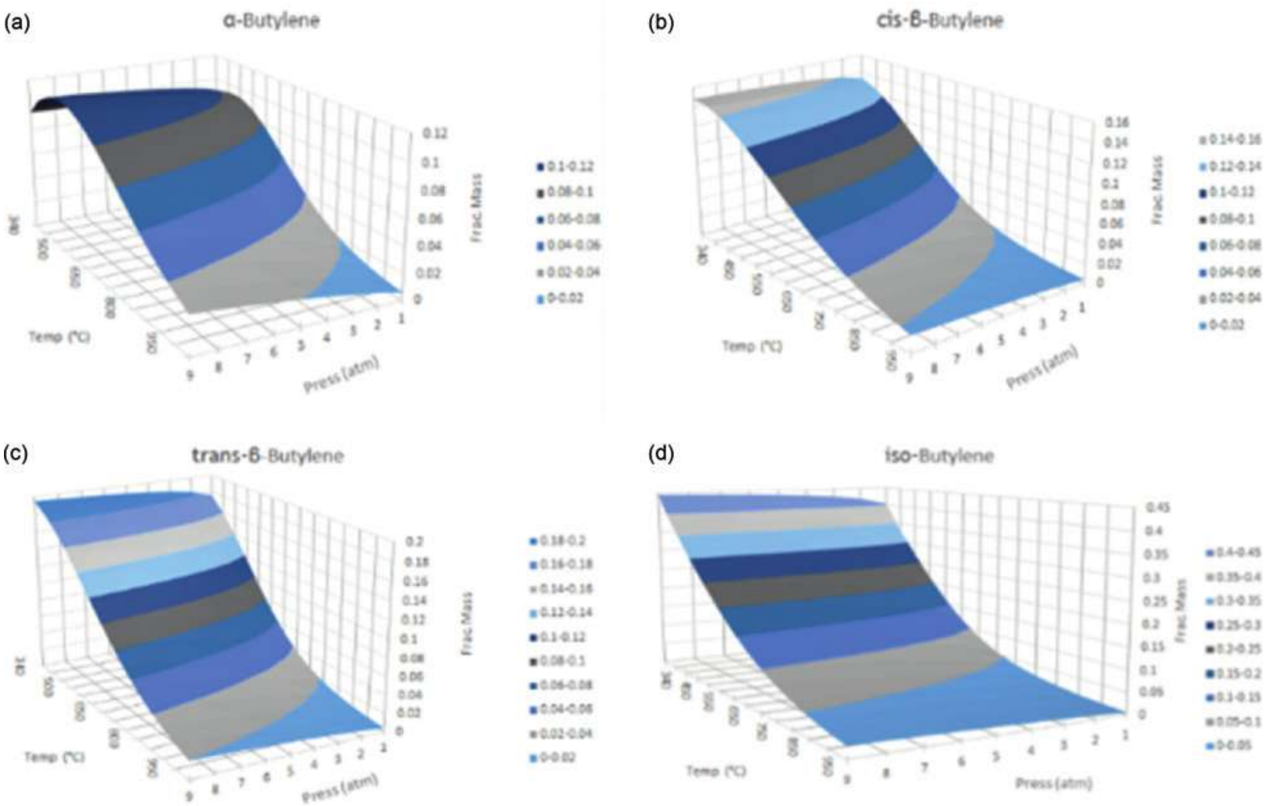


Figure 7: Output mass fraction (a) α-butylene (b) cis-β-butylene (c) trans-β-butylene (d) iso-butylene from pyrolysis reactor at different temperature and pressure.

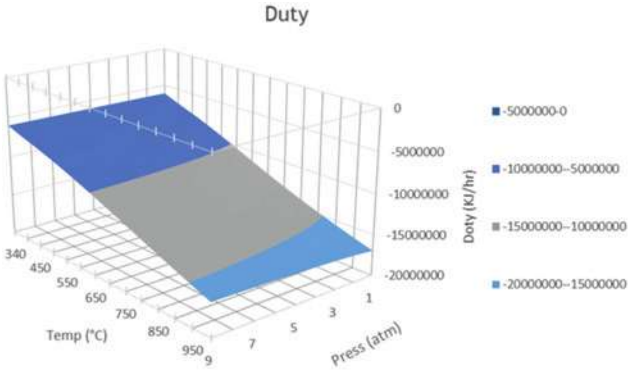


Figure 8: Input of energy (kJ/hr) to pyrolysis reactor at different pressure and temperature.

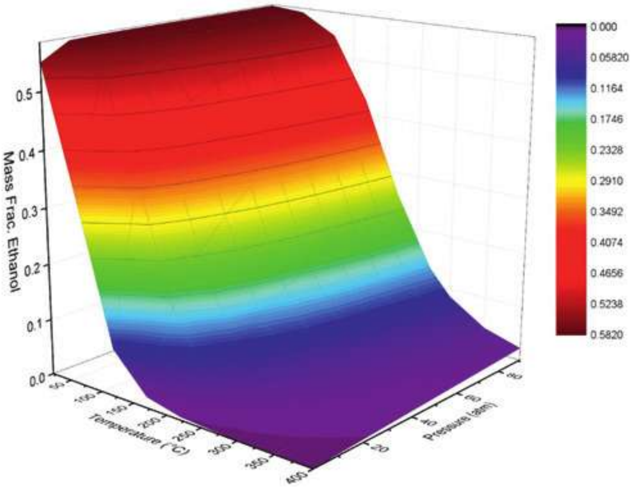


Figure 9: Ethanol production analysis.

of olefin/water molar relation, temperature and pressure. In this work, this relation was equimolar. it can be observed, that the pressure does not have a significant effect, but the temperature has to be low for get high ethylene conversion. It is difficult to compare with experimental results since in practice the direct process of hydration it is carried out in the gas phase over an acid catalysts, generally H_3PO_4 deposited on a solid support of SiO_2 or a resin. Working conditions are 300 °C and 70 atm (to promote the right balance) and a short time reaction to prevent the formation of other products (diethyl ether and oligomers). The ethylene conversion is only 5 % in a selectivity of 97 %; alcohol is condensed and the gases are recycled multiple times to achieve economic performance (97 %), for which it is necessary a high purity ethylene gas removal or to prevent an increase in the recycled inert gases. By the result obtained from simulation and the literature in which it was compered, the results obtained by Aspen Plus simulation were very similar (Sanz Tejedor 2008).

3.3 Process performance

In Mexico around 1,836,000 ton of polyethylene were apparently consumed in 2012 (Conde Ortiz 2013). As design basis is considered a plant capacity of 10 % of consumption of that year, equivalent to 500 tons/day de polyethylene to get 350,632.8 liters/day of ethanol (99.5 %wt), 201.6 ton/day of ethylene (99.85wt) and 91.2 ton/day of propylene (99.8 %wt).

Simulations of the three studied areas were used to generate their respective mass and energy balance sheets, which are the basic input for the techno-economic analysis. The simulation was validated by comparing yields of each product with data reported in literature for actual commercial processes including laboratory data for pyrolysis.

Olefins refinement is an important issue in order to produce good quality product, for that reason two separation columns are used to get pure ethylene and pure propylene; 3 columns to get anhydrous etanol from the ethylene stream. As it is known reflux ratio required energy in condenser and reboiler. The investment is another important issue to be considered. The bigger the column is the more expensive is the cost of the equipment then the investment could increase. For this reason the number of stages must be specified and the reflux ratio must be considered. With sensitivity analysis the RadFrac units are used to obtain the column conditions for each refinement. With a sensitivity analysis in the RadFrac units, the design specification of all of them were obtained considering the desire purity in each refinement possibility. Table 2 shows the different “RadFrac” units with his purity output and its operation conditions for each one.

One of the best alternatives for reducing production costs is to decrease the energy consumption during the production process by implementing more energy-efficient and better performing technologies (Moncada, El-Halwagi, and Cardona 2013). The Figure 10, shows the energy balance: the consumption is the thermic energy (utilities) required in the global process and the production is the electric energy produced in the Rankine cycle. It is almost in equilibrium. In the Figure 11 is reported the energy requirements for each step of the global process: It can be seen that ethanol is the highest energy consumer in this process with a 71.1 % of heat required, ethylene is the second one with 19.64 % and propylene with 8.89 %.

Table 2: Operating conditions of different distillation column.

Equipment	Operating conditions	Note
SEP1	N stages: 14 Feed stream: 8 Reflux Ratio: 1.8 Distillate rate: 15,980 kg/h Pressure: 16 atm	Cryogenic distillation separates ethylene 99.9% pure.
SEP2	N stages: 12 Feed stream: 11 Reflux Ratio: 1.5 Distillate rate: 3,713 kg/ Pressure: 5 atmh	Olefin distillation separate 99.9% propylene.
SEP3	N stages: 9 Feed stream: 3 Reflux Ratio: 1 Distillate rate: 114,000 kg/h Pressure: 13	Distillation column separates ethylene from ethanol-water mixing.
SEP4	N stages: 16 Feed stream: 14 Reflux Ratio: 3 Distillate rate 12,600 kg/h Pressure: 3 atm	Conventional distillation obtain 91.1% ethanol.
SEP5	N stages: 32 Feed stream: 31 Solvent Stream: 2 Reflux Ratio: 1 Distillate rate: 11,600 kg/h Pressure: 1 atm	Extractive distillation dehydrate ethanol reaching 99.5% pure.
SEP6	N stages: 6 Feed stream: 3 Reflux Ratio: 1 Bottoms rate: 15,000 Pressure: 1 atm	Solvent recovery distillation separate 99.9% ethylene-glycol.

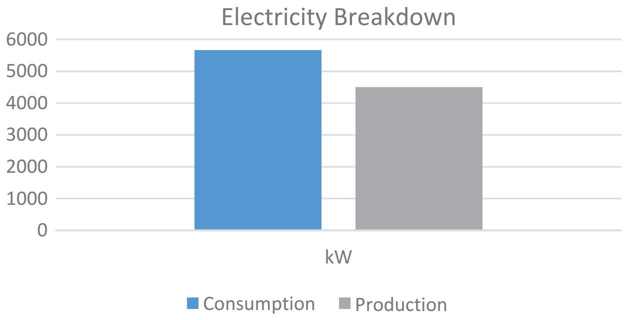


Figure 10: Energy balance of global process.

In this process the performance is similar to the real data that can be validated from different authors.

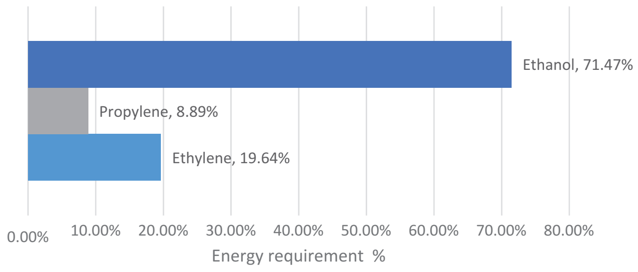


Figure 11: Energy requirement for process step.

By this knowledge the possibility for this project are technically viable to be build.

3.4 Process economics assessment

The ultimate purpose for developing such a process design, is to determine the economics benefits of ethanol, ethylene and propylene production. The operating cost includes various aspects inherent to the production process such as raw materials, utilities heat exchangers, labor and maintenance, general plant costs and general administrative costs. The comprehensive evaluation of each of these aspects leads to the plant's operating costs. This evaluation was based on the results obtained from the simulation and sent to Aspen Process Economic Analyzer, adapted to Mexican parameters.

For most industrial processes the raw material represents approximately more than 50 % of total production cost. Quantities of process were determined and compared with total products sales. Figure 12 shows the cash flow per year for this simulation. There is not a standard price for polyethylene price in Mexico. The reason to establish a point of reference to the price of PE is to make a possible economic analysis. Plastic materials price for this process is taken from an average cost of different suppliers in Mexico center zone. Moreover the polyethylene is almost pure in a mix of colors, it could be almost pure even with mixing other plastics and the cost could be reduce. For this reason the pyrolysis analysis of pure recycled polyethylene was chosen.

The process was analyzed according to their potential income, using a ratio called total sales/total production cost ratio as a preliminary sustainability assessment of a chemical process (Posada, Rincón, and Cardona 2012). The result was 2.54 for HDPE.

The total capital investment (TCI) is shown in Table 3. It should be emphasized that the investment is for 500 ton/day of HDPE.

Once the total capital investment and variable operating costs, a discounted cash flow rate of return (DCFROR)

Figure 12: Cost distribution.

Table 3: Total Capital investment plant USD.

Purchased equipment	12,912,700.00
Equipment setting	174,610.10
Piping	6,889,596.50
Civil	536,156.10
Steel	123,773.70
Instrumentation	2,183,556.80
Electrical	1,567,501.80
Insulation	932,443.10
Paint	111,904.70
Other	10,362,801.00
G and A overheads	929,992.30
Contract fee	1,209,097.50
Contingencies	6,828,144.00
Total project cost	$ 44,215,046.46

analysis can be used to determine the internal rate of return (IRR) of total process. The discounted cash flow analysis is calculated by iterating the internal rate of return until the net present value of the project is zero (Short, Packey, and Holt 1995). This analysis requires that the discount rate, depreciation method, income tax rates, plant life, and construction start-up duration must be specified in APEA. Table 4 shows the terms for this analysis.

Table 4: Discounted cash flow analysis parameters.

Depreciation method	**Straight Line**
Salvage value	20 %
Plant lifetime	20 years
Construction period	1 years
General plant recovery period	6 years
IRS	35 %

Analysis does not take into account any policy factors such as subsidies, mandates, or carbon credits because these would be purely speculative. The purpose of analysis is to demonstrate whether or not ethylene, propylene and ethanol from polymers can be cost-competitive. Table 5 shows the IRR from project, MIRR with an interest rate/desired rate of return of 50 % and project summary.

Practitioners often interpret internal rate of return as the annual equivalent return on a given investment; this easy analogy is the source of its intuitive appeal. But in fact, IRR is a true indication of a project's annual return on investment only when the project generates no different interim cash flows between the life expectancy of the project or when those interim cash flows really can be reinvested as long of this period. For this project the main IRR may be 68.96 % for HDPE. This prices may considered the freight, possible buying charges and any other fee that can be changed at any other moment with almost no transcendence in the outcome of the IRR. There is no interim cash flow forecast in the project so it could not have a realistic IRR. However the project must be prepared to any unexpected cash flow. The most straightforward way to avoid problems with IRR is to abstain from the idea of similar interim cash flow at any point it. Yet given its widespread use, it is unlikely that the IRR can be replaced easily. Executives should at least use a modified internal rate of return. While not perfect, MIRR at least allows users to set more realistic interim reinvestment rates, and therefore to calculate a true annual equivalent yield (Kelleher and MacCormack 2004). Since the interest rate/desired rate of return of 50 % was proposed as the first possibility even that can be any percentage that the investor desire, the calculation for 50 % were 51.95 % and 51.61 % MIRR respectively for HDPE and LDPE making the project as accepted. Any percentage lower than the desire rate of return is an unaccepted project.

Table 5: Summary economic analysis.

Total project capital cost	USD	44,215,046.46
Total products sales	USD/year	266,467,941.74
Total raw materials cost	USD/year	70,443,787.35
Total utilities cost	USD/year	25,631,792.43
Operating labor cost	USD/year	158,480.00
Maintenance cost	USD/year	362,000.00
Operating charges	USD/year	39,620.00
Plant overhead	USD/year	260,240.00
G and A cost	USD/year	7,751,673.58
Total operating cost	USD/year	104,647,593.37
IRR LDPE	%	73.83
MIRR LDPE	%	51.95
IRR HDPE	%	68.96
MIRR HDPE	%	51.61

4 Conclusions

A global process for production of olefins and ethanol from PSW was studied. For the first stage, pyrolysis, operating conditions to maximize the production of olefins are 1,000 °C and 1 atm.

The models that result from the combinations in the Aspen Plus simulation package concludes that 1,000 °C and 1 atm pressure are the best choices for real construction and process to produce olefins, giving as an extra bonus that fast pyrolysis kinetic reactions are favored to produce olefins. The direct hydration of the ethylene allow the production of ethanol with an almost equal purity as the input ethylene already refined in the previous pyrolysis process. The economic analysis provide the feasibility at 50 % desire rate of return.

From the results it can be concluded that a plant can be built in the center of Mexico for its closeness to the raw materials including PSW, or make an especial study to locate a better site. The benefit in job productions goes two sideways, being the first one the jobs in the process stage considering three turns of work each one of three supervisors and fifteen operators and all management requirements. The second are the side jobs, people required for the freight, selection, gathering and cleaning for all the raw materials and others requirements.

Even so the possible extensions for the plant goes from an increase of production to a diversification of other products like ethanal, ethylene oxide from ethylene produced, and acetone, 2-propenenitrile, epoxypropane, prop-2-enoic acid from propylene produced. Giving a great flexibility for new markets beside the three main products reviewed in this study.

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Production of Fermentable Sugars and Hydrogen-Rich Gas from Agave tequilana *Biomass*

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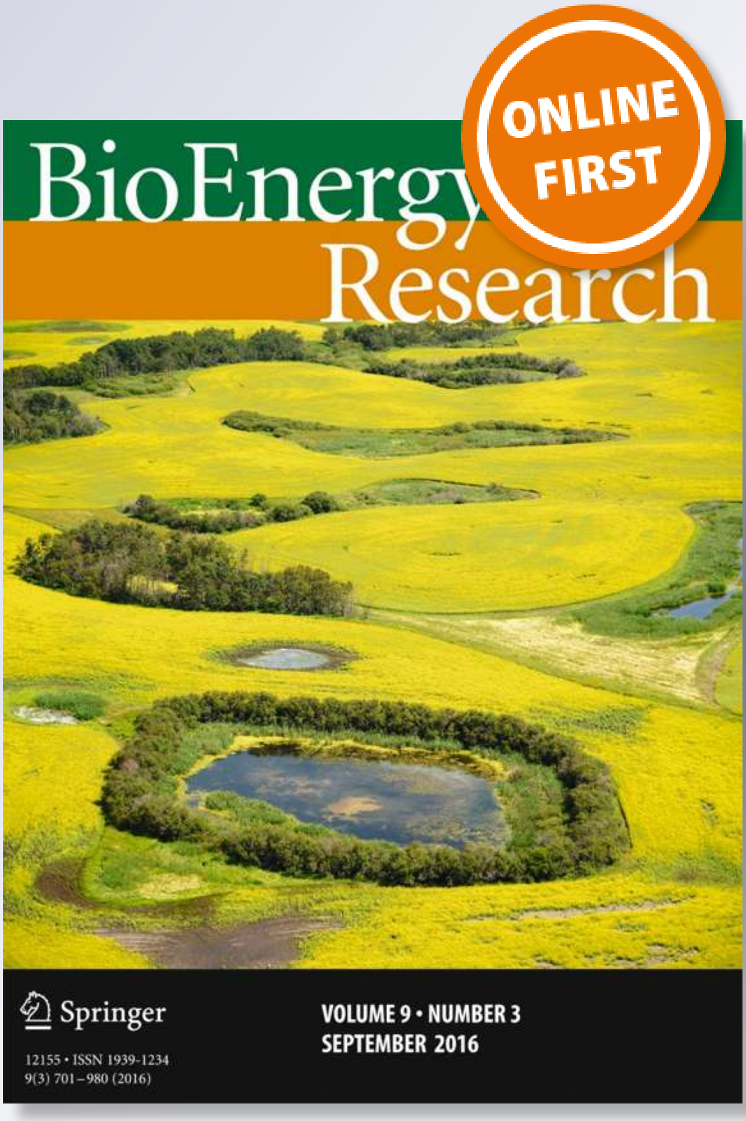
BioEnergy Research

ISSN 1939-1234

Bioenerg. Res.
DOI 10.1007/s12155-016-9799-y



Springer

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Production of Fermentable Sugars and Hydrogen-Rich Gas from *Agave tequilana* Biomass

Juan Carlos Farías-Sánchez[1] · **Ulises Velázquez-Valadez**[1] · **Alfonso Vargas-Santillán**[1] · **María Guadalupe Pineda-Pimentel**[1] · **Erick Alejandro Mendoza-Chávez**[1] · **José Guadalupe Rutiaga-Quiñones**[2] · **Jaime Saucedo-Luna**[1] · **Agustín Jaime Castro-Montoya**[1]

Abstract The Mexican tequila industry annually processes approximately 1×10^6 *Agave tequilana* plants, generating approximately 1.78×10^8 kg of bagasse per year. This biomass is considered an attractive alternative to fossil fuels as an energy source and to produce biofuels and/or chemical products because it is produced and used without adversely affecting the environment. The first aim of the present work was to determine the effect of temperature, the concentration of H_2SO_4, and reaction time on the hydrolysis of agave bagasse to maximize the fermentable sugars using a steam explosion. This step process generated 71.11 g/L of reducible sugars in the supernatant (59.29 % glucose, 29.05 % xylose, and 11.66 % fructose) and unconverted organic matter of enzymatic hydrolysis bagasse (35.4 % α-cellulose, 7.33 % hemicellulose, 49.91 % lignin, and 7.31 % ashes). A mathematical surface response analysis of the hydrolysis was used for process optimization. The second aim involves the study of the thermodynamics of the reforming of unconverted organic matter from enzymatic hydrolysis of *Agave tequilana* bagasse (ATB) evaluated by the Gibbs free energy minimization method for hydrogen production. The effect of the parameters on the system performance measures, such as reaction temperature (T), Water/Biomass ratio (WBR), and pressure (P), were also investigated. The maximum H_2 production obtained was 23.2 mol of H_2/271.5 g ATB with a WBR ≥ 11 and a temperature of 740 °C. These findings indicate that the temperature and WBR are essential factors in the production of H_2, which was reflected in the efficiency of the process.

Keywords Lignocellulose residue · Acid hydrolysis · *Agave tequilana* · Hydrogen · Thermodynamic analysis

Introduction

The energy sector is considered one of the most important because it contributes substantially to the national economy, stimulating essential factors for all productive activities, employment, and basic consumer goods. Globally, 88 % of the total energy consumption is produced from non-renewable sources, and only 12 % comes from renewable sources such as biogas, wind, geothermal, hydro, solar, and biomass [1], such is shown in Fig. 1.

A major challenge facing society today, due to exponential population growth in a very short time, the rapid and continuous reduction of deposits of fossil fuels and growing concern about the environmental effects of fossil fuel use, is to cover the increased demand for energy by identifying and developing alternative energy sources. In recent years, there has been more interest from the public and private sectors in exploring alternatives for power generation and other industrial products for consumption or export through biological processes and/or biomass resources.

Currently, in Mexico, there is a legal mandate to generate 35 % of electricity from non-fossil fuels in 2024 in order to promote the sustainability of the energy sector, increase energy security, and mitigate the negative impacts that the

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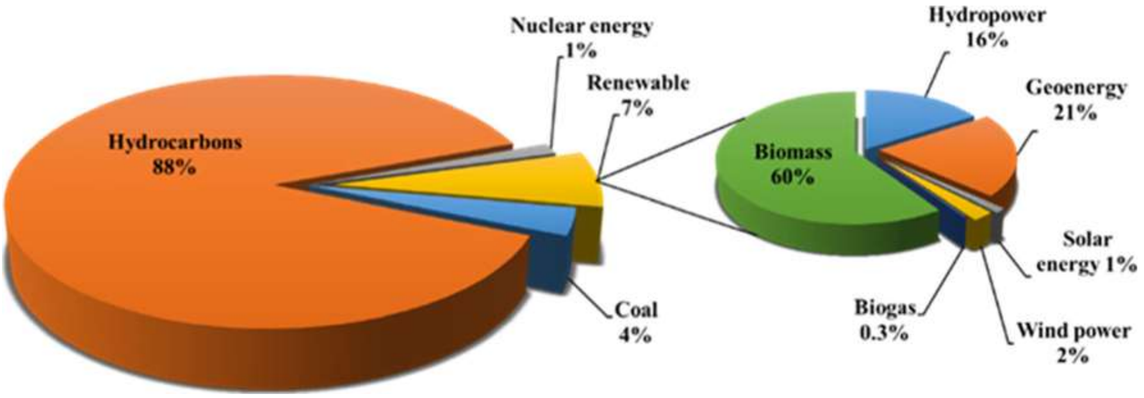


Fig. 1 Primary energy production

production and consumption of energy can have on health and the environment, including reducing emissions of greenhouse gases, according to SENER [2].

Reducing the dependence on oil, as the primary energy source in Mexico, and gradually incorporating new renewable energy sources are necessary to make better use of solar energy and its various secondary manifestations such as wind, hydropower, and various forms of biomass, the sustainable utilization of agricultural production and the high existing biodiversity as well as to promote the conditions that guarantee the food supply and protect the environment. For that reason, alternative fuels with a low environmental impact, especially those relating to emissions of greenhouse gases and health and safety considerations, such as bioethanol, biodiesel, and bio-hydrogen, play an important role in the energy future. Bio-ethanol and bio-hydrogen fuels will replace gasoline in the future [1].

Czernik et al. [3] indicated that the thermo-conversion of biomass is one of the leading near-term options for renewable hydrogen production, and it is possible to generate an output of approximately 130 million tons of hydrogen with 1.3 billion tons of biomass. In this context, the Mexican tequila industry annually processes approximately 1×10^6 *Agave tequilana* plants, generating approximately 1.78×10^8 kg of bagasse (ATB) per year [4, 5], which represents the potential for annual hydrogen production of 17.8 t. Furthermore, because this waste ATB is rich in carbon, it is composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin so that it is highly biologically degradable and emits methane and leachate. ATB is considered an attractive alternative to fossil fuels as an energy source and to produce biofuels and/or chemical products because it is produced and used without adversely affecting the environment [6]. Some advantages of the integrated use of ATB include the following: (i) use of ATB can significantly reduce emissions of greenhouse gases, especially carbon dioxide, CO_2; (ii) steam reforming can address the high water content of ATB; (iii) the conversion of ATB residues increases the value of *Agave tequilana* output; (iv) replacing fossil fuels with sustainable and ultraclean fuels; (v) a decrease in the costs of getting rid of the solid wastes of production; and (vi) the possible production of heat and power in addition to providing valuable fuels or chemical building blocks. Flowchart of integration of agave bagasse utilization can be seen in Fig. 2. It starts with the *Agave tequilana* plants, continues with process of tequila in

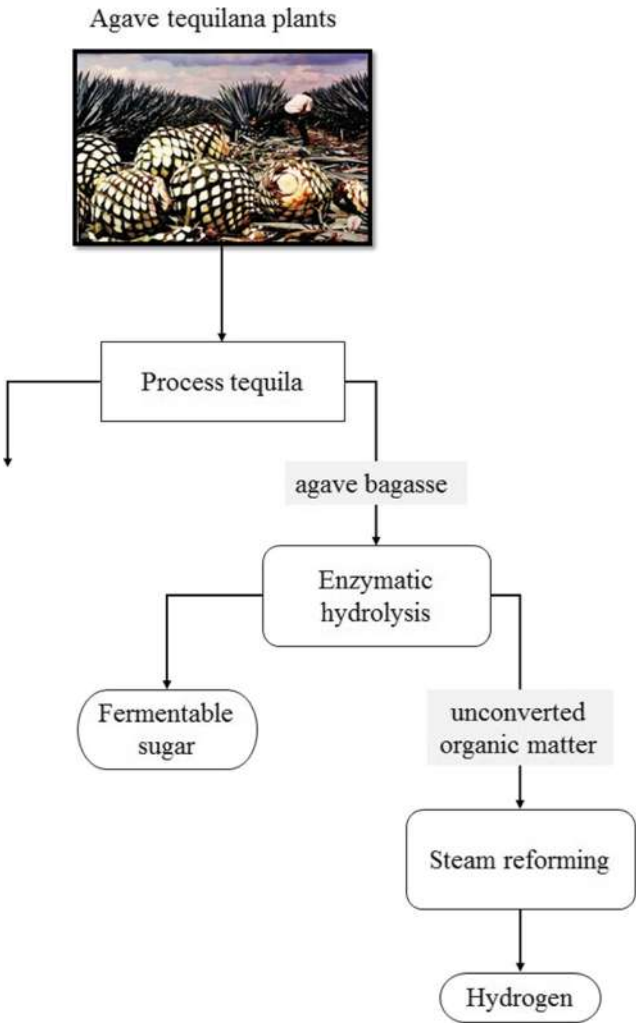


Fig. 2 Process flowchart

which we obtained agave bagasse as a waste, in that point the agave bagasse is pretreated and carried to an enzymatic hydrolysis then fermentable sugars can be separate from unconverted organic matter after being taken to reforming process which finally yields hydrogen.

In this work, it was found that it is possible to convert the organic fiber from bagasse into fermentable sugars through enzymatic hydrolysis, and other parts of the unconverted organic matter can be used to produce heat and power or valuable fuels, such as H_2. CO_2-neutral hydrogen that can be produced by the conversion of ATB via gasification, the pyrolysis of bio-oils, aqueous phase reforming of oxygenated hydrocarbons, and steam reforming of biomass-derived higher alkanes and alcohols [7]. For that reason, a process simulation has been used to determine the optimum process and operating conditions for effectively producing H_2, reducing the concentration of offgas and increasing the efficiency of the process.

Materials

Feedstock

Agave tequilana bagasse (ATB), was obtained from the mezcal industry "Ex hacienda de Zacapendo," located in the municipality of Indaparapeo, Michoacan, Mexico. It was dried at 80 °C for 24 h in a electric furnace and then partially reduced in size in a generic blade mill to obtain heterogeneous fibers ranging from 15 to 3 mm long with diameters of 0.3 mm. The experiments were performed with this fiber size, which is higher than the size usually used, mesh 40 spherical particles.

Fiber Composition Analysis

The material was analyzed to determine the lignin [6], holocellulose [7], alpha cellulose [8], and ash [9]. All analysis methodologies were carried out with extractive-free bagasse. The differences between holocelulose and alpha-cellulose was considered as hemicellulose. The free extractive raw material was done with a soxhlet extraction sequence with cyclohexane, acetone, methanol, and water.

Table 2 Experimental design results with sugars as response

Experiments	Sulfuric acid (%)	Pressure (kg/cm^2)	Ratio L/S	Sugars g/L
1	0.25	10.60	3.0	36.82
2	0.10	6.30	2.0	34.33
3	0.25	10.50	3.0	38.20
4	0.50	10.22	3.0	27.12
7	0.10	6.30	2.0	31.60
8	0.25	15.85	3.0	33.01
9	0.00	10.22	3.0	34.98
10	0.10	6.30	4.0	34.91
11	0.25	10.22	4.7	36.91
12	0.25	10.22	1.3	32.32
13	0.10	15.85	4.0	41.10
14	0.40	15.85	2.0	38.55
15	0.40	6.30	4.0	40.39
16	0.40	6.30	2.0	37.92
17	0.40	15.85	4.0	46.53
18	0.25	4.50	3.0	38.12

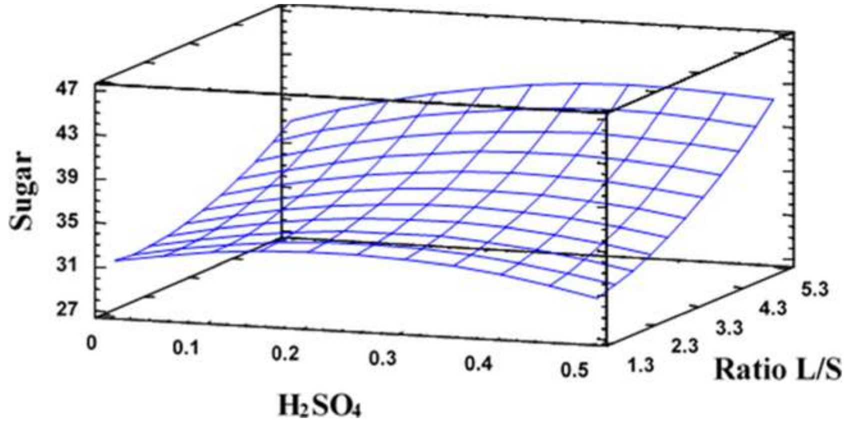
Pretreatment

The raw material was dried to 80 °C and no size reduction was applied, then it was pretreated using acidified steam-explosion equipment with a volume of 1300 mL designed for a maximum working pressure of 16 kg/cm^2. A experimental design of a response surface with 2 star points was carried out for experimentation with the agave bagasse that comes from industry. The highest (0.5 % H_2SO_4, liquid/solid ratio 4, pressure 15.85 kg/cm^2) and lowest (0.1 % H_2SO_4, liquid/solid ratio 2, pressure 6.3 kg/cm^2) values of the data are entered into the experimental program Statgraphics Centurion Version XV software ((Statistical Graphics Corp., Manugistics, Rockville,

Table 1 Main biomass steam reforming reactions

Name of reaction	Chemical equation	ΔH^0_{923} kJ/mol	Eq.
Biomass reforming	$C_nH_m + nH_2O \rightarrow nCO + \left(\frac{m}{2} + n\right)H_2$	+ΔH	(1)
Water-gas-shift	$CO + H_2O \leftrightarrow CO_2 + H_2$	−36 (Exo)	(2)
Water-gas (i)	$C + H_2O \leftrightarrow CO + H_2$	+136 (Endo)	(3)
Water-gas (ii)	$C + 2H_2O \leftrightarrow CO_2 + 2H_2$	+100 (Endo)	(4)
Carbon formation	$2CO \leftrightarrow C + CO_2$	−171 (Exo)	(5)
Methane formation	$CO + 3H_2 \leftrightarrow CH_4 + H_2O$	−224 (Exo)	(6)
Methane formation	$CO_2 + 4H_2 \leftrightarrow CH_4 + 2H_2O$	−188 (Exo)	(7)

Fig. 3 Graphic of response surface H_2SO_4 vs L/S ratio



MD), for the design response surface with 2 central points used for experimentation. A 40 g sample of dry bagasse was placed in an erlenmeyer flask of 500 mL, impregnated with sulfuric acid in the proportion given by the design, and kept for 24 h for impregnation. After the 24 h period, the mixture was treated by steam explosion at the pressure indicated by the design, arriving at the indicated pressure. After a sudden decompression of the system, the bagasse was cooled, detoxified with calcium hydroxide, and stored for later use in the enzymatic hydrolysis (MPD) [8]; the pretreated raw material was used moist to avoid changes in the structure by the effect of drying according to Luo, Zhu [9].

Enzymatic Hydrolysis

For the enzymatic hydrolysis, the enzymes Cellic Ctec 3 and Cellic Htec 3 were used at the rate of 6 % (the supplier Novozymes recommends using 6 % enzyme by dry biomass) of dry material each. The initial activity of this enzyme was between 217 FPU/ml 40 g sample of MPD, the enzyme solution, and a 0.1 M acetate buffer at pH = 5.0 at a ratio of 1:5 were added. This mixture was kept for 72 h at 50 °C with agitation at 150 rpm on a Polyscience orbital shaker. At the end of this period, the supernatant was analyzed using a standard technique on a Varian Pro Star HPLC using a column Metacarb 87 to 70 °C and a refractive index detector at 50 °C with isocratic water flow at 1 mL/min to determine simple sugars. After the hydrolysis, the sample was filtered, washed, and dried as a solid residue.

Thermodynamic Analysis

The thermodynamic analysis of the ATB reforming process was performed using the commercial software Aspen Plus 8.8 (Aspentech, Cambridge, USA). The unconverted organic matter from the enzymatic hydrolysis of the bagasse samples was used as a feedstock for the simulation. The effect of the reformer temperature (600 to 1300 °C) and the steam to biomass ratio on the production of hydrogen were studied. The equilibrium compositions were calculated with the Gibbs energy minimization method [10]. The water/biomass ratio (WBR) was varied from 0 to 12. The biomass stream was defined whit 35.7 % cellulose, 7.38 xylan, and 49.91 lignin. Cellulose and Xylan were set as "Oligomer" stream and the monomer cellulose-r and xylan-r is used to obtain the properties from Van Krevelen and Te Nijenhuis [11]. Lignin was set as Guaiacol as a conventional compound as is reported by Rodriguez et al. [10]. Cellulose and xylan are considered as polymer for that reason, polymer NRTL equation of state with Henry's law "POLYNRTL" was chosen as parameter models to calculate the thermophysical properties of the component. The RGibbs reactor subroutine was implemented in software for the gas production, which was fed with only lignocellulosic material and water steam.

The following assumptions are made in the current study for developing the process model.

1. All the chemical reactions were assumed to have reached equilibrium within the gasifier.

Table 3 Comparative isolated experiments

Pretreatment	Sugars (g/L)	Pressure (kg/cm^2)	H_2SO_4 (%)	Particle size (mesh)	Ratio L/S
1	23.06	6.30	0.5	40	5
2	49.14	10.22	0.5	40	5
3	71.11	15.85	0.5	40	5

2. Only methane, hydrogen, carbon monoxide, carbon dioxide, oxygen, and water were considered to be present in the product stream.
3. The primary components of char are only carbon and ash.

The reactions shown in the Table 1 were also involved, and in general, these reactions describe the main pathways to obtain the proposed products [12].

Results and Discussions

Fiber Composition Analysis

The chemical composition of the raw material (ATB) was: extractives 44.29 %, lignin13.57 %, alpha-cellulose 28.81 %, hemicellulose 11.33 %, and ash 2.00 %. The high value of extractives is because the ATB has adsorbed a large amount of soluble sugars.

Enzymatic Hydrolysis

In the Table 2, the complete experimental design is shown with the total sugars obtained after hydrolysis of the raw materials for each.

All MPD was hydrolyzed and analyzed, obtaining 46.53 g/L (45.17 % glucose, 21.77 % xylose, 33.05 % fructose) of maximum sugars, using 0.4 % H_2SO_4, a liquid/solid ratio (L/S) of 4, and a pressure of 15.85 kg/cm^2 for 72 h of hydrolysis, which corresponds to a yield of 52.78 % in enzymatic hydrolysis with respect to the raw material (ATB). Based on these results and Fig. 3, in which a trend toward 0.5 % acid and a L/S of 5 can be observed, it was decided to do additional tests to analyze the effect of particle size of ATB. For these further tests, a particle size that passes through 20 mesh but is retained on 40 mesh, was used, increasing the acid to 0.5 % H_2SO_4, the L/S to 5 and maintaining the pressure at 15.85 kg/cm^2. The results, as shown in Table 3.

With this improvement in the process, the concentration of reducing sugars was increased, resulting in 71.11 g/L (59.29 % glucose, 29.05 % xylose, and 11.66 % fructose) sugars and an efficiency of 71.15 %. These yields were close to those obtained by Negro et al. [13], who obtained a concentration of 72.8 g/L of sugar, an efficiency of 88 % with steam-explosion pretreatment at 195 °C, and phosphoric acid concentration of 1 % with material from olive trees. Also, there are other similar results as Sharma et al. [14] got an efficiency of 81.8 % for steam explosion pretreatment at 200 °C with 0.5 % sulfuric acid for rice straw and Zimbardi et al. [15] who used corn stover pretreated with steam-explosion and impregnated with 3 % sulfuric acid and a temperature of 190 °C to obtain a yield of 93 % sugars with an initial 48 h hydrolysis. Compared with Cara et al. [16], who used a steam-explosion pretreatment with olives at a temperature of 190 °C and obtained a yield of 52.6 % with a concentration of 51.3 g/L of reducing sugar after 72 h of enzymatic hydrolysis with 10 % (*w*/*v*) of pretreated material consisting of cellulolytic complex β-glucosidase. These results are better. In Fig. 4, the reader can see the actual process, since dry ATB until solid residue and hydrolyzate. Also, the total among of reagents and percent rate of effectiveness are showed. This raw material contains a large amount of extractives (60.8 %) which they are mostly inulin; which can be converted to monomers such as glucose and fructose. On the other hand, the solid residue is lignin rich, which can be used as a raw material to reforming process. The unconverted organic matter from pretreatment and enzymatic hydrolysis of the bagasse samples indicated that for each kg of ATB, it produces 271.5 g of solid waste, which has a composition as shown in Table 4. It can observe the large percentage of lignin, whereby it is considered a good raw material for

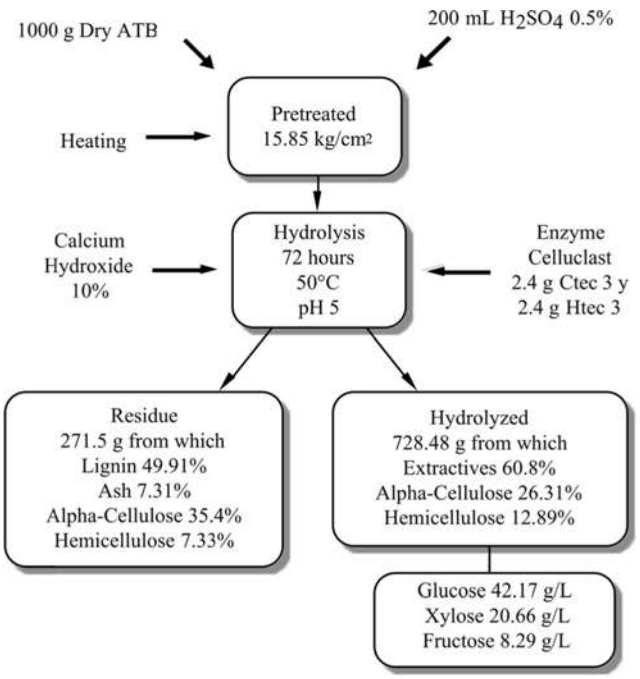


Fig. 4 Matter balance, raw material to hydrolyzed process

Table 4 Summary of the unconverted organic matter from enzymatic hydrolysis of bagasse

Component	Unconverted organic matter (%)
Cellulose	35.40 ± 1.32
Hemicellulose	7.33 ± 0.21
Lignin	49.91 ± 0.97
Ash	7.31 ± 0.11

the subsequent process of hydrogen production. This pretreatment has the advantage of a very low use of chemical reagents, have no polluting effluents to the environment, and since after pretreatment, the acid is neutralized with calcium hydroxide forming calcium sulfate which precipitates, is not toxic, and is suitable for a variety of uses.

Thermodynamic Analysis

After the pretreatment and enzymatic hydrolysis, the residual biomass is used in order to obtain a gaseous biofuel. Using a thermodynamic analysis, hydrogen generation was performed to evaluate the effects of two process variables, temperature and WBR, while estimating a biomass conversion of 100 % in the steam reforming process. The effect of each process variable on the products generated was analyzed using Gibbs Free Energy minimization with a basis of 275.1 g of waste solid. The effects of the variables on the amount of desired and undesired products obtained in the process were also determined. The products obtained from the steam reforming of ATB were hydrogen, methane, carbon monoxide, carbon dioxide, graphite, and water (Fig. 5).

In Fig. 5a, it shows H_2 yield tendencies as functions of the temperature for different water-to-ATB feed molar ratios (WBR). The results show that hydrogen exhibited a maximum at approximately 740 °C; this substantiate the results of the direct gasification. In addition, it can be seen a rise in hydrogen production when water is increased in the reactor. At 740 °C and a water relation 12:1 is the highest hydrogen production, and after this temperature its production gradually decrease until 1300 °C as top researched. The increase in the production of hydrogen when rising the temperature can be explained by the Eqs. 3 and 4 from the Table 1 in which the endothermic relation move toward hydrogen production as heat is added. Beside this, Eqs. 6 and 7 in Table 1 and taking in consideration, the Le Châtelier principle shows that

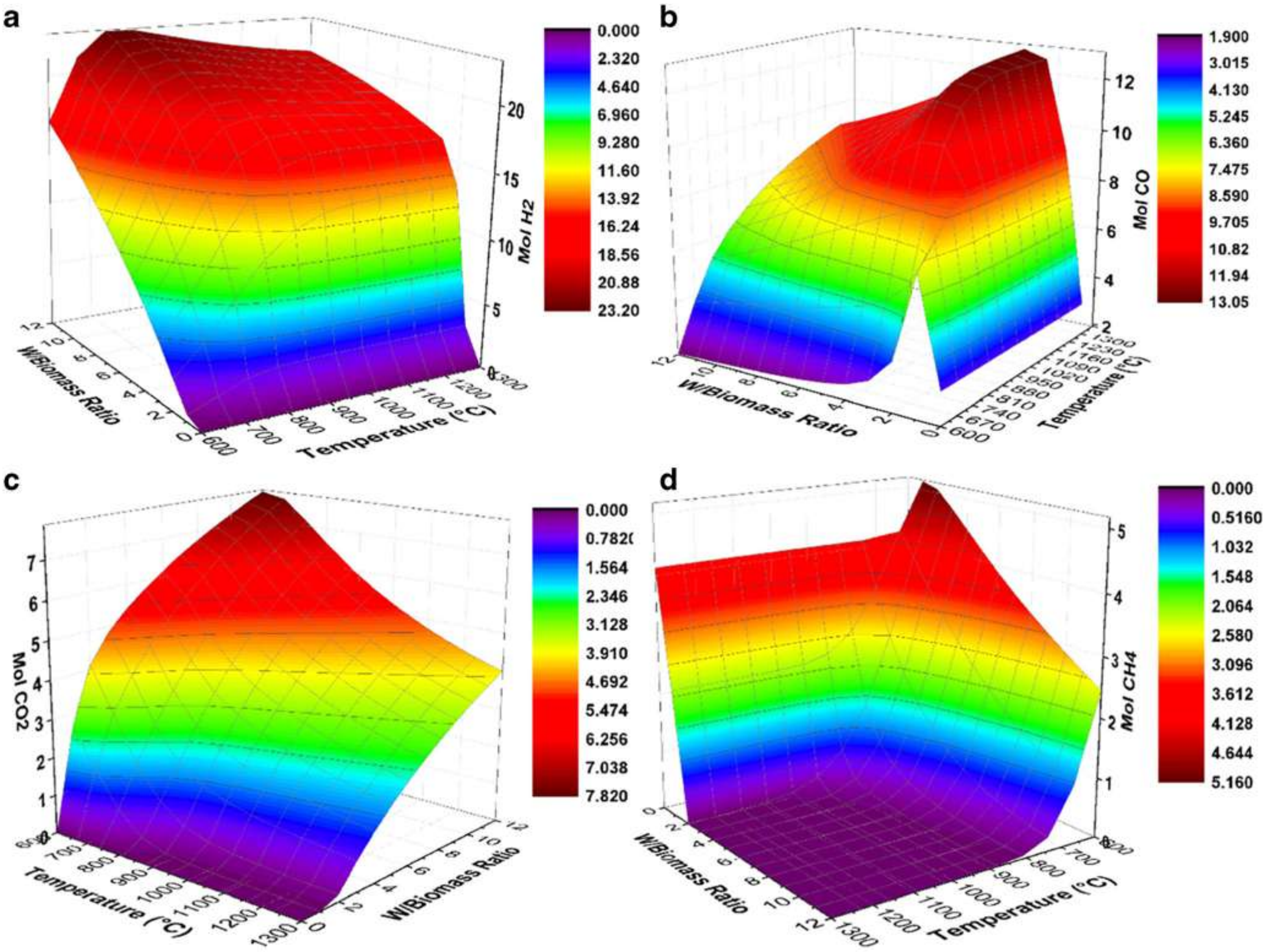
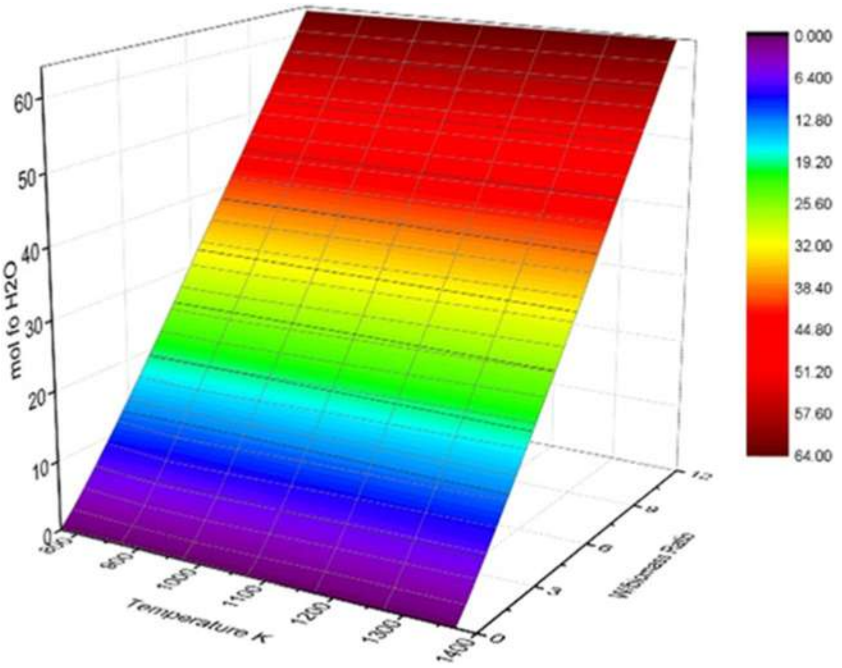


Fig. 5 **a** H_2 yield, **b** CO yield, **c** CO_2 yield, and **d** CH_4 yield from reforming at high temperatures (600 to 1300 °C) and using different H_2O/ATB ratios (0 to 12) at a pressure of 4 atm

Fig. 6 H_2O yield of reforming at high temperatures (600 to 1300 °C) and different H_2O/ATB ratios (0 to 12) at a pressure of 1 atm b)



equilibrium move to hydrogen conversion for being exothermic too, at a higher temperature, the equilibrium move toward the chemical reagents. About the supplied water in the system, the Eqs. 2, 3, 4, 6, and 7 in Table 1 show that with more water quantity, there will be a higher hydrogen production. Taking real attention to the Eq. 2, it is possible that the higher hydrogen may be determine at 740 °C and a high water/biomass relation. As it can be seen with the amount of 271.5 g of hydrolysis, residuum can produce 23.2 mol of hydrogen equivalent to 567.58 l at standard conditions.

In Fig. 5b, CO is shown as the second concerned compound and its behavior is different as H_2, having a better CO production at high temperature and poor at lower ones. This behavior can be explained because of the reactions (2), (3) and (6) from Table 1. Because (2) and (6) are exothermic, the equation move toward CO production favoring it when rising the temperature. The reaction (3) being endothermic helps the CO production moving the equation to the right as rising the heat. As the water in the system inreases, it helps the CO production as the reactions (2), (3) and (7) tells in the Fig. 1. This can explain the reaction (3) having more influence than reaction (2) but the two reactions are competing for the water consumption. The Fig. 5c shows the decrease in production of CO_2 related to heat and water adding to the reactor, as more heat and water is added, less CO_2 is outputted. Figure 5d shows the outlet methane from the reactor; the performance is inversely proportional to the temperature. It can be obtained 5.16 mol of methane from 1 for W/biomass ratio and 600 °C. On the other hand, Fig. 6a shows the output of non- reacted water from the reactor, temperature does not affect significantly, and the output water is the non-reacted water from the inlet.

Conclusions

The results of this research shows that *Agave tequilana* bagasse (ATB) is a very interesting raw material for bioethanol production and for development a biorefinery concept. Of natural form, it has a big amount of extractives (sugars) that could be used for the above purposes. With acidified steam explosion, pretreatment at 15.85 kg/cm^2, 0.5 % of sulfuric acid and mesh 40 size particles followed by enzymatic hydrolysis with Cellic Ctec 3 and Cellic Htec 3 was possible obtain a liquid hydrolyzed of 71.1 g/L of reducting sugars (59.29 % glucose, 29.05 % xylose, 11.66 % fructose). With the final residual, biomass (after pretreatment and hydrolysis) is thermodynamically possible to obtain hydrogen with a yield of 23.2 mol of H_2/271.5 g of ATB.

It can be concluded that a lignocellulosic material can be treated in order to obtain different compounds that can be used as raw material in other processes. In this study, sugars were obtained from ATB with which it is possible to produce bioethanol or other products such as like lactic acid, pyruvic acid, citric acid, furfural, sorbitol, xylitol, etc. Furthermore from de hydrolysis waste material, it could be obtained syngas and theoretically produce another add value products.

Acknowledgments This work was supported by the Mexican Council of Science and Technology (CONACYT) through the Bioenergy Thematic Network ("Red Temática de Bioenergía").

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Mater. Res. Soc. Symp. Proc. Vol. 1817 © 2016 Materials Research Society
DOI: 10.1557/opl.2016.45

Conformational and Morphological Study of Chitosan Nanohydrogels by MD Simulation and SEM

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ABSTRACT

Chitosan is biocompatible polymer has a great commercial interest because it can be processed in a sort of devices varying in shape and size, such as membranes, gels and nanoparticles. Mostly, the cell's attachment and proliferation are very positive on nanostructurated materials with a three-dimensional formation. An irreversible network can be produced by covalently binding the polymer to the cross-linker molecules. Chitosan nanoparticles were prepared using glutaraldehyde as cross-linker. This crosss-liker mostly reacts with chitosan amino groups. In order to control and understand the physical characteristics of chitosan nanoparticle, in this work is showed the molecular behavior of chitosan/glutaraldehyde from the viewpoint of molecular interactions base in a series of molecular dynamics (MD) computer simulation. The results indicated the conformations of both molecules, which had a significant influence on the molecular association. The chitosan chains were uniformly distributed presenting a high flexibility and preference for the relaxed two-fold helix. This was due to the various associations such as intramolecular chitosan interactions –O-H···O-C-. While the chitosan-glutaraldehyde associations were due to the positive net charge density of hydrogens in the chitosan plus - $H_2N\cdots C=O$ associations. In solid state chitosan nano and microparticles were analyzed by scanning electron microscopy (SEM). According to the micrographs results, the nanoparticles presented a monomorphism with piles of particles arranged in linear order which was consistent with the conformations determined by simulation.

INTRODUCTION

Chitosan is a natural cationic biopolymer commercially produced by deacetylation of chitin, which is present in the exoskeletons of crustacean, insects and some fungi [1]. It has received great attention as a functional biopolymer due to its high biodegradability, nontoxicity and antimicrobial properties. Additionally, features such as positive charge and mucoadhesive properties make this polysaccharide attractive for biomedical applications [2].

In recent years, nanoparticles of chitosan have been investigated as a potential carrier system for the nasal delivery of insulin. The mucoadhesive properties of the chitosan are based on the formation of noncovalent bonds such as hydrogen bonds, ionic interactions and Van der

Waals forces, or physical interchains [3]. In this regard, due to a large surface area, nanoparticles of chitosan can penetrate into tissues, reaching the bloodstream, thereby increasing the release of drugs [4]. Some researchers have optimized formulations of chitosan with glutaraldehyde as an amine-reactive bifunctional crosslinker. These formulations pretend increase the thermal and chemical properties of chitosan [5].

This study focuses to understand the mechanism of aggregation of chitosan molecules as well as the chitosan/glutaraldehyde, from a study of molecular simulation. Thus, dynamics simulations were performed to analyze the behavior between chitosan and glutaraldehyde. Chitosan /glutaraldehyde nanoparticles obtained on laboratory scale were also investigated in this work to provide insights into the mechanism of the atomic structures of polymeric structures and present a new perspective for the design of desirable nanostructures.

EXPERIMENT

Molecular simulations

Towards exploring the interaction mechanisms, molecular mechanics (MM) and dynamics (MD) simulations were employed to reproduce the *in situ* processes of the interaction between chitosan and glutaraldehyde. Materials studio from Accelrys Inc. was the software used in this study. All the atomistic simulations were performed using the COMPASS (condensed-phase optimized molecular potential for atomistic simulation studies) forcefield. COMPASS is a general all-atom force field for atomistic simulation parametrized by *ab initio* and empirical parametrization techniques and validated for a wide variety of systems [6].

Three conformational models were used for CS-1 (chitosan of one ring), CS-20 (chitosan of twenty rings) and glutaraldehyde. These were subject to calculations in order to arrive at the optimized geometry. These optimized structures were obtained by using the geometry optimization with a Smart algorithm (cascade of steepest descent, conjugate gradient, and quasi-Newton methods) with 50,000 main steps.

Amorphous cell and Discover modules were used to build an amorphous cell to implement the MD calculation by employing COMPASS forcefield. Two chains of chitosan and two chains of chitosan with glutaraldehyde were packed into the cubic cell with a density value of 1.00 g/cm^3 to obtain an appropriate relaxed structure, where the estimation for a specific volume through the NTP (25 °C) ensemble was used. In the present work, the amorphous cell after relaxation treatment was used in all calculations.

The relaxation time for MD simulation was determined in accordance with literature value, since generation of the initial molecular configurations.

Preparation of nanoparticles

Low molecular weight chitosan (85% deacetylation), acetic acid (ACS reagent, ≥99.7%) and glutaraldehyde solution (50% in H_2O) were supplied by Sigma- Aldrich. All the chemicals reagents were used without further purification. Deionized water was used throughout the study.

Chitosan (0.5 g) was dissolved in 1.5% (w/v) (2M) acetic acid to obtain a solution. Additionally, glutaraldehyde (25%) was added to chitosan solution with mild stirring until an opalescent suspension was obtained. The chitosan/glutaraldehyde solution (pH 3) was poured into a basic solution (1.5 M NaOH at 20 °C) maintained at 2,000 rpm, and after 15 min, the beads were obtained. In order to remove both the unreacted glutaraldehyde and the excess of NaOH, the solution containing the polymers beads was washed for 3 days with purified water, [7]. The experiment was carried out in a completely randomized design, with five repetitions.

Characterization

Uncoated cross-linked chitosan nanoparticles were analyzed with a JEOL JSM-7600F FEG-SEM 6400 scanning electron microscope (SEM) using the tungsten filament and operated with a voltage of 15 kV. Previous the SEM analysis, the samples were dyed at 60 °C, during 24 hr, under vacuum pressure.

DISCUSSION

The results of optimized energies for the three distinct conformational structures are listed in Table I. The chitosan containing 20 rings (CS-20) exhibited the higher level of energy from the optimized molecular structure, while glutaraldehyde is a structure with the lower energy level.

Table I. Optimized energy of chitosan and glutaraldehyde from conformational models

Structures	Rings	Molecular weight in g/mol.	Optimized energy in kcal/mol
CS-1	1	161.15	14.76
CS-20	20	3223.00	109.20
Glutaraldehyde	1	100.11	-2.43

Figure 1 shows the molecular structures of CS-1, CS-20 and glutaraldehyde. The chitosan is essentially a linear chain molecule consisting of alpha 1,4 linked glucose units, while glutaraldehyde is a symmetric structure.

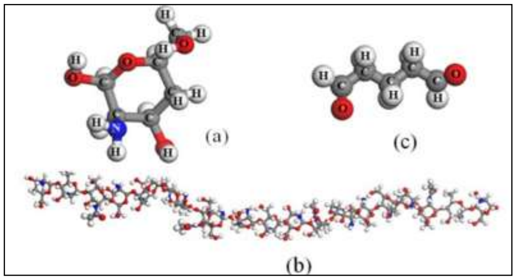

Figure 1. Optimized structures of (a) CS-1, (b) CS-20 and (c) glutaraldehyde structures, based on molecular mechanics (MM) simulations.

Figure 2 shows an amorphous cell where two CS-20 were integrated. Thus, Figure 2a presents the interaction of two CS-20. Figure 2b shows a larger vision of Figure 2a. In this image was possible to observe the associations between chitosan chains (CS-20 with CS-20). The intermolecular hydrogen bonding between the chains resulted from the forces of attraction between groups as C-OH···NH- and C-OH···C-O-C. Thus, the chitosan chains presented a high flexibility helical structure with amine groups, which offer a multitude of possible inter- and intra-molecular interactions.

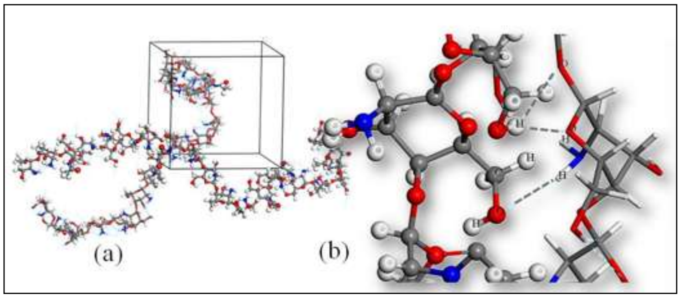

Figure 2. Amorphous cell with (a) two chains of CS-20, and (b) a magnified image of the contact of two CS-20 chains.

Figure 3 presents the visualization of the interaction at lowest energy configuration between the CS-20 and glutaraldehyde. The average life time of this conformational state was 20 ps. As it can see in this figure, the chain presents an aggregation, which is consistent with the behavior of natural polymers. In this sense, chitosan is known for a tendency to self-aggregate [8]. Figures 3a and 3b show the conformation of CS-20 chain underwent changes. It promoted contacts between CS-20 chains, which were close to the middle of the chains; in agreement with intramolecular hydrogen bonding, hydrophobic interactions and electrostatic forces [9]. Particularly, the effect of opposite charges, N-terminus (with $-NH_3{}^+$) and deprotonated C-terminus (with $-COO^-$) attract them in the middle of the chain, where the movements are more limited.

Figure 3b shows the association between two species of molecules, CS-20 and glutaraldehyde. The CS-20 chains were aligned along the x-axis (defined orientation) passing through the aldehyde sites of glutaraldehyde. The intramolecular hydrogen bonding (attractive forces) between chitosan and glutaraldehyde result from forces of attraction between the amine and carbonyl groups (- H_2N···C=O) as well as the hydroxyl and the carbonyl groups (-C-OH O=C). Thus, theoretically, a chemical reaction is possible to produce different products under acid conditions [10].

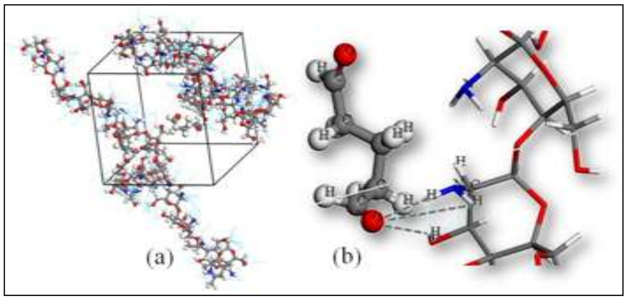

Figure 3. Amorphous cell with (a) two chains of CS-20 with a glutaraldehyde molecule, and (b) a magnified image of the contact of CS-20 with a glutaraldehyde molecule.

Figure 4a and 4b show images by SEM of the morphology of original chitosan (flakes) and particles of chitosan respectively. Figure 4a exhibits flakes of chitosan composed of fibers which were used for the chitosan particles formation. The analyzed flakes presented a longitudinal size around 350 μm.

Figure 4b displays micro and nanoparticles of chitosan. It was possible to consider the creation of hydrogels, because of particles were prepared by using glutaraldehyde as crosslinking agent. In this sense, due to hydrogels were prepared under standard stirring conditions (2000 rpm) a high grade of reproducibility of size and shape were obtained. Particularly no high size dispersion was achieved. The nano and micro sizes were in the range of 900 nm to 1.3 μm.

Figure 4b′ is a magnified image of the area marked with a white square in Figure 4b. The Figure shows with more detail a nearly spherical particle (hydrogel) where the internal vapor pressure forms dimples during the dried process [11].

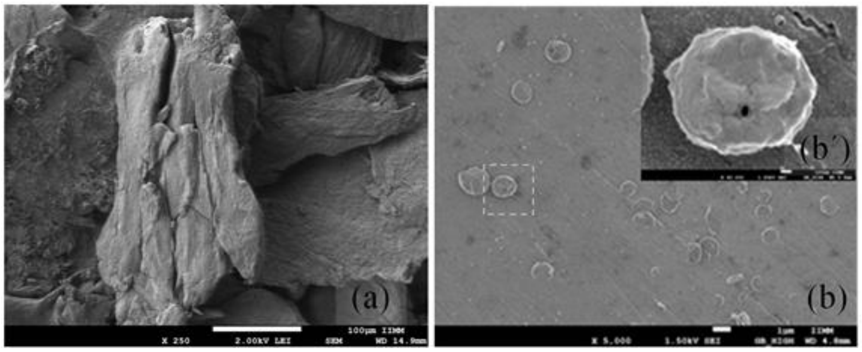

Figure 4. SEM micrographs of (a) chitosan flakes at 250x, (b) chitosan hydrogels at 5000x and (b′) magnified image of a single hydrogel.

CONCLUSIONS

Molecular dynamics (MD) simulations and experimental studies were performance to investigate the chitosan interacting with glutaraldehyde. By considering chitosan with a molecular weight of 3223 (twenty rings). In an amorphous cell It was found the strength of interaction between two chitosan molecules was obtained by the number of attractions between C-OH···NH- and C-OH···C-O-C sites. Although, chitosan present a high number of these groups, the majority of them are not capable of forming interactions, due to the helical conformation of the backbone decrease the contact sites between both molecules. This lack of interaction is positive in the association with the glutaraldehyde which takes place between - H_2N···C=O and -C-OH O=C sites. During the experimental studies, the crosslinked chitosan nano and microparticles (hydrogels) presented a high grade elastic deformation, which was identified by a dimple in the particle. This radial deformation was reported after the removal of water molecules during a drying process. In this case, the theoretical-experimental information provided a much more clear understanding of chitosan particle deformation.

ACKNOWLEDGMENTS

The authors thank the financial support from PROMEP Collaborative Thematic Networks. Coordination of Scientific Research (UMSNH) and National Council of Science and Technology (CONACyT-Mexico) under the projects approved No. 104259, 61414.

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Simultaneous Saccharification and Fermentation of Pine Sawdust (*Pinus pseudostrobus* L.) Pretreated with Nitric Acid and Sodium Hydroxide for Bioethanol Production

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With the purpose of taking advantage of pine sawdust residue coming from a sawmill located in Michoacán, México, a pretreatment with nitric acid and sodium hydroxide was performed. Also, the production of bioethanol by enzymatic hydrolysis was investigated. Using a response surface method, the intermediate points for the optimal HNO_3 concentration were determined. Results showed that using HNO_3 as a pretreatment leads to higher ethanol yields at an optimal concentration of 10.90% HNO_3. After a 30-min pretreatment with 10.90% HNO_3 at 114.32 °C, followed by 1% NaOH and enzymatic hydrolysis performed in shaker at a pH of 4.8 and 150 rpm for 72 h, with an enzyme loading of 25 FPU/g of total carbohydrates, the reducing sugars concentration was 99.2% (conversion of polysaccharides to monomers). On the other hand, the ethanol yield obtained from the simultaneous saccharification and fermentation treatment was 15.0 g/L, and the separate hydrolysis and fermentation was 17.1 g/L at a pH of 4.8 and 150 rpm with $1X10^7$ Cel/mL of *Saccharomyces cerevisiae* and an enzymatic loading of 25 FPU/g of total carbohydrates. When comparing the results obtained with literature data, it is concluded that this procedure is suitable to exploit the lignocellulosic wastes from the Indigenous Community of San Juan Nuevo Parangaricutiro, Michoacán, Mexico.

Keywords: Reducing sugars; Enzymatic hydrolysis; Pine sawdust; Pinus pseudostrobus

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INTRODUCTION

A potential petroleum shortage in the near future, increased levels of atmospheric CO_2, global warming, and the pressing need to tap into readily renewable lignocellulosic material that is produced and wasted worldwide are all factors that have spurred the search for technologies that foster the production of alternative fuels for the transport sector.

A variety of studies to identify raw materials that are likely to be economically turned into ethanol of first, second, and third generation have been conducted, providing the development of new technologies that enable the use of these resources to the utmost. Some of the sources for first generation fuel production are of agricultural origin and are confirmed by the nutritional parts of plants, which have a high content of cellulose, sugars, and oils. Examples of these sources of ethanol include organic solid waste, animal fat, and residual oil and grease used in cooking and food preparation (Álvarez 2009).

Second-generation biofuels have further advantages, such as the fact that they do not compete directly with food production market and, because all the plant biomass above the ground can be used, it is possible to achieve better and more efficient land use. On the other hand, compared with first-generation fuels, second-generation fuels demand major investments (Stevens *et al.* 2004).

Third-generation fuels are produced from fast-growing non-food crops with high energetic density stored in their chemical components, called "energy crops," such as perennial grass, trees, fast-growing plants, and green and blue-green seaweed. The advantage of these biofuels is that they capture carbonic anhydride (CO_2) for growth, thus generating a positive greenhouse gas balance. Their disadvantage, with the exception of green seaweed, is that the growing of such energy crops would occupy farm land (Atev *et al.* 1983).

The most widely studied processes for wood biomass pretreatment include alkaline, acid, steam explosion, organosolv, and SPORL sulfite pretreatment (Zhu *et al.* 2009). Because the enzymatic procedure is limited by the presence of lignin and cellulose crystallinity (Millett *et al.* 1976; Åkerholm and Salmén 2001; McLean *et al.* 2002), several pretreatment procedures have been used to expose cellulose more easily. For instance, sodium hydroxide (NaOH) pretreatment breaks down lignin-carbohydrate bonds, partially withdraws lignin and hemicelluloses, opens the material structures, increases interface area, and reduces the polymerization degree and crystallinity (Tuor *et al.* 1995). Pretreating the biomass with organosolv pretreatment can effectively remove recalcitrant lignin from wood biomass, resulting in an enzymatic saccharification of cellulose (Pan *et al.* 2005, 2006; Pan 2008). Using HNO_3, which is a strong oxidizer pretreatment, results in a limited hydrolysis usually referred to as *prehydrolysis.* Prehydrolysis consists of hydrolyzing the hemicellulose fraction, while the cellulose and the lignin fractions remain with almost no alteration. Each pretreatment has specific characteristics that have been developed to maximize hydrolysis yields. The main advantage of a simultaneous saccharification and fermentation process is that it reduces the inhibition per final product that takes place in the two-stage operation because the presence of fermenting microorganisms and cellulose enzymes reduces sugar accumulation in the fermenter. The combination of both methods leads to higher rates of hydrolysis than the separate use of hydrolysis and fermentation processes, requiring a smaller amount of enzymes and obtaining higher ethanol yields (Ballesteros 2001).

The objective of this study was to optimize the pretreatment conditions with nitric acid and sodium hydroxide of pine sawdust (*Pinus pseudostrobus* L.). It has been found that the hemicellulose and lignin removal is enhanced with the consecutive use of these chemicals. Being the nitric acid a strong oxidizing agent, and the sodium hydroxide action removes part of the lignin and the remained hemicellulose after the acid pretreatment. Followed by a comparison between simultaneous saccharification and fermentation (SSF) and Separate Hydrolysis and Fermentation (SHF), to use it as raw material for bioethanol production.

EXPERIMENTAL

Materials

Pinus pseudostrobus sawdust from the Indigenous Community of San Juan Parangaricutiro, in the state of Michoacán, Mexico, was used for this study. The sawdust was dried at 80 °C for 24 h in a Novatech electric furnace (Mexico, Tlaquepaque, Jal.) and then partially reduced in size using a generic blade mill at 1500 W to obtain heterogeneous fibers. The fibers were then sieved using a 20-mesh screen (841 μm) and retained on a 40-mesh screen.

The chemical composition of this biomaterial was determined and reported in a previous work (Farías 2014). Commercial cellulase enzyme preparations of *Trichoderma reesei*, including Celluclast 1.5 L by Sigma Aldrich, were used in this study. This enzyme preparation can be used to break down cellulose into glucose as shown in the page of manufacturer. *Saccharomyces cerevisiae* strain (Instituto Tecnológico de Durango, ITD-00185, Mexico).

Chemical Composition of Pretreated Material

The chemical features of the pretreated material determined in this study were moisture content, that was determined by Technical Association of the Pulp & Paper Industry (TAPPI) TAPPI T 210 cm-93 (1993), and ash content by TAPPI T 211 om–93 (1993). The total amount of extractive substances was determined by a sequence of Soxhlet extractions with cyclohexane, acetone, methanol, and water. Lignin by TAPPI T 222 om-88 (1998), holocellulose (Wise *et al.* 1946), and alpha cellulose by ASTM D 1103-60 (1978) contents were determined in the procedure for wood meal without extractable substances.

Combined Pretreatment Preliminary

To evaluate the sugar yield (the response variable), a 2^3 factorial design was performed, determining the following factors and levels: HNO_3 concentration (3% and 6%), temperature (50 and 100 °C), and time (30 and 60 min). In each experiment, 10 g of dry sawdust was placed in a 250-mL round-bottom flask followed by the addition of 60 mL of HNO_3 solution. The flask was then connected to a condenser, and the mixture was boiled for 30 min under reflux in an oil bath kept at a specific temperature. Next, the mixture was filtered and washed with 500 mL of distilled water (each experiment was done in triplicate). Finally, the three experiments was dried to 100 °C for yield determination.

The dry solids of the experiment with HNO_3 was put into a 250-mL round-bottom flask, followed by the addition of 120 mL of 1% NaOH solution. The residue was heated again for 30 min at reflux by means of an oil bath at 90 °C. After the chemical reactions took place, the raw material was filtered, washed with 500 mL of distilled water, and dried to 100 °C for yield determination.

Enzymatic Hydrolysis

Samples of 1.0 g of the pretreated material (HNO_3, followed by NaOH) were placed into 20-mL plastic containers and mixed with 2 mL of 1% m/v sodium azide and Celluclast enzyme cocktail 1.5 L (25 FPU/g of total carbohydrates). The reaction volume was then adjusted to 10 mL using 0.1 M acetate buffer solution of a pH 4.5. All samples were incubated under enzymatic hydrolysis at 48 °C and 150 rpm.

Optimization of the Pretreatment

A compound central design with three intermediate points was used to optimize the process and, at this point, the factors and levels were as follows: HNO_3 concentration (6.5% and 12%), temperature (100 and 130 °C), and time (constant time of 30 min). The content of reducing sugars in the hydrolyzed pine sawdust was the response variable in this study, was determined using the Ghose method (1987).

Separate Hydrolysis and Fermentation (SHF)

This process was carried out with the strain *Saccharomyces cerevisiae* ITD-00185, for which the fermentation process was kept in YPG (yeast, peptone, glucose) medium at pH 5.5. Once the hydrolysis process was optimized, the fermentation of the hydrolyzates obtained with the optimal procedure was carried out.

The mixture was centrifuged at 8000 rpm for 5 min to separate the residual solids. Then, yeast extract (10 g/L) and peptone (5 g/L) were added to the centrifuged liquid. Finally, a *Saccharomyces cerevisiae* strain with a microbial charge of 1×10^7 was inoculated (the proportion of microbial culture fluid added was approximately 2% of the total volume of the fermentation).

The inoculated medium was incubated at 30 °C for 72 h, taking samples every 8 h. The ethanol content was determined by a high-performance liquid chromatography (HPLC), using a Metacarb 87 C (Agilent Technologies, Santa Clara, CA) device at 65 °C, with an isocratic flux of water and a refractive index detector at 50 °C. The working pressure was 58 bar. The reducing sugars content was determined using the Ghose method (1987).

Simultaneous Saccharification and Fermentation (SSF)

Each experiment consisted of 2 g of the pretreated material (resulting from the optimal conditions) placed in 50-mL Erlenmeyer-flasks, and then shaken. Afterwards the contents were mixed with 15 mL of YP growing medium (10 g/L yeast extract and 5 g/L peptone). Then the final reaction volume was adjusted to 20 mL with a 0.05 M acetate buffer of pH 5.0.

The mixture was sterilized at 120 °C for 15 min and then inoculated with a 2% v/v of a culture fluid containing 1×10^7 Cel/mL of *S. cerevisiae* and 25 FPU/g of enzymatic cellulase solution (Celluclast 1.5 L, an enzyme preparation of *Trichoderma reesei*). The whole of the mixture was incubated to 150 rpm at 35 °C for 72 h, taking samples every 8 h, under aerobic conditions. In this case the temperature needs to be higher in order to allow the enzymatic activity without decrease the growing of the strain. For this reason, one can consider that this is the compromise variable. The ethanol content was determined by HPLC at 65 °C, with an isocratic flux of water and a refractive index detector at 50 °C. The working pressure was 58 bar.

Statistical Analysis

A 2^3 factorial design was performed, and with the results of this was done a compound central design the following factors and levels: HNO_3 concentration (9.25% and 12%), and temperature (100 and 130 °C). All the experiments were performed by triplicate, and the obtained data were analyzed with 95% statistical confidence using STATGRAPHICS Centurion XVI version 16.1.18 software (Warrenton, Virginia).

RESULTS AND DISCUSSION

Chemical Composition of Pretreated Material

The chemical composition of *Pinus pseudostrobus* sawdust was determined and reported in a previous work (Farías 2014). The chemical analysis results (Table 1) of the pretreated sawdust indicated a 5.67% lignin content and, as expected, a higher content of polysaccharides. It follows that approximately 81% of the lignin was eliminated by the pretreatment performed.

Table 1. Chemical Composition of the Pretreated Sawdust and Residues of the Hydrolyzed Material (%)

	Sawdust **	Pretreated sawdust
Ash	0.19±0.06	0,18±0.04
Extractives	5.11±0.32	-
Lignin	28.94±0.17	5,67±0.18
*Holocellulose	66.53±0.85	94,5±0.77
Hemicellulose	23.55±0.85	28,74±0.77
α-cellulose	42.98±4.96	65,76±1.37
	100.77±1.4	100,35±2.36
* Holocellulose is made up of α-cellulose and hemicellulose content. **Farías-Sánchez *et al.* (2015)		

Combined Pretreatment Preliminary

According to the 2^3 experimental design applied in this study, it was observed that the pretreatment time did not significantly influence the hydrolysis process yield (p = 0.8167), while the acid concentration and temperature of the process did influence the sugar yield significantly ($p < 0.0001$). It can be concluded that the best yield was obtained under the pretreatment conditions of 6% nitric acid at 100 °C, followed by 1% NaOH at 90 °C, as shown by the means graphs (Figs. 1 and 2). As time did not influence the yield significantly, it was decided to keep the time factor constant at 30 min for the composed central design.

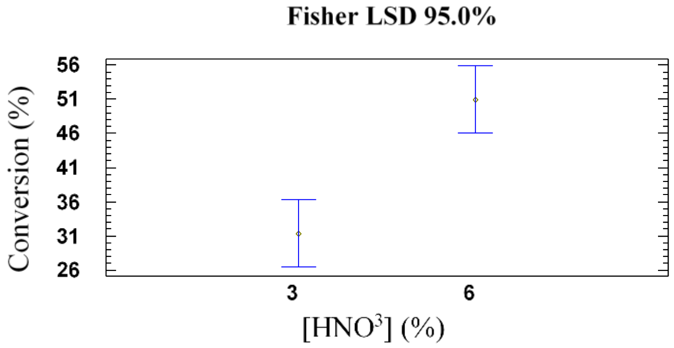

Fig. 1. Means of 2^3 experimental design for acid concentration factor at 100°C

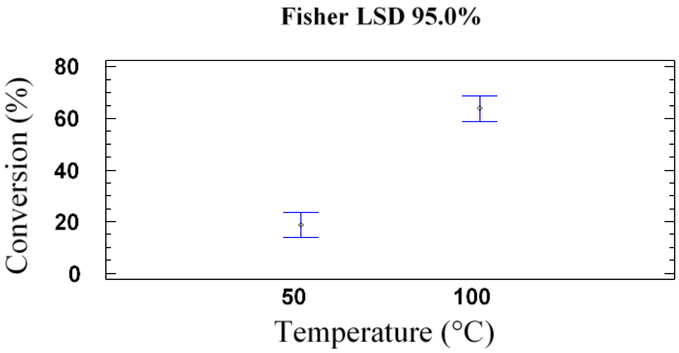

Fig. 2. Means of 2^3 experimental design for temperature factor with 6% of nitric acid

Pretreatment Optimization

Table 2 shows the optimal conditions and results obtained when applying compound central design. The conversion column indicates the amount of reducing sugars obtained by the hydrolysis with respect to the total amount of cellulose in the pretreated material. The analysis of variance performed indicates that the acid concentration (p = 0.0315) and the temperature (p = 0.0013) had significant effects on the yield. The average values of each experiment are shown in Table 2. The highest yield (98.61 ± 2.12%) was obtained at an acid concentration of 9.25%, temperature of 115 °C (Table 2), 30 min of pretreatment. The experimental procedure and material balance are shown in Fig. 3.

Table 2. Average Values of the Compound Central Design Matrix

Run	[HNO_3] (%)	Temperature (°C)	Conversion (%)
1	9.25	94	70.63 ± 1.80
2	6.25	100	73.71 ± 2.21
3	12.00	100	97.23 ± 1.94
4	5.36	115	93.67 ± 1.80
5*	9.25	115	98.61 ± 2.12
6	13.13	115	92.93 ± 1.71
7	6.5	130	92.93 ± 1.92
8	12.00	130	87.23 ± 2.03
9	9.25	136	83.75 ± 1.82

* This analysis was repeated six times, according to the compound central design.

The optimization of the acid treatment using the response surface method showed that the optimal conditions were as follows: pretreatment time of 30 min, HNO_3 concentration of 10.90%, pretreatment temperature of 114.32 °C, and hydrolysis time of 72 h. The graph of the response surface results is shown in Fig. 4.

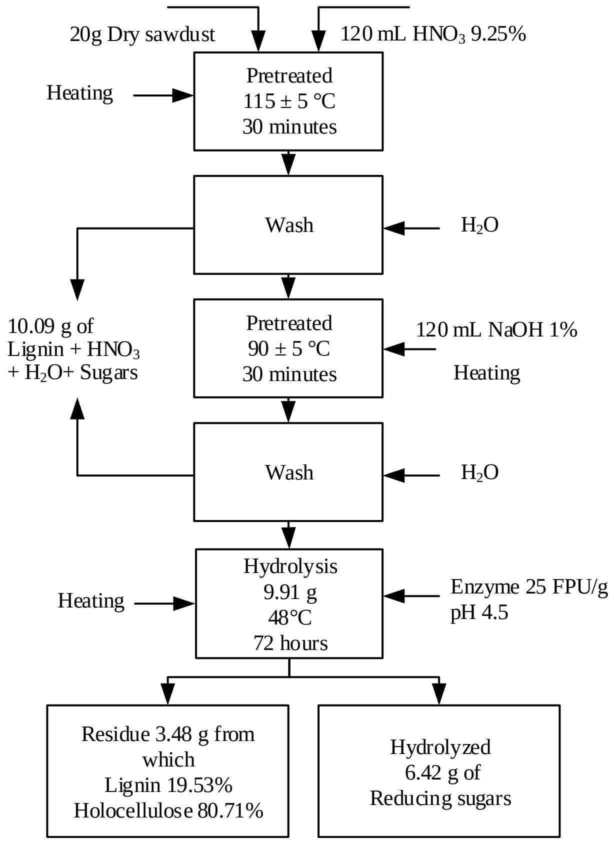

Fig. 3. Material balance and process flow diagram

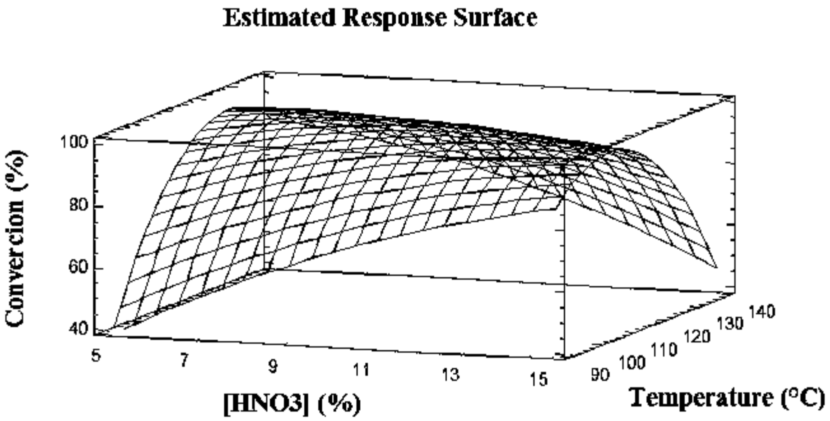

Fig. 4. Compound central design of pretreatment conditions optimization

Figure 4 shows the behavior of experiments. This was done by taking the data of Table 2, the columns [HNO3] (%) *vs.* Temperature (° C) *vs.* Conversion (%) of each of the experiments, which is given by the STATGRAPHICS Centurion XVI software version 1.16.18 (Warrenton, Virginia). Results can be expressed as Eq. 1, for which the coefficients, based on data regression for reducing sugar concentration, are given in Table 3.

Table 3. Regression Coefficients for [Conversion]

Coefficient	Estimated
Constant	-738.52
A: [HNO_3]	26.065
B: Temperature	12.1695
AA	-0.267105
AB	-0.177061
BB	-0.0447837

$$Y\,(\%) = Constant + A * X_1 + B * X_2 - A * X_1^2 + AB * X_1 * X_2 + BB * X_2^2 \qquad (1)$$

In Eq. 1, Y is the reducing sugar concentration (%), X_1 is [HNO_3] (%), and X_2 is temperature (°C) and constants A, B, AB, and BB are replaced by the values given by the column "Estimated".

Entering the optimized conditions for X_1 as acid concentration of 10.90% and X_2 as temperature of 114.32 °C into Eq. 1 provides a reducing sugar yield of 99.15% with relation to the total cellulose contained in the pretreated biomass. These results might lead to the development of a technology to exploit pine sawdust for the generation of an alternative fuel for the automotive industry and other value-added products.

Separate Hydrolysis and Fermentation (SHF)

The numerical results obtained from the separate treatments of hydrolysis and fermentation are shown in Fig. 5, which is a plot based on these results.

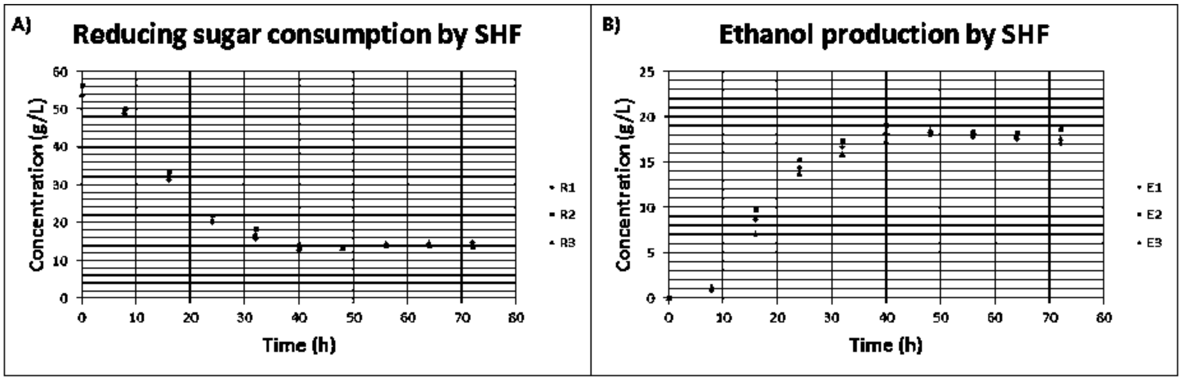

Fig. 5. Kinetics data for separate hydrolysis and fermentation treatments

The maximum production of ethanol was 17.01 g/L, at 40 h. This result is equal to 88% of the theoretical conversion of the alcoholic fermentation process. Also, after 40 h, the reducing sugar concentration did not show any noticeable change. Therefore, the residual sugar could be xylose, as the *S. cerevisiae* strain used in this study cannot ferment xylose.

Simultaneous Saccharification and Fermentation (SSF)

The numerical results for the SSF treatment are shown in Fig. 6.

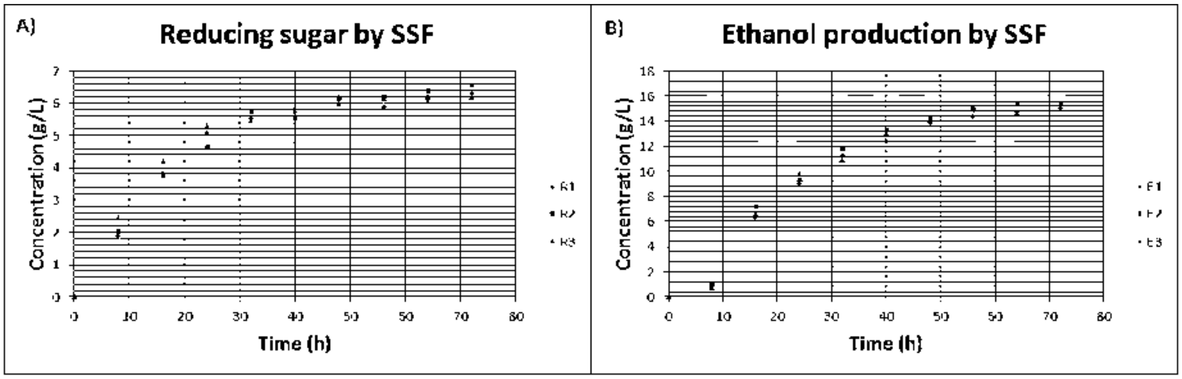

Fig. 6. Kinetics data for simultaneous saccharification and fermentation

The aim of the integrated hydrolysis and fermentation treatment was to improve the yield of ethanol. This is not supported by the results shown in Figs. 5 and 6, for which the concentration of ethanol was almost the same in both process configurations. In the SSF process there is not a high concentration of glucose at any time, and for this reason the ethanol production was lower than the SHF process. On the other hand, during SHF process the hydrolysis continues until the fermentation stage. So, the pentose concentration can be greater than that in the SSF process, which explains the higher value of residual reducing sugars in SHF.

For both SSF and SHF, the equivalent of 537.2 kg of reducing sugars per ton of pretreated material was transformed, resulting in 235.3 L of ethanol in the SHF and 167.0 L of ethanol in the SSF.

The results obtained in this research were relatively high when compared with the results reported in other works. Tang *et al.* (2013) obtained from corn starch a ratio of 86.9% by a polysaccharide utilization with commercial thermostable α-amylase and amyloglucosidase for saccharification and *Streptococcus thermophiles*, *Lactobacillus bulgaricus*, and *Saccharomyces cerevisiae* for SSF. Luo *et al.* (2010) obtained 69% of the theoretical ethanol yield with the SPORL procedure. In their work, these authors used 2.21% sulfuric acid gas charges and 8% sodium bisulfite on oven-dried wood, and an enzymatic load composed of Celluclast 1.5 L (15 FPU/g substrate) and Novozyme 188 (22.5 CBU/g substrate). Ballesteros *et al.* (2004) treated wheat straw and obtained an ethanol concentration of 18.1g/L and a yield of 62.5%. Vázquez-Ortega (2013) worked with bean straw and obtained an ethanol concentration of 10.50 g/L and a yield of 50.59%. Saucedo-Luna *et al.* (2010) used 2% sulfuric acid at 151 °C and a reaction time of 10 min, obtaining a yield of 48.5% and, when using *Agave tequilana* bagasse as the raw material, the ethanol concentration was 22.02 g/L. Nevertheless, the results in the present work are slightly lower than the data reported by Shuai *et al.* (2010), who reported a 77.7% yield at 180 °C using steam explosion for 30 min and a 5% sulfuric acid load on oven-dried spruce wood with a 1:5 wood-liquor ratio and an enzymatic load of 15 FPU. Velázquez-Valadez *et al.* (2016) worked with *Tequilana weber* agave bagasse using 6% w/w sodium hydroxide solution, 6% w/w hydrogen peroxide solution, 6% Cellic CTec3, and 6% Cellic HTec3 and obtained a concentration of 165.67 g/L of reducing sugars and 84.49 g/L of theoretical ethanol yield.

CONCLUSIONS

1. Optimization of acid treatment using the response surface method showed that the optimal conditions were: pretreatment time, 30 min; HNO_3 concentration, 10.9%; pretreatment temperature, 114.8 °C; and hydrolysis time, 72 h. Under these conditions, a 99.2% yield of reducing sugars was obtained with respect to the cellulose contained in the pretreated biomass.
2. A higher yield of fermenting sugar was observed with the use of nitric acid and sodium hydroxide as well as a shorter fermentation time of solids when compared with previous pretreatment conditions and to sawdust without any pretreatment. It was also observed that the hydrolysis efficiency with nitric acid pretreatment was even higher than that of untreated cellulose.
3. Results suggest that combined pretreatment of the sawdust withdraws lignin and part of the hemicelluloses, making the conversion of crystalline cellulose into amorphous cellulose easier. Therefore, the combined pretreatment aids the enzymes to increase the final yield by means of a more efficient hydrolysis.
4. Finally, based on the calculated yields obtained in this research work, it may be concluded that for each ton of sawdust processed, 235.3 L of ethanol can be produced.
5. To sum up, sawdust (a potentially useful wood waste) can be used for the production of reducing sugars, and thus, for bioethanol production.

ACKNOWLEDGEMENTS

The authors are grateful to CONACYT (Project 166444) for the financial support, and deeply appreciate the study material donated by the Indigenous Community of San Juan Nuevo Parangaricutiro, Michoacán, Mexico.

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Article submitted: September 15, 2016; Peer review completed: November 4, 2016; Revised version received and accepted: December 8, 2016; Published: December 14, 2016.
DOI: 10.15376/biores.12.1.1052-1063

Evaluación del pretratamiento alcalino-oxidativo previo a la hidrólisis enzimática de bagazo de Agave tequilana.

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Resumen

Se realizó un estudio para determinar una combinación de reactivos en el pretratamiento alcalino-oxidativo que minimice la cantidad de lignina residual a la vez que maximice la producción de azúcares fermentables después de la hidrólisis enzimática para lo cual se planteó un diseño factorial de dos factores y tres niveles. El resultado de este análisis llevó a la conclusión de que la mejor combinación de reactivos es 6% de hidróxido de sodio y 6% de peróxido de hidrogeno, Obteniendo un 8.24% de lignina en el material pretratado y 56.02 g/L de azúcares totales después de la hidrólisis lo que equivale a una conversión de 55.50%, esto nos indica que se pueden obtener 344 kilogramos de azúcares por tonelada de biomasa lignocelulósica libre de extraibles.

Palabras clave

Azúcares, lignina, hidrólisis, alcalino, oxidativo.

1. Introducción

El consumo de energía se ha incrementado a lo largo del último siglo, a medida que la población en las grandes ciudades se ha multiplicado; debido a que la principal fuente de energía es el petróleo, se tiene una acumulación de gases contaminantes que en los últimos años ha llegado a niveles peligrosos, provocando efectos nocivos para el medio ambiente, tales como el cambio climático. Por otro lado, los combustibles fósiles están distribuidos de manera no uniforme en la tierra, se calcula que el 63% de las reservas de hidrocarburos se encuentran en el territorio donde vive solo el 2% de la población mundial [1], lo cual acarrea problemas geopolíticos y desabasto de energéticos en algunas regiones. Como una manera de combatir estos efectos nocivos se han desarrollado los combustibles alternativos como el etanol, el cual ha llegado a sustituir el 3% del consumo global de energéticos para el transporte en 2011 [2]. Sin embargo, esa cantidad de etanol es considerado de primera generación debido a que se obtiene del almidón de las semillas de maíz o de jugo de caña de azúcar. Como consecuencia de la utilización de cultivos alimenticios para la obtención de combustibles podemos citar el encarecimiento de los alimentos básicos y la sobre explotación de tierras de cultivo. Es por estas razones que se plantea la alternativa de utilizar los azúcares contenidos en forma de polímeros (como celulosa y hemicelulosa) en la biomasa residual de la agroindustria para obtener etanol. Por otro lado, para lograr que los cultivos sean sustentables se requiere que las plantas destinadas para este fin se puedan desarrollar en tierras abandonadas, semi-aridas o degradadas [3]. El *Agave tequilana* es una opción viable que cumple con esa característica, además de que tiene un ritmo de crecimiento relativamente rápido. Las piñas de *Agave tequilana* son utilizadas en la región occidente de México en la elaboración de bebidas espirituosas como el tequila o el mezcal. Posteriormente, el material residual (libre de fructanos) es un material que en el mejor de los casos es usado como combustible directo en las calderas. Dicho residuo es biomasa lignocelulósica que presenta alrededor de 21% de lignina y un 73% de holocelulosa [4] por lo cual es posible utilizar este material para la producción de etanol por medio de la fermentación alcohólica. No obstante, para posibilitar este proceso biológico es necesario liberar los azúcares que forman parte de la estructura de la holocelulosa y retirar el contenido de lignina que juega un papel antagónico en el crecimiento bacteriano y la actividad enzimática [5]. El pretratamiento es la operación que disgrega la estructura de la biomasa lignocelulósica al romper los puentes de hidrogeno existentes entre las cadenas de celulosa y además elimina la lignina. Adicionalmente a la efectividad de esta operación, se deben cumplir otros requisitos económico-ambientales; no debe hacer uso de químicos demasiado caros, ni gran cantidad de estos y tampoco debe consumir una gran cantidad de energía. Por este motivo existe una gran diversidad de configuraciones que han sido probadas en la práctica. Para este fin existe una gran cantidad de procedimientos, que van desde los tratamientos mecánicos como la simple reducción de tamaño, o la extrusión; los fisicoquímicos como la explosión con vapor o con amonio (AFEX, "*amonia fiber explosión*"); y los químicos, como el tratamiento con ácido diluido, ozonolisis, tratamiento con solventes orgánicos, líquidos iónicos y el tratamiento alcalino. En dicha técnica se aprovecha el efecto que tienen las bases como el hidróxido de sodio, sobre la materia lignocelulósica. Puede ser llevado a cabo incluso a temperatura ambiente, y su tiempo de acción va desde unos cuantos segundos hasta varios días dependiendo de la temperatura. Funciona mejor para residuos agroindustriales que para maderas. Tiene la desventaja de que puede haber perdidas de azúcares fermentables. Produce aumento de volumen del substrato a tratar y por ende aumento el área expuesta de las cadenas de celulosa, también disminuye el grado de cristalinidad y polimerización de las fibras [6]. Si se combina este tipo de pretratamiento con la acción de un agente oxidante como el peróxido de hidrogeno es posible lograr que más de la mitad de la lignina y casi toda la hemicelulosa se solubilicen. Esta operación es fuertemente dependiente del pH y su máximo de efectividad esta dado entre 11.5 y 11.6 [7,8].

2. Materiales y Métodos

2.1 Obtención del material y tratamiento previo

El bagazo de agave se obtuvo de la tequilera "Ex Hacienda Zacapendo" como residuo de la cocción y prensado de piñas de *Agave tequilana.* Posteriormente se secó a una temperatura de 80°C por 24 horas en un horno eléctrico Novatech, posteriormente se redujo su tamaño parcialmente en un molino de cuchillas genérico de 1500 W para obtener tamaños de fibras heterogéneos que van de 3 a 15 mm de largo con diámetros de 0.7 mm. Cabe señalar que no se utilizó ninguna clase de tamiz para reducir al máximo el consumo de energía de la molienda. Se determinó la composición química del material pretratado: porcentaje de humedad [9], cenizas [10], lignina Runkel [11] y holocelulosa [12], fueron determinados en la harina del material libre de solubles.

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2.2 Pretratamiento

El material seco y molido se trató con diferentes combinaciones de reactivos, las cuales se estudiaron en un diseño factorial 2^3 como se observa en la tabla 2. En todos los casos se utilizaron 10g de bagazo seco y molido, se colocaron en un matraz Erlenmeyer de 500 mL, se le agregaron 100 mL de hidróxido de sodio a la concentración especificada por el diseño (Tabla 2). Se trató dicha mezcla a 121°C en autoclave por 60 minutos. Transcurrido ese tiempo se dejó enfriar a temperatura ambiente y se le agregaron 100 mL de la solución de peróxido de hidrogeno especificada por el diseño. Esta mezcla se dejó reposar por 24 horas a 30°C en una estufa bacteriológica marca Precision; Una vez transcurrido ese tiempo, se acidifico la mezcla con ácido clorhídrico concentrado (al 36.9%) hasta un pH de 5.0, para el proceso de hidrólisis, la materia prima se almaceno húmeda para evitar los cambios de estructura por efecto del secado [13]. A cada uno de los materiales resultantes de los pretratamientos se les determinó su contenido de lignina por el método antes mencionado.

2.3 Hidrólisis enzimática

Para la hidrólisis enzimática, se utilizó la enzima Celluclast 1.5L de Novozymes. Se agregaron 2 gramos en base seca de material pretratado, 1 mL de enzima y solución buffer de citratos 0.05 M pH = 5.0 hasta completar 20 mL. Dicha mezcla se conservó 48 horas a 50°C con una agitación de 150 rpm en un shaker orbital marca Polyscience; al final de ese lapso se analizó el sobrenadante por un método estandarizado en un equipo HPLC marca Varian Pro Star, utilizando una columna Metacarb 87 C a 70°C y un detector de índice de refracción a 50°C con flujo isocrático de agua a 1 mL/min para determinar azúcares simples.

3. Resultados y discusión

3.1 Composición del bagazo de *Agave tequilana*

La composición del material lignocelulósico se muestra en la tabla 1 realizada con la metodología antes mencionada.

Tabla 1. Composición del material lignocelulósico inicial.

Componente	Cantidad (% w/w)
Material Soluble	44.29 ± 0.93
Lignina	13.57 ± 0.18
Cenizas	2.07 ± 0.08
Holocelulosa	40.07 ± 0.77

Se observa que en el bagazo de agave obtenido como residuo de la producción tequilera hay una gran cantidad de material soluble, el cual se puede remover por simple ebullición con agua; Esta materia soluble está constituida principalmente por inulina, esto se corroboro analizando la muestra del sobrenadante del proceso de remoción de material soluble por medio de HPLC con el método estandarizado. Los resultados obtenidos experimentalmente de la composición del bagazo de agave corresponden con los datos obtenidos por otros autores como Yang et al. [3] quien reporta 13.8±1.3% de lignina, 43.8 ± 1.3 % de holocelulosa y 6 % de cenizas y Flores-Sahagun et al. [4] quien reporta 21.10 ± 1.13 % de lignina, 73.6 ± 0.01 % de holocelulosa y 5.3 ± 0.07 % de cenizas en base seca y libre de solubles, estas pequeñas variaciones pueden ser atribuidas a la zona de crecimiento y época de cosecha del Agave tequilana.

3.2 Pretratamiento alcalino-oxidativo.

Realizando el procedimiento establecido para el pretratamiento explicado con anterioridad (alcalino-oxidativo) para el bagazo de *Agave tequilana*, se realizó la cuantificación de lignina residual y se llegó a los resultados mostrados en la tabla 2, la tendencia de eliminación de lignina con respecto a la concentración de peróxido de hidrogeno, muestra un máximo a una concentración de 10% (w/v), eliminando un 63.1% de la lignina original y conteniendo una lignina final del 5.01%, lo cual es una cantidad muy buena comparado con Cheng et al. [14] quien solo obtuvo una remoción del 12.9% de la lignina original con un tratamiento de 0.6% de H_2O_2 por 50 minutos con bagazo de caña de azúcar; tal como lo mencionan Wilkinson et al. [8] la cantidad de lignina después del pretratamiento está entre 4 y 6 %. Los sobrenadantes resultantes se analizaron por HPLC en con el método antes mencionado. En todos los casos se encontraron trazas de azúcares simples y una gran cantidad de inulina.

3.3 Hidrólisis enzimática.

Todos los experimentos se corrieron por triplicado y los resultados de la hidrólisis de los experimentos relacionados al método alcalino-oxidativo se muestran en la tabla 2.

Nótese que el máximo de hidrólisis se encuentra en una concentración intermedia de hidróxido de sodio y de peróxido de hidrogeno. Esto se debe a que a bajas concentraciones de reactivos hay poca disgregación de la estructura del material lo que impide la acción de las enzimas. Sin embargo, si la concentración de reactivos es muy alta, entonces se consigue una gran destrucción de la matriz lignocelulósica pero a la par también se producen compuestos nocivos que inhiben la actividad enzimática. Según Wilkinson et al. [8], la cantidad de azúcares reductores obtenidos esta alrededor de 30 a 40 g/L, para la hidrólisis de residuos de granos de malta pretratada con un método alcalino oxidativo similar, también podemos comparar los rendimientos con Caspeta et al. [15] el cual obtuvo con un pretratamiento combinado de ácido y etanol en un proceso etanosolv e hidrólisis enzimática una concentración de azúcares de 56.4 g/L con la relación del 10% de sólido usando como materia prima bagazo de agave y con rendimientos de azúcares ligeramente más altos con Saha, Cotta [16] quien reporta una concentración de azúcares de 66 g/L usando un preparado comercial de enzimas (celulasa, α-glucosidasa, y xylanasa) con un pretratamiento alcalino/oxidativo de 8.6%, w/v de NaOH y 2.15% H_2O_2, v/v a 35 °C por 24 h con paja de trigo.

Tabla 2. Tabla del diseño factorial 2^3 para el pretratamiento alcalino-oxidativo: Porcentaje de lignina residual y conversión del proceso de hidrólisis.

Número	NaOH (% p/v)	H_2O_2 (% p/v)	Lignina residual (% p/p)	Concentración de azúcares (g/L)
1	2	2	11.13 ± 1.01	33.82 ± 1.04
2	2	6	14.18 ± 0.89	38.30 ± 0.74
3	2	10	12.64 ± 0.77	35.65 ± 0.57
4	6	2	6.34 ± 0.21	49.04 ± 0.67
5	6	6	8.24 ± 0.32	56.02 ± 0.92
6	6	10	5.01 ± 0.17	54.92 ± 1.16
7	10	2	5.24 ± 0.12	12.33 ± 0.97
8	10	6	6.31 ± 0.06	16.78 ± 0.82
9	10	10	5.3 ± 0.08	13.68 ± 0.77

3.4 Análisis estadístico y balance de materia.

En primera instancia se realizó un análisis del experimento factorial, para lo cual se utilizó la Tabla 3, que representa la ANOVA. Obsérvese que los dos factores principales tienen un efecto significativo ($p<0.01$) en el grado de despolimerización de la fibra de *Agave tequilana*; la interacción de estos factores también resulto ser significativa ($p=0.025$) sin embargo se concluye que es débil en comparación con los efectos de las concentraciones de los reactivos.

Tabla 3. Tabla ANOVA para el análisis del diseño factorial 2^3 del pretratamiento alcalino oxidativo.

						FT	
F	**GL**	**SS**	**MS**	**F0**	**0.05**	**0.025**	**0.01**
A	2	6887.69	3443.85	2405.30	3.55	4.56	6.01
B	2	133.88	66.94	46.75	3.55	4.56	6.01
AB	4	21.02	5.26	3.67	2.93	3.61	4.58
ERROR	18	25.77	1.43				
TOTAL	26	7068.37	271.86				

Para optimizar el proceso del pretratamiento, se recurrió al análisis de superficie de respuesta, la cual se muestra en la Figura 1. Del mismo modo se obtuvo el modelo matemático del pretratamiento, el cual queda representado en la ecuación 1. Obsérvese que el término combinado, tiene un coeficiente de valor numérico bajo, debido a la poca interacción de los factores estudiados.

$$y = ax_1^2 + bx_2^2 + cx_1 + dx_2 + ex_1x_2 \qquad (1)$$

-2.49444 + 18.5125*NaOH + 3.2675*H2O2 - 1.76458*NaOH^2 - 0.0075*NaOH*H2O2 - 0.237083*H2O2^2

Donde y es la concentración de azúcares reductores en g/L; x_1, es la concentración de hidróxido de sodio; x_2, es la concentración de peróxido de hidrógeno, ambas en porcentaje peso-volumen; las constantes a, b, c, d y e son dimensionalmente congruentes y se obtuvieron por medio de una regresión utilizando el método de diferencias cuadradas. En la Tabla 4, se resumen los valores de estas constantes. Finalmente, con la utilización del modelo obtenido y la técnica de máximos y mínimos, se llegó a la conclusión de que el pretratamiento óptimo se lleva a cabo con 5.21% de hidróxido de sodio y 6.78% de peróxido de hidrógeno.

Obsérvese que el término combinado, tiene un coeficiente de valor numérico bajo, debido a la poca interacción de los factores estudiados, por otro lado, obsérvese que los coeficientes de x_2 tienen un valor más alto, por lo cual se concluye que la etapa determinante del proceso es el tratamiento con hidróxido de sodio.

Tabla 4. Valor numérico de los coeficientes de correlación para el modelo del pretratamiento alcalino-oxidativo propuesto.

Coeficientes de correlación	
a	-1.7324
b	-0.2092
c	18.0147
d	2.8109
e	0.0062

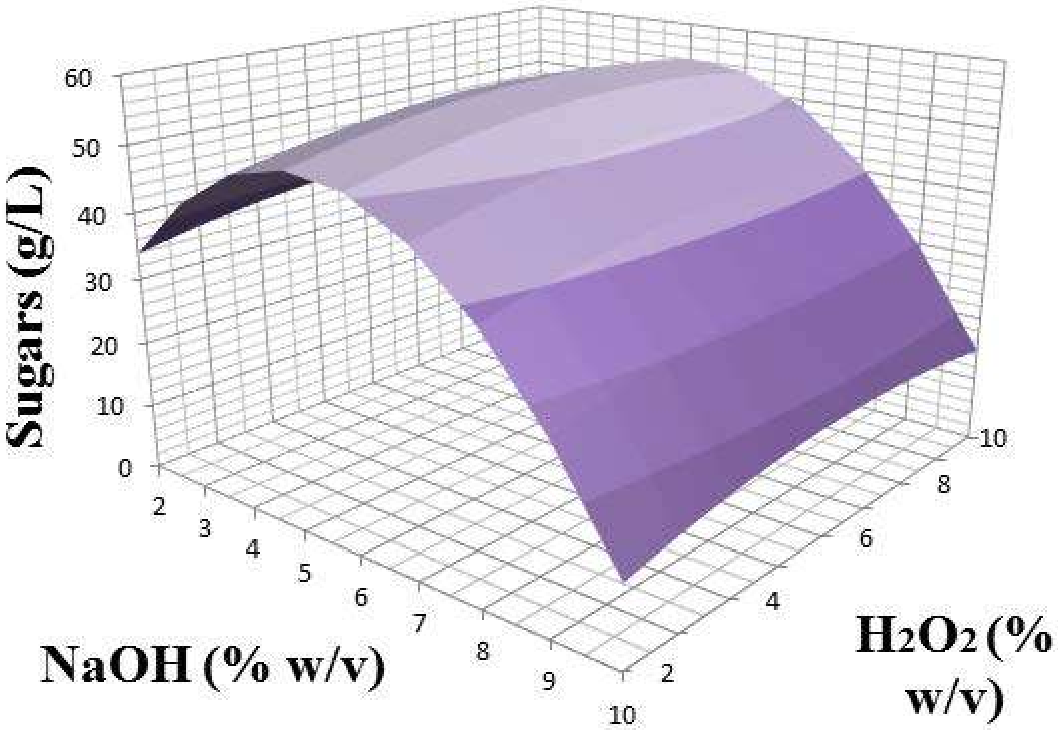

Figura 1. Superficie de respuesta para el diseño experimental del pretratamiento alcalino-oxidativo.

Finalmente se realizó un balance de materia global del mejor pretratamiento e hidrólisis, este balance incluye las tres corrientes resultantes:

- El efluente del pretratamiento, el cual consta básicamente de lignina disuelta (y probablemente despolimerizada), inulina, productos de la degradación de polisacáridos y otros compuestos solubles. La cantidad total de materia removida por el pretratamiento incluye a estos cuatro componentes, sin embargo, en el balance se muestran valores en base libre de material soluble.
- El sobrenadante del proceso de hidrólisis, el cual consta de una solución de azúcares simples (principalmente glucosa y solo una pequeña parte de xilosa) y pequeños oligosacáridos (principalmente celobiosa).

- El sólido residual después de la hidrólisis, el cual consta de toda la lignina que el pretratamiento no pudo remover y los polisacáridos no hidrolizados.

Para una base de 10 g de material lignocelulósico libre de solubles, utilizando el pretratamiento elegido y posteriormente hidrolizando con el procedimiento estándar; se obtuvieron 3.78 gramos de azúcares simples y 1.06 g de oligosacáridos (determinados como celobiosa), resultando 2.24 gramos de solido residual. El efluente del pretratamiento contiene todo el material restante, el cual se calculó por la diferencia del material sólido posterior al pretratamiento y el material inicial, el cual corresponde a 3.24 gramos. De esta manera es posible calcular la cantidad de azúcares reductores obtenidos, 378.57 kg de azúcares por tonelada de material lignocelulósico libre de solubles, estos rendimientos son altos comparados con Farías-Sánchez et al. [17] quien obtuvo un rendimiento de 273.46 kg de azúcares por tonelada de biomasa lignocelulósica con un pretratamiento de 7.5 % de ácido nítrico a 120 °C por 30 minutos, seguido de 1 % hidróxido de sodio a 90 °C y una hidrólisis enzimática con 25 UPF de Celluclast 1.5 L, de NOVOZYME por gramo de muestra pretratada a partir de aserrín de pino. En el estudio de Mathew et al. [18] se logra un rendimiento de 440 g/Kg de azúcares reductores, partiendo de paja de semillas oleaginosas pretratadas con un método alcalino, sin embargo para la hidrólisis utilizaron además de Celluclast 1.5, Novozyme 188, demostrando que con la utilización de diferentes tipos de enzimas es posible lograr sinergias entre ellas que redunden en un aumento en la productividad de azúcares, tal y como lo mencionan Bals et al. [19] quienes hidrolizaron pasto de pradera pretratado por medio de explosión de amoniaco y la combinación de las enzimas Novozyme 188, Accelerase, Multiefect Xilanase y Multiefect Pectinase obteniendo hasta 520 kg/ton.

4. Conclusiones.

El pretratamiento alcalino-oxidativo propuesto en el presente trabajo es adecuado para su uso en bagazo de Agave tequilana. Las condiciones que presentaron los mejores resultados en un proceso de dos etapas son: Una primera etapa de tratamiento alcalino a 121°C por 60 minutos con una concentración de hidróxido de sodio del 6 % y una relación solido a líquido del 10%. Una segunda etapa oxidativa a 30°C por 24 horas, concentración de peróxido de hidrogeno del 6 %, relación solido a liquido del 10%.

Con dichas condiciones y la utilización de Celluclast 1.5 L, se obtuvo una concentración de azúcares reductores de 56.02 ± 0.92 g/L y por ende con un rendimiento de fermentación teórico del 51% podría ser factible la obtención de 28.57 ± 0.92 g/L de etanol. Por otro lado, con las condiciones estudiadas es posible obtener 378.57 kg de azúcares simples por tonelada de bagazo de *Agave tequilana* seco y libre de solubles. Adicionalmente si el efluente del pretratamiento se recupera, es posible obtener fructosa derivada de la gran cantidad de inulina presente en el bagazo. Considerando que alrededor del 40% del peso inicial del material utilizado es inulina, si esta se hidroliza, se estima que el rendimiento total de azúcares simples podría rebasar los 500 kg de azúcares por tonelada de bagazo seco. A pesar de que el pretratamiento utilizado es efectivo, hecho que se demuestra por la gran cantidad de lignina removida; la obtención de azúcares simples es pobre. Sobre la base de este hecho y las experiencias de otros investigadores, se propone el empleo de un coctel enzimático para mejorar el rendimiento de la hidrólisis.

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